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ORIGINAL ARTICLE

Deletions of *IKZF1* and *SPRED1* are associated with poor prognosis in a population-based series of pediatric B-cell precursor acute lymphoblastic leukemia diagnosed between 1992 and 2011

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Despite the favorable prognosis of childhood acute lymphoblastic leukemia (ALL), a substantial subset of patients relapses. As this occurs not only in the high risk but also in the standard/intermediate groups, the presently used risk stratification is suboptimal. The underlying mechanisms for treatment failure include the presence of genetic changes causing insensitivity to the therapy administered. To identify relapse-associated aberrations, we performed single-nucleotide polymorphism array analyses of 307 uniformly treated, consecutive pediatric ALL cases accrued during 1992–2011. Recurrent aberrations of 14 genes in patients who subsequently relapsed or had induction failure were detected. Of these, deletions/uniparental isodisomies of *ADD3*, *ATP10A*, *EBF1*, *IKZF1*, *PAN3*, *RAG1*, *SPRED1* and *TBL1XR1* were significantly more common in B-cell precursor ALL patients who relapsed compared with those remaining in complete remission. In univariate analyses, age ($\geqslant$10 years), white blood cell counts ($>100\times10^9$/l), t(9;22)(q34;q11), *MLL* rearrangements, near-haploidy and deletions of *ATP10A*, *IKZF1*, *SPRED1* and the pseudoautosomal 1 regions on Xp/Yp were significantly associated with decreased 10-year event-free survival, with *IKZF1* abnormalities being an independent risk factor in multivariate analysis irrespective of the risk group. Older age and deletions of *IKZF1* and *SPRED1* were also associated with poor overall survival. Thus, analyses of these genes provide clinically important information.

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Keywords: pediatric acute lymphoblastic leukemia; single-nucleotide polymorphism array analyses; relapse; *IKZF1*; *SPRED1*

INTRODUCTION

The cure rate for pediatric acute lymphoblastic leukemia (ALL) has improved dramatically during the past few decades, with disease-free survival at 5 years now exceeding 80% in most current treatment protocols.[1,2] However, the one-fifth who relapses still fares poorly, with overall survival (OS) rates of only approximately 30% despite aggressive chemotherapy and stem cell transplantation, indicating that the blasts at relapse are refractory to presently available therapeutic regimes in most instances.[1,3–5] Hence, it is vital to identify 'relapse-prone' clones already at the time of diagnosis in order to ensure proper risk stratification and treatment decisions. Today, several clinical and genetic factors are routinely used to stratify patients into different risk groups/treatment intensities, such as white blood cell (WBC) count, age and immunophenotypic and genetic features.[1,2,5,6] However, as relapses occur not only in the high-risk group but also in the standard and intermediate groups, these factors are suboptimal.

A number of studies aiming at elucidating the underlying mechanisms for relapse has compared genetic features in paired diagnostic and relapse samples using conventional cytogenetic,[7,8] clone-specific PCR,[9,10] fluorescence *in situ* hybridization (FISH)[11,12] and single-nucleotide polymorphism (SNP) array analyses,[4,13–17] revealing that the relapsed clones can be identical to the ones seen at diagnosis, display additional changes (clonal evolution) or harbor both additional, identical as well as fewer changes (evolution from a preleukemic/ancestral clone). The two latter evolution patterns indicate outgrowth of therapy-resistant minor subclones.[4,9–12,16,17] In addition, high-resolution genomic profiling has identified genes, for example, *CDKN2A*, *ETV6*, *IKZF1* and *EBF1*, that are more frequently deleted in samples from patients who subsequently relapse than in samples from those remaining in first complete remission (CR1).[4,13,14,18] However, whether these deletions serve as independent prognostic markers is still unclear.[13,14,18,19]

In the present study, SNP array analyses were performed on diagnostic, CR1, relapse and induction failure (IF) samples from pediatric ALL patients to identify copy number aberrations and uniparental isodisomies (UPDs) associated with relapse/IF. In a next step, all identified recurrent abnormalities were screened for in diagnostic samples from patients without relapse/IF to ascertain whether they may be associated with event-free survival (EFS) and/or OS in a large patient cohort uniformly treated according to the Nordic Society of Pediatric Hematology and Oncology (NOPHO) ALL protocols.

