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# Genomic landscape of pediatric B-other acute lymphoblastic leukemia in a consecutive European cohort

Marketa Zaliova,<sup>1,2,3</sup> Jan Stuchly,<sup>1,2</sup> Lucie Winkowska,<sup>1,2</sup> Alena Musilova,<sup>1,2</sup> Karel Fiser,<sup>1,2</sup> Martina Slamova,<sup>1,2</sup> Julia Starkova,<sup>1,2</sup> Martina Vaskova,<sup>1,2</sup> Ondrej Hrusak,<sup>1,2,3</sup> Lucie Sramkova,<sup>1,2,3</sup> Jan Stary,<sup>2,3</sup> Jan Zuna<sup>1,2,3</sup> and Jan Trka<sup>1,2,3</sup>

<sup>1</sup>CLIP - Childhood Leukaemia Investigation Prague; <sup>2</sup>Department of Paediatric Haematology and Oncology, Second Faculty of Medicine, Charles University and

<sup>3</sup>University Hospital Motol, Prague, Czech Republic

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## ABSTRACT

Novel biological subtypes and clinically important genetic aberrations (druggable lesions, prognostic factors) have been described in B-other acute lymphoblastic leukemia (ALL) during the last decade; however, due to a lack of studies on unselected cohorts, their population frequency and mutual associations still have to be established. We studied 110 consecutively diagnosed and uniformly treated childhood B-other patients using single nucleotide polymorphism arrays and whole exome/transcriptome sequencing. The frequency of *DUX4*-rearranged, *BCR-ABL1*-like, *ZNF384*-rearranged, *ETV6-RUNX1*-like, *iAMP21* and *MEF2D*-rearranged subtypes was 27%, 15%, 5%, 5%, 4%, and 2%, respectively; 43% of cases were not classified into any of these subtypes (B-rest). We found worse early response to treatment in *DUX4*-rearranged leukemia and a strong association of *ZNF384*-rearranged leukemia with B-myeloid immunophenotype. Of the druggable lesions, JAK/STAT-class and RAS/RAF/MAPK-class aberrations were found in 21% and 43% of patients, respectively; an ABL-class aberration was found in one patient. A recently described negative prognostic factor, *IKZF1<sup>plus</sup>*, was found in 14% of patients and was enriched in (but not exclusive for) *BCR-ABL1*-like subtype. *PAX5* fusions (including 4 novel), intragenic amplifications and P80R mutations were mutually exclusive and only occurred in the B-rest subset, altogether accounting for 20% of the B-other group. *PAX5* P80R was associated with a specific gene expression signature, potentially defining a novel leukemia subtype. Our study shows unbiased European population-based frequencies of novel ALL subtypes, recurrent (cyto)genetic aberrations and their mutual associations. This study also strengthens and widens the current knowledge of B-other ALL and provides an objective basis for optimization of current genetic diagnostics.

## Correspondence:

JAN TRKA/MARKETA ZALIOVA  
jan.trka@lfmotol.cuni.cz/  
marketa.zaliova@lfmotol.cuni.cz

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## Introduction

Knowledge about the genetics of pediatric B-cell precursor (BCP) acute lymphoblastic leukemia (ALL) has grown substantially in the past decade due to the boom in genome-wide technologies. Some of the most important novel findings concern B-other ALL, a heterogeneous group of BCP-ALL with previously unknown genetic backgrounds, usually defined by the absence of all routinely assessed classifying aberrations. Several subtypes have been described within B-other ALL based on the presence of unique (presumably primary) genetic aberrations [*iAMP21* ALL,<sup>1</sup> *DUX4* rearranged (r) ALL,<sup>2</sup> *ZNF384r* ALL,<sup>3,5,6</sup> *MEF2Dr* ALL<sup>3,6,7</sup>] or gene expression signatures (*BCR-ABL1*-like ALL<sup>8-10</sup> and *ETV6-RUNX1*-like ALL<sup>2,11</sup>). Although the classification of BCP-ALL has been substantially refined, it is complicated by a partial overlap of some of these subtypes and/or their imperfect definitions. It has been shown that a significant proportion of *iAMP21* ALL have

**Table 1.** Early response to treatment of B-other acute lymphoblastic leukemia patients stratified by *DUX4r*.

	Total B-other (n=110) n (%)	<i>DUX4r</i> ALL (n=30) n (%)	non- <i>DUX4r</i> ALL (n=80) n (%)	P <i>DUX4r</i> vs. non- <i>DUX4r</i>
Prednisone response <sup>1</sup>				
Good	102 (93%)	24 (80%)	78 (98%)	
Poor	8 (7%)	6 (20%)	2 (2%)	0.005
FC MRD d15 <sup>2</sup>				
<10%	86 (82%)	19 (68%)	67 (87%)	
≥10%	19 (18%)	9 (32%)	10 (13%)	0.04
NA	5	2	3	
PCR MRD <sup>3</sup>				
TP1 + TP2 neg.	28 (26%)	3 (10%)	25 (32%)	
Other	56 (53%)	17 (59%)	39 (51%)	
TP1 ≥10-3 + TP2 pos. or TP2 ≥10-3	22 (21%)	9 (31%)	13 (17%)	0.04
NA	4 <sup>5</sup>	1	3	
Final risk group <sup>4</sup>				
SR	25 (23%)	3 (10%)	22 (28%)	
MR	52 (49%)	13 (45%)	39 (50%)	
HR	30 (28%)	13 (45%)	17 (22%)	0.03
NA	3 <sup>5</sup>	1	2	

<sup>1</sup>Good: <1000 leukemic blood blasts/μL on treatment day (d) 8; poor: ≥1000/μL. <sup>2</sup>Minimal residual disease (MRD) at d15 of treatment measured by flow cytometry (FC). <sup>3</sup>MRD measured by polymerase chain reaction (PCR). n: number; TP: time point; TP1: treatment day 33; TP2: treatment day 78. <sup>4</sup>Standard risk (SR), medium risk (MR), high risk (HR); for definitions see *Online Supplementary Methods*. P-value by Fisher exact test. <sup>5</sup>Patients died before TP2 or no MRD target; neg.: negative; pos.: positive (any positive value); NA: not applicable/not available. Unknown values are not considered for percentage and statistics.

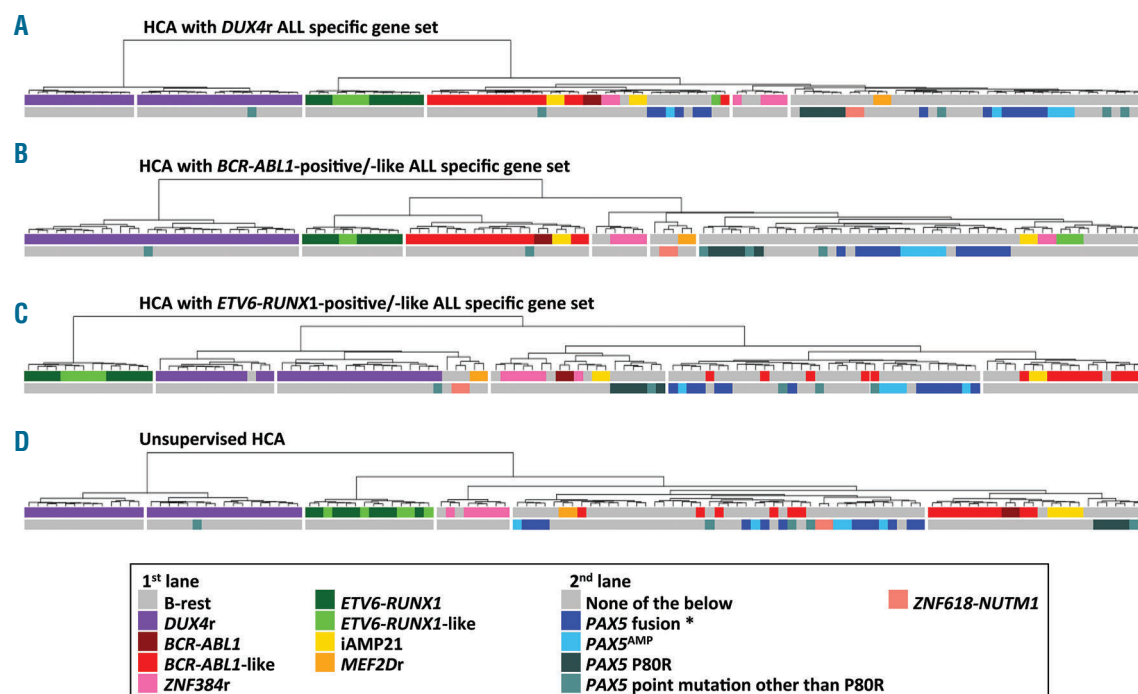
the *BCR-ABL1*-like phenotype<sup>12</sup> or may coincide with the *ETV6-RUNX1*-like phenotype.<sup>11</sup> Moreover, it has been demonstrated that the definition of *BCR-ABL1*-like ALL varies significantly among studies;<sup>12</sup> this likely results from the limited specificity of *BCR-ABL1*-associated gene expression signature. Thus, not only a complex genetic and gene expression characterization but also a hierarchical approach is needed in order to classify BCP-ALL into non-overlapping subsets.

In parallel to aberrations that define subtypes, a wide range of additional genetic aberrations has been identified. Among them, two groups of aberrations have already become therapeutically relevant either as novel prognostic factors or as therapy targets. The first group is represented by *IKZF1* gene deletion (*IKZF1*del) and *IKZF1*<sup>plus</sup> deletion pattern. *IKZF1*del was repeatedly shown to confer a higher risk of relapse<sup>9,13-15</sup> and is used in therapy adjustment algorithms in some contemporary treatment protocols (*clinicaltrials.gov* identifier: 03020030; DCOG ALL11<sup>16</sup>). However, the Italian Association of Pediatric Hematology and Oncology-Berlin-Frankfurt-Muenster (AIEOP-BFM) study group has recently shown that *IKZF1*<sup>plus</sup>, defined by a combination of *IKZF1*del with other aberrations (deletions of *CDKN2A*, *CDKN2B*, *PAX5*, or *PAR1* in the absence of *ERG* deletion), confers a significantly higher risk of relapse compared to *IKZF1*del alone, and the newly defined *IKZF1*<sup>plus</sup> pattern will be used to improve risk prediction accuracy in the upcoming BFM ALL protocol.<sup>17</sup> The second group of therapeutically relevant aberrations largely comprises aberrations affecting genes encoding kinases, cytokine receptors and signaling regulators, resulting in activation of kinase signaling<sup>10,18-23</sup> (“kinase aberrations”). These aberrations occur especially, but not

exclusively, in *BCR-ABL1*-like ALL. A wide range of kinase aberrations has already been described, and novel ones still continue to be reported. These aberrations vary by type (gene fusions/juxtapositions, copy number changes, mutations) and frequency; *CRLF2*, *NRAS* or *KRAS* lesions are relatively common and occur in various ALL subtypes,<sup>2,6,24-27</sup> while, for example, fusions involving *ABL1*, *ABL2*, *EPOR*, *PDGFRB* or *JAK2* are less frequent and occur predominantly in *BCR-ABL1*-like ALL.<sup>28-30</sup> Two functional classes of aberrations, ABL-class and JAK/STAT-class, are currently used for targeted ALL therapy in some ongoing clinical trials (*clinicaltrials.gov* identifiers: 03117751, 02883049, 03020030).

The ongoing translation of these genetic findings into treatment is bringing new diagnostic demands and a dilemma as to which genetic methods should be incorporated into diagnostic algorithms and how this should be achieved. In order to explore this, well-defined consecutive cohorts need to be analyzed to assess the real incidence of various ALL subtypes and genetic aberrations. Unfortunately, so far, such data have been very scarce, and virtually all the published cohorts were in some way selected, enriched, biased, or not analyzed completely.

Here, we present a genetic study of all pediatric B-other ALL cases consecutively diagnosed and uniformly treated in the Czech Republic over the last seven years. We performed this study to facilitate the use of optimal treatment strategies by extending and refining diagnostic algorithms, and to assess the feasibility and the benefits of introducing genome-wide technologies into routine diagnostics. A further study aim was to determine unbiased population frequencies of known ALL subtypes derived from B-other



**Figure 1. Results of hierarchical clustering analyses (HCA).** A total of 110 B-other acute lymphoblastic leukemia (ALL) were clustered hierarchically based on the expression of genes belonging to defined gene sets (A-C) or based on the expression of the most variably expressed transcripts (409 transcripts with standard deviations  $\geq 35\%$  of the maximal standard deviation) (D) and resulting dendrograms are shown. Gene sets are described in *Online Supplementary Tables S4-S5*. Genetic annotation is split in two lanes: 1<sup>st</sup> lane shows classification into already established subtypes, 2<sup>nd</sup> lane shows selected recurrent genetic aberrations (mutually exclusive with each other and with the exception of *PAX5* mutations other than P80R also with established subtypes). \*Does not include *ZCCHC7-PAX5* fusion.

ALL and genetic aberrations, their mutual associations, and associations with demographic and clinical parameters.

## Methods

### Patients

The present study included 110 children (aged 1-18 years) diagnosed with B-other ALL [BCP-ALL negative for *ETV6-RUNX1*, *BCR-ABL1*, *TCF3-PBX1*, *KMT2A*-rearrangements, hyperdiploidy (>50 chromosomes), and hypodiploidy (<44 chromosomes)] between December 2010 and December 2017 treated according to the AIEOP-BFM ALL 2009 trial (*clinicaltrials.gov identifier: 01117441*). In total, 410 BCP-ALL and/or BCP-myeloid mixed phenotype acute leukemia (MPAL) cases were diagnosed and treated according to this protocol within the period of study (*Online Supplementary Table S1*). The study was approved by the local Institutional Review Board. Informed consent was obtained in accordance with the Declaration of Helsinki.

### Analysis of *ERG* gene deletion and *P2RY8-CRLF2* by polymerase chain reaction

Presence of the *ERG* gene deletion (*ERGdel*) was analyzed by DNA-based multiplex polymerase chain reaction (PCR).<sup>31</sup> Two primers corresponding to additional centromere breakpoint sites<sup>32</sup> were added: 5'-GCGGCTACTTGTGGTCCAAGAA-3' and 5'-CTATCCTGAACATTGCTGCCAG-3'.

Presence of the *P2RY8-CRLF2* transcript was analyzed by real time (RT)-PCR and quantified by quantitative real time (qRT)-PCR as described previously<sup>18</sup> with replacement of the reverse primer for qRT-PCR (5'-AGCCTCCCAGCAGAAAGAC-3').