PATIENTS AND METHODS

Patient cohort

Between 1992 and 2011, 307 children/adolescents (<18 years) were diagnosed with ALL at the Departments of Pediatric Oncology and Hematology, Lund and Linköping University Hospitals, Sweden. The vast majority ($n=288$; 94%) were treated according to the NOPHO ALL 1992

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($n = 138$), 2000 ($n = 94$) or 2008 ($n = 56$) protocols.[2] The median age was 4 years, the male/female ratio 1.4 and the median WBC count 10.8×10^9/l (range 0.8–802). Of the 307 patients, 269 (88%) had B-cell precursor (BCP) ALL, 33 (11%) T-ALL, 4 (1.3%) mature B-cell ALL and 1 (0.3%) biphenotypic leukemia. All bone marrow/peripheral blood samples were analyzed using conventional chromosome banding and targeted analyses (reverse transcriptase–PCR, FISH or Southern blot analyses). Among the BCP ALL cases, 5.6% were positive for 11q23/*MLL* rearrangements (251 analyzed cases), 5.2% for t(1;19)(q23;p13) (*TCF3/PBX1*; $n = 212$), 2.7% for t(9;22)(q34;q11) (*BCR/ABL1*; $n = 219$) and 24% for t(12;21)(p13;q22) (*ETV6/RUNX1*; $n = 230$), and 70 (26%) cases were high hyperdiploid. None of the T-ALL or mature B-cell ALL cases analyzed was positive for any of these rearrangements. The single case with biphenotypic leukemia harbored an *MLL* rearrangement. Basic clinical and genetic features are provided in Supplementary Table S1.

Among the 307 patients, 60 (20%) relapsed, 44 (73%) of which were bone marrow relapses. In addition, 17 (5.5%) had IF (defined as >5% abnormal nuclei as ascertained by locus-specific FISH analyses at days 15 or 29). The median age of all the 77 patients with relapse/IF was 3.5 years (range 0–17), the male/female ratio 1.7 and the median WBC count 14.1×10^9/l (range 0.8–802). Sixty-seven (87%) of the patients had BCP ALL; the remaining 10 (13%) had T-ALL (Table 1).

The genetic subgroups represented among the 67 BCP ALL samples, obtained from patients with subsequent relapse/IF, comprised high hyperdiploidy (HeH, 51–67 chromosomes; 25%), t(12;21) (19%), *MLL* rearrangements (13%), normal karyotype (NK; 4.5%), t(9;22) (6.0%) and near-haploidy (23–29 chromosomes; 6.0%); the remaining cases (25%) had other abnormalities or were cytogenetic failures. Among the 10 T-ALL cases, 3 had NK, 4 harbored *CDKN2A* deletions and 3 carried various, non-recurrent abnormalities. The clinical and cytogenetic data of the 77 relapse/IF patients are given in Supplementary Table S2.

SNP array analysis

DNA was extracted, using standard methods, from bone marrow or peripheral blood cells at the time of diagnosis, CR1 and at relapse/IF. The analyses were performed using the HumanOmni1-Quid and Human1M-Duo array systems (Illumina, San Diego, CA, USA), covering >1 000 000 SNPs. The Genomestudio software 2011.1 (Illumina) was applied for analysis of copy number aberrations and UPDs; some samples were analyzed in build GRCh36.1, whereas the majority was analyzed in build GRCh37 (Supplementary Table S2). All recurrent aberrations detected were converted to GRCh37 coordinates (http://www.ensembl.org/Homo_sapiens/Info/Index) to find the smallest overlap. Imbalances seen in remission samples or that overlapped with copy number polymorphisms listed in the Database of Genomic Variants (http://projects.tcag.ca/variation/) were excluded from further analysis, and so were deletions corresponding to somatic rearrangements of the T-cell receptor and immunoglobulin loci.[16] A flowchart depicting the cases analyzed by SNP arrays is shown in Supplementary Figure S1.

Identification of recurrent gene targets and genomic imbalances

Gene targets (in all instances, deletions) and genomic imbalances, the latter comprising partial gains and losses not involving single genes, identified in diagnostic samples from at least two patients with subsequent relapse/IF were considered recurrent. To be denoted a gene target, at least one of the samples had to display a focal deletion involving only the specified gene. All recurrent aberrations were subsequently ascertained in the entire patient cohort without relapse/IF successfully analyzed by SNP arrays ($n = 170$).

Statistical analyses

The PASW Statistics 20 software for Windows (SPSS Inc., Chicago, IL, USA) was used for all analyses. The significance limit for two-sided *P*-values was set to <0.05. The frequencies of gene targets/genomic imbalances in diagnostic and relapse/IF samples were compared using the Wilcoxon signed-rank and two-tailed Fisher's exact probability tests. The 10-year (yr) probabilities of EFS (pEFS) and OS (pOS) in relation to clinical (sex, age, risk group and WBC count) and genetic features (modal chromosome number, cytogenetic subgroup and gene targets/genomic imbalances) were calculated using the Kaplan–Meier method for patients treated only according to the NOPHO ALL 1992 and 2000 protocols, as these protocols were virtually identical with regards to risk group assignment and therapy (the study cohorts are shown in Supplementary Figure S2). Multivariate