### Single-nucleotide polymorphism array

Single-nucleotide polymorphism array (SNPa) was performed in 103 out of 110 B-other patients. Copy number aberrations (CNA) and regions of uniparental disomy (UPD) were identified using HumanOmni Express BeadChip (Illumina, USA) or CytoScan HD arrays (Affymetrix, USA).

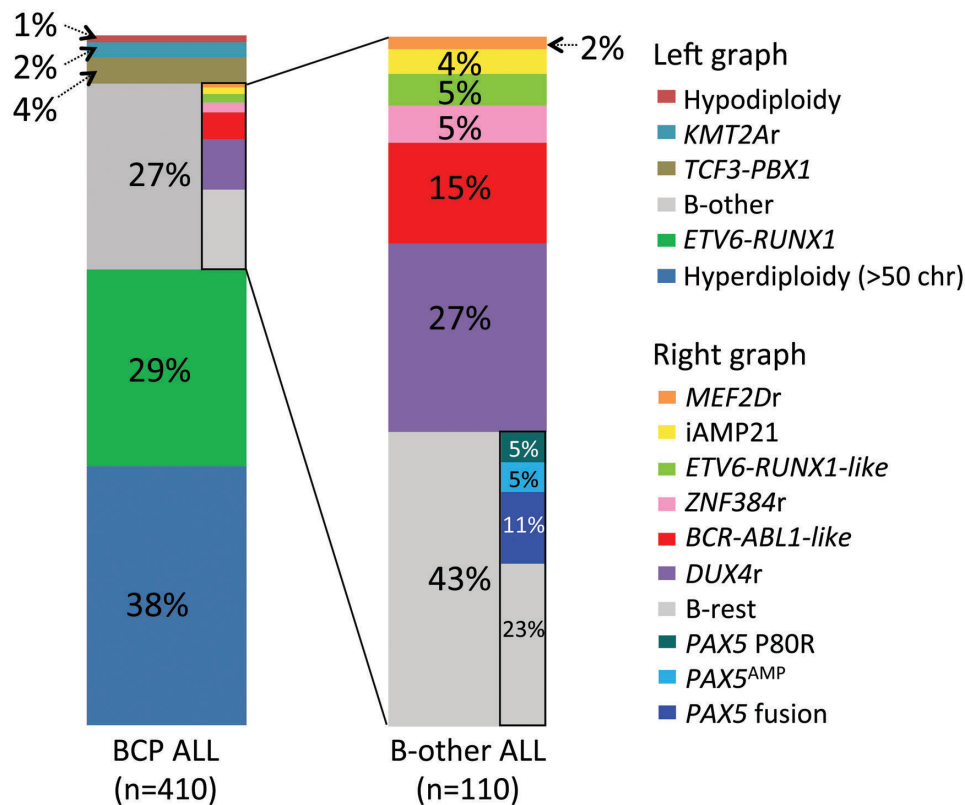
### Whole-transcriptome and whole-exome sequencing

Whole-transcriptome (RNA-sequencing) and whole-exome (WES) sequencing were performed using diagnostic samples in 109 out of 110 and 69 out of 110 B-other patients, respectively, as described previously.<sup>11</sup> In one patient without material from initial diagnosis, the relapse sample was used for subtype classification. Sequencing libraries were prepared from DNA and total RNA using Agilent SureSelectXT HumanAllExon V4, V5 or V6 and Agilent SureSelect mRNA Strand Specific kits, respectively, according to the manufacturer's instructions (Agilent Technologies Inc., Santa Clara, CA, USA). High-throughput sequencing was performed on HiSeq2500 (1x50, 2x50 or 2x100 bp) or NextSeq500 (2x75 bp) using TruSeq Rapid SBS and PE Cluster kits and High Output Kit (Illumina).

Selected data metrics are shown in *Online Supplementary Tables S2 and S3*.

### Analysis of fusion transcripts and *IGH*, *DUX4* and *CRLF2* rearrangements

RNA-sequencing data were analyzed using TopHat<sup>33</sup> and deFuse.<sup>34</sup> In-frame fusion transcripts and those out-of-frame fusion transcripts that disrupted known ALL-associated genes and/or correlated with boundaries of CNAs found by SNPa were selected for further verification. Fusions verified by RT-PCR as leukemia-spe-



**Figure 2.** Frequency of acute lymphoblastic leukemia (ALL) subtypes among 410 B-cell precursor (BCP) ALL patients consecutively diagnosed and uniformly treated in the Czech Republic from December 2010 to December 2017. In addition to the five established B-other ALL subtypes, frequencies of specific *PAX5* aberrations which were mutually exclusive with these subtypes are shown. Aberrations which are not mutually exclusive with the established subtypes [e.g. *CRLF2r*, *dic(9;20)*] are not shown. Infants and *BCR-ABL1*-positive patients were treated according to different protocols and are not included. Chr: chromosomes. *PAX5* fusions do not include *ZCCHC7-PAX5*.

cific (absent in healthy mononuclear blood cells) are reported.

To analyze the presence of *IGH-DUX4* and *IGH-CRLF2* fusion transcripts that may not be revealed by TopHat/deFuse, reads mapped to fusion partner genes were analyzed again manually.

### Analysis of genomic variants

Read pairs were aligned to hg19 using BWA<sup>35</sup> (WES) and STAR<sup>36</sup> (RNA-sequencing) and further processed by Picard tools (<http://broadinstitute.github.io/picard/>). Variant calling was performed using VarScan<sup>37</sup> and Samtools (<http://samtools.sourceforge.net/>). To distinguish somatic and germline variants, WES results from ALL diagnosis were compared to the remission sample.

### Gene expression profiling, hierarchical clustering analysis, *DUX4* gene expression analysis

Genome-wide gene expression analysis was performed using RNA-sequencing data of the 110 B-other study patients plus two *BCR-ABL1*-positive and nine *ETV6-RUNX1*-positive patients. Alignment and counting were performed as described previously.<sup>38</sup> Data normalization and hierarchical clustering analysis (HCA) based on the expression of the most variably expressed genes or of genes belonging to particular gene sets were performed using R package Deseq2<sup>39</sup> (vst normalization, ward.D method and Euclidean distance linkage for HCA). The list of gene sets and included genes used for HCA are shown in *Online Supplementary Tables S4* and *S5*.

To assess *DUX4* gene expression, reads mapped to the *DUX4* reference were counted as described previously.<sup>3</sup> To correct for uneven sequencing depth, counts were normalized using size factors computed by Deseq2.

### Flow cytometry

Routine flow cytometry was used for the diagnosis of BCP-ALL and mixed-phenotype acute leukemia (MPAL) using criteria of the

World Health Organization and/or of the European Group for Immunophenotyping of Leukemia. *CRLF2* expression was assessed using PE anti-human TSLPR Antibody (clone 1D3; BioLegend, USA). In general, a 50% (10%) cut-off was used to assign strong (weak) positivity. A small *CRLF2*-positive subclone (<10%), distinct from the major negative population and clearly distinguishable from the technical background, was reported in one patient.

For more details of the methods used see the *Online Supplementary Methods*.

## Results

### Frequency of the B-other-derived acute lymphoblastic leukemia subtypes and associations with clinical parameters

To classify B-other patients into iAMP21, *ZNF384r*, *MEF2Dr*, *DUX4r*, *ETV6-RUNX1-like* and *BCR-ABL1-like* subtypes, we used data from SNPa and RNA-sequencing. Our cohort included six cases with *ZNF384* fusions (*EP300-ZNF384*, n=4; *TCF3-ZNF384*, n=1; *TAF15-ZNF384*, n=1), four cases with iAMP21, and two cases with *MEF2Dr* (*MEF2D-BCL9*, n=2). The *BCR-ABL1-like*, *ETV6-RUNX1-like* and *DUX4r* ALLs were classified using HCA based on the expression of gene sets representative of subtype-specific gene expression signatures (subtype-specific gene sets) from published studies<sup>4,10,11</sup> (Figure 1A-C). Using the *DUX4r* gene set, 30 cases were classified as *DUX4r* ALL. In accordance with published studies, *DUX4r* patients had significantly higher *DUX4* expression compared to remaining ALLs (*Online Supplementary Figure S1*) and fusion transcripts supporting the presence of *DUX4r* were found in 29 out of 30 patients. To verify co-clustering

Table 2. Frequency of selected genetic lesions in total cohort and across acute lymphoblastic leukemia subsets.

	Percentage of (number of total analyzed) cases positive for genetic lesions within total B-other cohort / individual ALL subsets							
	Total (n=103-110†)	B-rest (n=47)	DUX4r (n=30)	BCR-ABL1-like (n=16)	ZNF384r (n=6)	ETV6-RUNX1-like (n=5)	iAMP21 (n=4)	MEF2Dr (n=2)
PAX5 any aberration	53% (55/104)	82% (36/44)****	21% (6/28)	50% (8/16)	17% (1/6)	40% (2/5)	33% (1/3)	50% (1/2)
PAX5 fusion <sup>1</sup>	11% (12/109)	26% (12/47)****	0% (0/30)	0% (0/16)	0% (0/6)	0% (0/5)	0% (0/3)	0% (0/2)
PAX5 AMP	5% (5/109)	11% (5/47)*	0% (0/30)	0% (0/16)	0% (0/6)	0% (0/5)	0% (0/3)	0% (0/2)
PAX5 P80R	5% (5/109)	11% (5/47)*	0% (0/30)	0% (0/16)	0% (0/6)	0% (0/5)	0% (0/3)	0% (0/2)
CDKN2A/B deletion	51% (53/103)	72% (31/43)***	32% (9/28)	50% (8/16)	33% (2/6)	20% (1/5)	33% (1/3)	50% (1/2)
dic(9;20)	9% (9/103)	19% (8/43)**	0% (0/28)	6% (1/16)	0% (0/6)	0% (0/5)	0% (0/3)	0% (0/2)
TOX deletion	8% (8/103)	19% (8/43)***	0% (0/28)	0% (0/16)	0% (0/6)	0% (0/5)	0% (0/3)	0% (0/2)
trisomy 5	4% (4/103)	9% (4/43)*	0% (0/28)	0% (0/16)	0% (0/6)	0% (0/5)	0% (0/3)	0% (0/2)
ERG deletion <sup>2</sup>	18% (19/108)	0% (0/46)	63% (19/30)****	0% (0/16)	0% (0/6)	0% (0/5)	0% (0/3)	0% (0/2)
TBLXR1 any aberration	12% (12/103)	5% (2/43)	24% (7/29)*	6% (1/16)	0% (0/6)	20% (1/5)	0% (0/3)	50% (1/2)
DMD deletion	6% (6/103)	2% (1/43)	18% (5/28)**	0% (0/16)	0% (0/6)	0% (0/5)	0% (0/3)	0% (0/2)
CRLF2 fusion <sup>3</sup>	24% (26/109)	28% (13/47)	0% (0/30)	69% (11/16)****	0% (0/6)	20% (1/5)	33% (1/3)	0% (0/2)
CRLF2r	15% (16/109)	6% (3/47)	0% (0/30)	69% (11/16)****	0% (0/6)	20% (1/5)	33% (1/3)	0% (0/2)
P2RY8-CRLF2-low	9% (10/108)	21% (10/47)***	0% (0/29)	0% (0/15)	0% (0/6)	0% (0/5)	0% (0/4)	0% (0/2)
JAK-STAT pathway lesion	21% (23/109)	15% (7/47)	0% (0/30)	75% (12/16)****	17% (1/6)	20% (1/5)	66% (2/3)	0% (0/2)
IKZF1 deletion	26% (27/103)	12% (5/43)	21% (6/28)	69% (11/16)****	17% (1/6)	60% (3/5)	33% (1/3)	0% (0/2)
IKZF1 any aberration	28% (29/103)	16% (7/43)	21% (6/28)	69% (11/16)***	17% (1/6)	60% (3/5)	33% (1/3)	0% (0/2)
IKZF1 <sup>plus</sup>	14% (14/103)	4% (4/43)	0% (0/28)	50% (8/16)***	0% (0/6)	40% (2/5)	0% (0/3)	0% (0/2)
trisomy 21 acquired	8% (8/103)	7% (3/43)	4% (1/28)	25% (4/16)*	0% (0/6)	0% (0/5)	0% (0/3)	0% (0/2)
trisomy 21 germline	4% (4/110)	2% (1/47)	0% (0/30)	19% (3/16)**	0% (0/6)	0% (0/5)	0% (0/4)	0% (0/2)
EBF1 deletion	6% (6/103)	2% (1/43)	0% (0/28)	25% (4/16)**	17% (1/6)	0% (0/5)	0% (0/3)	0% (0/2)
KMT2D any aberration	8% (9/109)	2% (1/47)	17% (5/30)	0% (0/16)	50% (3/6)**	0% (0/5)	0% (0/3)	0% (0/2)
SETD2 any aberration	6% (6/109)	6% (3/47)	0% (0/30)	6% (1/16)	33% (2/6)*	0% (0/5)	0% (0/3)	0% (0/2)
ETV6 any aberration	17% (17/103)	16% (7/43)	4% (1/28)	19% (3/16)	0% (0/6)	100% (5/5)****	0% (0/3)	50% (1/2)
ETV6 deletion	13% (13/103)	14% (6/43)	0% (0/28)	6% (1/16)	0% (0/6)	100% (5/5)****	0% (0/3)	50% (1/2)
ARPP21 deletion	5% (5/103)	5% (2/43)	0% (0/28)	6% (1/16)	0% (0/6)	40% (2/5)*	0% (0/3)	0% (0/2)
BTG1 any aberration	8% (8/103)	7% (3/43)	4% (1/28)	6% (1/16)	0% (0/6)	60% (3/5)**	0% (0/3)	0% (0/2)
HIST1H genes deletion	7% (7/103)	2% (1/43)	0% (0/28)	19% (3/16)	0% (0/6)	40% (2/5)*	33% (1/3)	0% (0/2)

Only genetic lesions with uneven distribution across acute lymphoblastic leukemia (ALL) subtypes are included. The numbers of analyzed patients vary for individual genetic lesions based on the detection method: 103 patients were analyzed by single-nucleotide polymorphism array (SNPa), 109 were analyzed by RNA-sequencing in diagnostic sample, one patient who was analyzed by RNA-sequencing in relapse sample is not included in this analysis. <sup>1</sup>Does not include ZCCHC7-PAX5 fusion. <sup>2</sup>Does not include uncommon type of ERGdel identified by SNPa but negative by polymerase chain reaction (PCR) in a single DUX4r-positive case. <sup>3</sup>Includes CRLF2r and P2RY8-CRLF2-low. \*P≤0.05; \*\*P≤0.01; \*\*\*P≤0.001; \*\*\*\*P≤0.0001; by Fisher Exact Probability test for the significant enrichment of genetic lesion in respective subtype compared to all remaining cases.

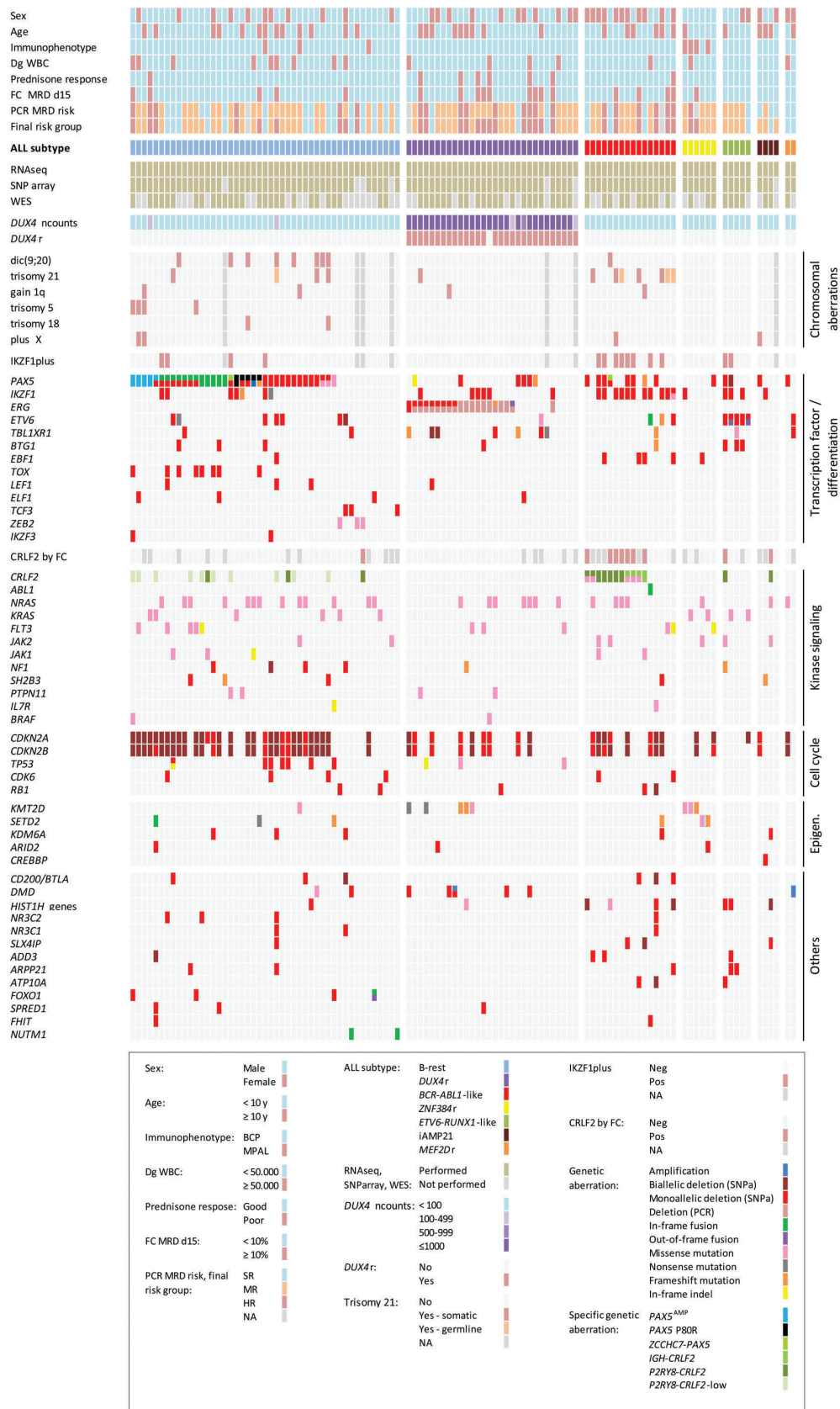
of BCR-ABL1-like and ETV6-RUNX1-like subtypes with respective genetic groups, we amended the cohort with two BCR-ABL1-positive and nine ETV6-RUNX1-positive ALLs. Eighteen patients had the BCR-ABL1-like phenotype according to HCA; two of them had iAMP21 and were not included in BCR-ABL1-like ALL for the subsequent analyses, and the remaining 16 patients were classified as BCR-ABL1-like ALL. Five patients were classified as ETV6-RUNX1-like ALL. Unlike DUX4r and ETV6-RUNX1-positive/-like ALL, BCR-ABL1-like ALL did not co-cluster together in unsupervised HCA, confirming that the gene expression profile of BCR-ABL1(-like) ALL is less unique or specific (Figure 1D).

Overall, the most common B-other ALL subtype was DUX4r ALL (27%), followed by BCR-ABL1-like (15%), ZNF384r (5%), ETV6-RUNX1-like (5%), iAMP21 (4%), and MEF2Dr ALL (2%); the largest proportion of cases

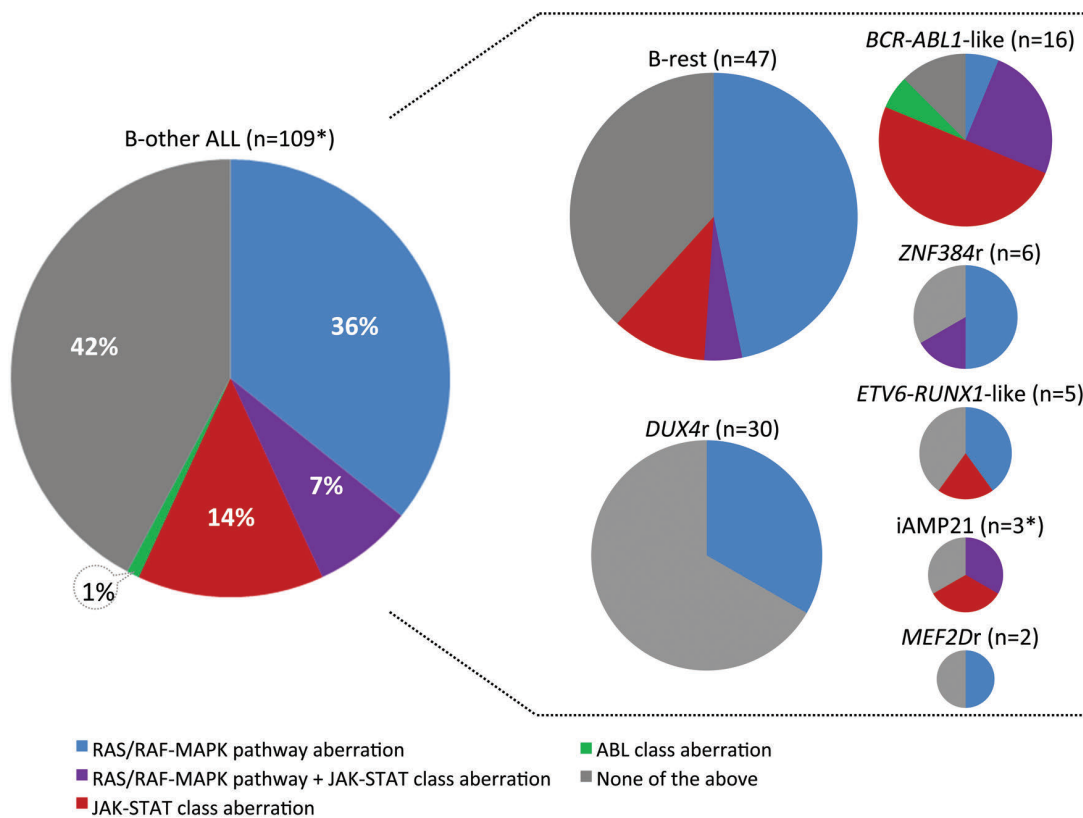
(“B-rest”; n=47, 43%) did not belong to any of these subtypes (Figure 2).

Compared to remaining BCP-ALLs, B-others were more frequently males, older, had higher white blood cell (WBC) count, and worse early response to therapy (Online Supplementary Table S1). Analysis of demographic and clinical parameters in the two largest B-other-derived subtypes, DUX4r, BCR-ABL1-like, and B-rest subgroup showed a significantly higher proportion of females in BCR-ABL1-like compared to non-BCR-ABL1-like ALL, while there were no significant differences in age or WBC (Online Supplementary Table S1). Importantly, we found a significantly worse early response to treatment assessed by several criteria in the DUX4r ALL compared to non-DUX4r ALL, resulting in a higher proportion of high-risk patients among the DUX4r ALL (Table 1).

In total, 7 out of 110 B-other ALLs had an MPAL



**Figure 3. Graphical overview of clinical characteristics, the analyses performed and the genetic findings in 110 B-other acute lymphoblastic leukemia (ALL) patients grouped by ALL subtype.** Patients are ordered according to the ALL subtype; demographic and clinical data are shown above, while genetic data are displayed below the ALL subtype track. Recurrently affected genes are arranged according to functional categories. Epigen.: epigenetic regulators/modifiers; MPAL: BCP-myeloid mixed phenotype acute leukemia; Dg WBC; initial white blood cell count (cells/ $\mu$ L); y; years; FC: flow cytometry; SR: standard risk; MR: medium risk; HR: high risk; Neg: negative; Pos: positive; ncounts: normalized counts. For definitions of good and poor prednisone response, polymerase chain reaction (PCR) minimal residual disease (MRD) risk, and final risk group stratification see Table 1, *Online Supplementary Methods* and *Online Supplementary Table S6*.



**Figure 4. Frequency and distribution of kinase aberrations.** The graphs are proportional to (sub)cohort sizes (number of patients in the total B-other cohort and in individual subtypes). \*The one patient who was analyzed by RNA-sequencing only at relapse is not included in this analysis.

immunophenotype and these were significantly enriched in *ZNF384r* ALL [MPALs comprised 67% (4 out of 6) of *ZNF384r* vs. 3% (3 out of 104) of non-*ZNF384r* ALLs;  $P < 0.0001$ ].

#### Genomic characterization of the study cohort

Genomic aberrations were analyzed by SNP<sub>a</sub>, WES, RNA-sequencing and PCR. On average, we found 9 CNAs/UPDs per patient by SNP<sub>a</sub> (range 0-35) and 14 mutations [single nucleotide variants (SNVs), insertions/deletions (indels)] by WES (range 1-65) (*Online Supplementary Tables S7 and S8*). In total, we identified 18 recurrently (in  $\geq 2$  patients) mutated genes by WES, and 43 recurrent chromosomal imbalances and CNAs subtracted to genes by SNP<sub>a</sub>; all had already been reported to occur in ALL. In addition to *IGH-DUX4*, we found 41 different fusion transcripts including 23 that were novel: 24 were in-frame (13 out of 24 novel), 9 contained a full coding sequence of one partner gene (3 out of 9 novel), and 8 were out-of-frame (7 out of 8 novel) (*Online Supplementary Table S9*). Subclonal deletions of *ERG* and *P2RY8-CRLF2* fusions were detected by PCR in 11 and ten patients negative for these lesions by SNP<sub>a</sub> and RNA-sequencing, respectively. All recurrent findings are shown together with clinical parameters in Figure 3 and *Online Supplementary Table S6*.

#### Specific genetic features of the B-other-derived acute lymphoblastic leukemia subtypes

No significant differences were observed in numbers of CNAs/UPDs or SNVs/indels comparing individual ALL

subtypes except for a significantly lower number of CNAs/UPDs in *DUX4r* versus remaining ALL cases ( $4 \pm 0.5$  vs.  $10 \pm 0.9$ ;  $P < 0.0001$ ) (*Online Supplementary Figure S2*). Apart from the *MEF2Dr* and *iAMP21* ALL, which were represented by a very low number of patients, each of the remaining subtypes was significantly enriched for specific genetic aberrations (Table 2). *ERGdel* occurred in 63% of *DUX4r* ALL but not in non-*DUX4r* ALL patients. Aberrations of *TBLXR1* and *DMD* genes were also enriched in *DUX4r* ALL. *BCR-ABL1*-like ALL was enriched for the *CRLF2* fusions, *IKZF1del*, both constitutional and somatic trisomy 21, and deletions of *EBF1*. Mutations in the *KMT2D* and *SETD2* genes (encoding histone methyltransferases) were mutually exclusive and occurred in 5 out of 6 *ZNF384r* ALL patients. In agreement with our previous study, all *ETV6-RUNX1*-like patients harbored an *ETV6* aberration (5 out of 5 had a deletion and 2 out of 5 had a fusion). This subtype was also enriched for *ARPP21* deletions, deletions of histone gene cluster on 6p22.2, and *BTG1* aberrations.

#### Specific genetic features of the B-rest acute lymphoblastic leukemia subset

The B-rest subset was significantly enriched for the *PAX5* fusions, *PAX5* amplifications, *PAX5* P80R mutation, *CDKN2A/B* deletions, *dic(9;20)*, and *TOX* deletions compared to remaining B-other ALLs. It is worthy of note that the *PAX5* fusions, amplifications and P80R mutations and *TOX* deletions occurred exclusively in the B-rest subset; 6 out of 8 *TOX* deletions occurred together with the *PAX5* fusions (Table 2).

Table 3. PAX5-involving fusion genes.

UPN	PAX5 exons (NM_016734)	Fusion partner			Fusion-related chromosomal imbalances	
		Gene symbol	Reference	Exons		
1768	1-6	<i>PML</i>	NM_033238	3-9	15q24	None
1826	1-8	<b><i>PROC</i></b>	NM_000312	3-9	2q14	None
2188	1-5	<i>NOLAL</i>	NM_080616	3-8	20q11.21	dic(9;20)
2319	1-5	<i>CBFA2T3</i>	NM_005187	2-12	16q24	deletion of 3' part of <i>PAX5</i>
2415	1-7	<i>CBFA2T2</i>	NM_005093	8-12	20q11.21	deletion of 3' part of <i>PAX5</i> and deletion of 5' part of <i>CBFA2T2</i>
2544	1-5	<i>CBFA2T3</i>	NM_005187	4-12	16q24	None at PAX5 locus, <i>CBFA2T3</i> locus unclear (multiple CNA across 16q)
2548	1-8	<b><i>PIK3AP1</i></b>	NM_152309	14-17	10q24	deletion of 5' part of <i>PIK3AP1</i>
2564	1-7	<i>ESRRB</i>	NM_004452	5-11	14q24	deletion of 3' part of <i>PAX5</i> and deletion of 5' part of <i>ESRRB</i>
2578	1-6	<b><i>MPRIP</i></b>	NM_015134	11-23	17p11.2	deletion of 3' part of <i>PAX5</i> and deletion of 5' part of <i>MPRIP</i>
2621	1-8	<i>DNAI1</i>	NM_001539	4-9	9p13.3	deletion of 5' part of <i>DNAI1</i>
2663	1-8	<b><i>ARHGAP22</i></b>	NM_021226	4-10	10q11.23	deletion of 3' part of <i>PAX5</i>
2701	1-5	<b><i>ARHGAP22</i></b>	NM_021226	5-10	10q11.23	deletion of 3' part of <i>PAX5</i>
2486	2-10	<i>ZCCHC7</i>	NM_032226	1-2	9p21.1	None
2596	2-10	<i>ZCCHC7</i>	NM_032226	1-2	9p21.1	None

PAX5 fusion partners newly identified in the present study are shown in bold.

In total, we found 11 different PAX5-involving in-frame fusions in 14 patients (Table 3). There were 2 types of fusions (Online Supplementary Figure S3). The more common type was comprised of the 5' part of PAX5 (exons 1-5/6/7/8) with the (partially) absent PAX5 transactivation domain (TAD), fused to various partner genes. This type was present in 12 patients, representing 11% of the total B-other cohort (Figure 2). The second type, represented only by the ZCCHC7-PAX5 fusion [generated by intra-chromosomal inversion (Online Supplementary Figure S3)], involved the 3' part of PAX5 including both the DNA-binding domain and complete TAD (exons 2-10). We identified four novel PAX5 partner genes: ARHGAP22 (n=2), MPRIP, PIK3AP1, and PROC. To the best of our knowledge, except for PIK3AP1 (whose deletions were reported in adult ALL<sup>40</sup>), none of these genes have previously been reported as recurrently affected in BCP-ALL. Interestingly, we found rearrangement of the second PIK3AP1 allele resulting in an in-frame PIK3AP1-WDR5 fusion in the PAX5-PIK3AP1-positive patient.

Intragenic PAX5 amplification was found in six B-rest ALL patients, representing 5% of total B-other cohort (Figure 2). In five out of six patients, amplification encompassed exons 2-5 (n=4) or exon 5 (n=1), and thus corresponded to the recurrent type of amplification, PAX5<sup>AMP</sup>, which was recently characterized in detail;<sup>41</sup> the remaining patient had atypical amplification encompassing exons 4-8 (Online Supplementary Figure S5). RNA-sequencing revealed aberrant PAX5 transcripts containing amplified exons in all six patients (Online Supplementary Figure S6). In line with the previous study,<sup>41</sup> PAX5<sup>AMP</sup> was accompanied by the trisomy 5 in three out of five patients.

The PAX5 P80R mutation was found in five patients from the B-rest subset who co-clustered tightly together in

unsupervised HCA (Figure 1D) and represented 5% of the total B-other cohort (Figure 2). The variant allele frequency (VAF) of PAX5 P80R ranged from 32% to 100%. The second PAX5 allele was deleted/lost in four out of five patients and likely inactivated in the remaining patient, with VAF 32% at the genomic but 98% at the mRNA level. We did not find any other genetic lesion which could explain co-clustering of these patients, which suggests that the specific expression signature was triggered by PAX5 P80R.

Unlike those with PAX5 P80R, patients with PAX5 fusions or PAX5<sup>AMP</sup> did not form clear clusters in unsupervised HCA; however, via an analysis of differential gene expression, we identified a gene set which distinguished patients with PAX5<sup>AMP</sup> from the remaining patients in supervised HCA (Online Supplementary Figure S7).

Two and three patients from the B-rest subset co-clustered together with ZNF384r and MEF2Dr ALL subtypes, respectively (Figure 1 and Online Supplementary Figure S7). We did not find any common genetic lesion in patients co-clustering with ZNF384r ALL; however, two out of three patients co-clustering with MEF2Dr ALL harbored the ZNF618-NUTM1 fusion. The NUTM1-gene involving fusions were recently shown to occur recurrently in childhood ALL, outside the established ALL subtypes.<sup>7</sup>

#### Frequency and distribution of therapeutically relevant aberrations

The IKZF1<sup>plus</sup> pattern was found in 14 out of 103 B-other ALLs (BCR-ABL1-like, n=8; ETV6-RUNX1-like, n=2; B-rest ALLs, n=4) and was significantly enriched in the BCR-ABL1-like subtype (Table 2). It has been shown that IKZF1<sup>plus</sup> has a negative prognostic impact only in patients with detectable MRD at the end of induction.<sup>17</sup> All but two



*IKZF1*<sup>plus</sup>-positive patients in the present study were MRD-positive at day 33 and thus predicted to have a high-risk of relapse.

The frequency and distribution of kinase aberrations is shown in Figure 4. Kinase aberrations that can be grouped into JAK/STAT-class were found in 23 patients who harbored lesions of *CRLF2* (n=21), *JAK2* (n=7), *JAK1* (n=6), *IL7R* (n=2), and *SH2B3* (n=1). The most common aberrations were *CRLF2* gene rearrangements which had previously been shown to frequently occur at subclonal level.<sup>42</sup> Accordingly, in addition to 12 *P2RY8-CRLF2* and four *IGH-CRLF2* fusions affecting a major leukemic clone (*CRLF2r*, n=16), *P2RY8-CRLF2* fusions affecting a minor leukemic clone (*P2RY8-CRLF2-low*) were detected by RT-PCR in ten patients. The *CRLF2r* always resulted in surface *CRLF2* positivity assessed by flow cytometry, was identified by RNA-sequencing, in the case of *P2RY8-CRLF2* evidenced by *PAR1* deletion on SNPa and was frequently accompanied by the activating *CRLF2* mutation F232C (5 out of 16 cases) or by *JAK1/2* mutation (6 out of 16 cases). On the other hand, all patients with *P2RY8-CRLF2-low* were negative for fusion transcript and *PAR1* region deletion by RNA-sequencing and SNPa, respectively, and all but one were negative for surface *CRLF2* by flow cytometry (a small *CRLF2*-positive subclone was reported in one patient). qPCR showed a clear separation (>1 log distance) of the two groups of *P2RY8-CRLF2*-positive patients (*Online Supplementary Figure S8*). While *CRLF2r* (and JAK/STAT-class aberrations in general) were significantly associated with a *BCR-ABL1*-like subtype, *P2RY8-CRLF2-low* occurred exclusively in the B-rest ALL subset (Table 2).

In the total B-other cohort, we found only one ABL-class aberration. The *ETV6-ABL1* fusion was found in a *BCR-ABL1*-like ALL patient and was accompanied by another two in-frame fusions resulting from a single chromosomal rearrangement that we recently characterized in a separate report.<sup>45</sup>

Aberrations activating RAS/RAF-MAPK signaling (aberrations of *NRAS*, *KRAS*, *FLT3*, *BRAF*, *PTPN11*, *NF1*) which may not be considered as suitable therapeutic targets because of their frequent subclonality, were found in 47 patients spread across all ALL subtypes.

Importantly, 50 out of 51 kinase aberrations detected by WES were also detected by RNA-sequencing.

## Discussion

Although several large genomic studies have been published over the last few years, these were mostly based on non-consecutive cohorts of patients.<sup>4,6,7,21</sup> Here, we present the first European population-based genomic study of pediatric B-other ALL based on a consecutive cohort of uniformly treated patients with a 100% inclusion rate. The only other consecutive study, the Swedish study by Lilljebjörn *et al.*,<sup>2</sup> based on 54 B-other ALLs (considering the B-other definition used herein) with an inclusion rate of 69%, showed a similarly high proportion of the “B-rest” ALL (40-45%) and similarly infrequent *ZNF384r* and *MEF2Dr* ALL subgroups. Although the frequency of the remaining three subtypes differs between both studies (*ETV6-RUNX1*-like ALL 5% vs. 11%, *DUX4r* ALL 27% vs. 15%, *BCR-ABL1*-like ALL 15% vs. 28%), these differences are not statistically significant. Moreover, in the case of *BCR-ABL1*-like ALL, the difference might be influenced

by heterogeneity in this subtype definition, which represents a broader phenomenon<sup>12</sup> and reflects the low specificity of a *BCR-ABL1*-positive/-like gene expression signature.

Despite the limitations of our analysis of clinical parameters imposed by the small size of the individual subtypes, we do present two important observations. First, we show a strong association of *ZNF384r* with mixed, B-myeloid immunophenotype. Although this association had already been established,<sup>5,44-46</sup> here we demonstrate that *ZNF384r* ALL represents the majority of all consecutively diagnosed non-infant pediatric B-other B-myeloid MPALs (4 out of 7; 57%). Second, we show a significant association of *DUX4r* ALL with worse early response to therapy. This ALL subtype is now often considered equivalent to *ERGdel*-positive ALL. However, an important proportion of *DUX4r* ALL lacks the *ERGdel*. We have shown previously that despite the association of *ERGdel* with worse early therapy response, this did not translate into worse outcome in the MRD-based BFM ALL 2000 protocol.<sup>31</sup> Although two recent studies reported a favorable outcome of *DUX4r* ALL,<sup>2,4</sup> its potential heterogeneity with respect to *ERGdel* has still not been studied. Thus, the outcome of *DUX4r* ALL in BFM studies, both in general and stratified by *ERGdel*, still remains to be addressed.

Previous studies demonstrated the frequent occurrence and remarkable diversity of *PAX5* aberrations in childhood BCP ALL.<sup>9,47-49</sup> We focused here on three types of *PAX5* aberrations that, in our study, were mutually exclusive with established ALL subtypes and altogether represented a large proportion of B-other ALL (20%). Moreover, we suggest here that one of them, the *PAX5* P80R mutation, likely represents a primary lesion that defines a novel biological ALL subtype, whose existence and character, however, remain to be confirmed and defined in detail by future studies. Among *PAX5* fusions, we identified four novel *PAX5* fusion partners further broadening an already sizeable *PAX5* recombinome.<sup>50</sup> Although the *PAX5* fusions in our study lacked a specific gene expression signature, which questions their potential to define a unique biological subset, with respect to their frequency (which exceeds that of some already established subtypes) future efforts to evaluate their clinical impact are to be expected. Interestingly, the patients with *PAX5* fusions included in our study had a significantly better early treatment response compared to the other patients; none was stratified as high risk (*Online Supplementary Table S10*).

*IKZF1*<sup>plus</sup> genotype has been recently described to confer poor outcome (30-40% 5-year event-free survival) in non-standard risk patients on the ALL BFM 2000 study.<sup>17</sup> We have identified 14 *IKZF1*<sup>plus</sup>-positive patients, representing 14% of B-other ALLs. Because *BCR-ABL1*-like ALL is enriched for *IKZF1del* and *PAR1* deletion (included in the *IKZF1*<sup>plus</sup> definition), it has been assumed that *IKZF1*<sup>plus</sup> and *BCR-ABL1*-like cases may largely overlap, although this has still not been demonstrated. In our study, 57% of patients with the *IKZF1*<sup>plus</sup> genotype were classified as *BCR-ABL1*-like. Thus, although *IKZF1*<sup>plus</sup> was significantly enriched in *BCR-ABL1*-like ALL, a large proportion of *IKZF1*<sup>plus</sup>-positive patients were still not classified into this subtype. Only 2 out of 14 *IKZF1*<sup>plus</sup>-positive ALLs were stratified into standard risk, both belonging to the B-rest subset. Larger cohorts will be needed to determine whether a refined ALL subtype classification could help to

further specify the prognostic impact of *IKZF1*<sup>plus</sup> and explain its absence in standard risk patients.<sup>17</sup>

One of the most striking findings of our study is the low frequency of kinase/cytokine receptor gene fusions other than those involving *CRLF2*. Except for *CRLF2* fusions and a single case with ABL-class fusion, no other kinase-activating fusions were detected among all B-other patients diagnosed in the Czech Republic over a 7-year period. The low frequency of non-*CRLF2* fusions, supported also by the result of the Swedish study,<sup>2</sup> seems to contrast with some American genomic studies;<sup>21,30</sup> however, a direct comparison of frequencies between the European and American populations is not possible at the moment, as only the two European studies refer to consecutive, unselected cohorts. Considering the low costs and the excellent performance of flow cytometry in detecting *CRLF2*-positive patients, it remains questionable whether any benefit can be gained from testing the *BCR-ABL1*-like signature in all B-other ALL patients (irrespective of their treatment response) in order to identify patients with a higher probability of having druggable kinase/cytokine receptor gene fusions. Instead, identification of targetable lesions (guiding the choice of appropriate drug) can be performed in patients with poor response to treatment upfront and irrespective of potential presence/absence of *BCR-ABL1*-like phenotype.

In our study, we also aimed to assess the feasibility and benefit of incorporating genome-wide technologies into routine diagnostics. Thanks to centralization in a single laboratory, we successfully standardized all processes and minimized the time to integrate results of all analyses. Although we see some benefit in performing all three genome-wide analyses (SNP<sub>a</sub>, RNA-sequencing, WES) to obtain the most complex genomic profile, considering the cost-benefit ratio, we found the combination of SNP<sub>a</sub> and RNA-sequencing to be the most efficient. This combination enables the therapeutically most relevant aberrations (*IKZF1*<sup>plus</sup> and kinase-activating lesions) to be detected.

Moreover, RNA-sequencing enables patients to be classified into novel subtypes; this would not be possible with targeted approaches, which are methodologically and analytically less demanding and possibly cheaper. However, these advantages fade with the increasing number of aberrations that need to be tested and, in addition, undescribed/private, but druggable aberrations, cannot always be detected. Furthermore, the complex data gained through genome-wide methods are extremely valuable for retrospective discovery/validation analyses. Nevertheless, it should be noted that genome-wide methods also have additional limitations (e.g. lower sensitivity in fusion transcript detection than PCR), and these need to be considered and tested during the implementation process.

In conclusion, our study shows unbiased European population-based frequency of novel B-other ALL subtypes, recurrent (cyto)genetic aberrations as well as their mutual associations, some of which have not yet been reported. We believe that these findings not only help to strengthen and deepen the current knowledge of B-other ALL, but also provide an objective basis on which other groups/countries can decide how to optimize their current diagnostic routine.

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