Table 1. Clinical and genetic features of the 77 ALL cases with relapse/induction failure

Clinical features	N *(%)*	*Genetic features*	N *(%)*
Sex		*Subgroup*	
Female	29 (38)	HeH/(51–67 chromosomes)	17 (22)
Male	48 (62)	NH/(23–29 chromosomes)	4 (5.2)
Age (years)		t(1;19)(q23;p13)/*TCF3-PBX1*	0 (0)
<1	8 (10)	t(9;22)(q34;q11)/*BCR-ABL1*[a]	4 (5.2)
1–9	52 (68)	11q23/*MLL* rearrangement[b]	9 (12)
10–17	17 (22)	t(12;21)(p13;q22)/*ETV6-RUNX1*	13 (17)
Immunophenotype		Normal karyotype	6 (7.8)
B lineage	67 (87)	Other	24 (31)
T lineage	10 (13)	*SNP array analysis of diagnostic samples*	
WBC count ($\times 10^9$/l)		Yes	58 (75)
0–9	31 (40)	No	12 (16)
10–49	15 (19)	Failure	7 (9.1)
50+	31 (40)	*SNP array analysis of remission samples*	
Events		Yes	33 (43)
R	60 (78)	No	41 (53)
BM	44 (73)	Failure	3 (3.9)
CNS	8 (13)	*SNP array analysis of R/IF samples*	
Testis	5 (8.3)	Yes	21 (34)
CNS + testis	2 (3.3)	No	46 (60)
Ovary	1 (1.7)	Failure	10 (6.5)
IF	17 (22)		
Alive			
Yes	40 (52)		
No	37 (48)		

Abbreviations: ALL, acute lymphoblastic leukemia; BM, bone marrow; CNS, central nervous system; HeH, high hyperdiploidy; IF, induction failure; *N*, number; NH, near-haploidy; R, relapse; SNP, single-nucleotide polymorphism; WBC, white blood cell. [a]Of the four cases with *BCR-ABL1* fusion, two had the P190 transcript and one had the P210 transcript; the type of transcript was unknown in one case. [b]Of the nine cases with *MLL* rearrangement, four had *MLL-AFF1* [t(4;11)(q21;q23)], three had *MLL-MLLT1* [t(11;19)(q23;p13.3)], one had *MLL-GAS7* [t(11;17)(q23;p13)] and one had an unknown partner gene.

analysis using a Cox's regression model was performed to identify genetic/clinical factors that had an independent impact on pEFS. The median observation time for patients in CR1 was 128 months (range 43–227 months). The investigation was approved by the Research Ethics Committee of Lund University, and informed consent was provided according to the Declaration of Helsinki.

RESULTS

SNP array findings in the paired diagnostic and relapse/IF samples

Among the 77 patients with subsequent relapse/IF (Table 1), 58 diagnostic and 21 relapse/IF samples could be successfully analyzed. The remaining samples could not be investigated due to lack of samples (12 diagnostic and 46 relapse/IF), failed SNP arrays (6 diagnostic and 5 relapse/IF) or presence of only non-malignant cells in the stored samples (1 diagnostic and 5 relapse/IF). In total, 17 paired diagnostic and relapse/IF samples (Nos. 1–17; Supplementary Table S2), 41 diagnostic samples without relapse/IF samples (Nos. 18–58) and 4 relapse samples without diagnostic samples (Nos. 59–62) were analyzed in this study.

At diagnosis, the median number of genomic imbalances per case was 7 (range 0–24), with 3 (range 0–11) duplications, 2 (range 0–12) losses (hemizygous/homozygous deletions as well as monosomies), 0 (range 0–20) whole-chromosome UPDs, 0 (range 0–2) partial UPDs (pUPDs) and 3 (range 0–20) structural changes, including pUPDs. The corresponding frequencies for the relapse/IF samples were 8 (range 0–27), 3 (range 0–11), 2 (range 0–17), 0 (range 0–20), 0 (range 0–2) and 3 (range 0–27). There were no significant differences in the total number of imbalances, duplications, whole-chromosome UPDs, pUPDs and structural changes between the paired samples, whereas losses were slightly more common at relapse/IF ($P = 0.048$).

Genetic evolution patterns in the paired diagnostic and relapse/IF samples

Seven (41%) of the 17 paired cases had identical genetic changes or harbored no genomic imbalances at diagnosis and relapse/IF. Six (35%) cases displayed additional changes at relapse/IF, indicating clonal evolution from the major diagnostic clone. An additional two (12%) cases showed clonal evolution, but in these cases the SNP array findings clearly showed that they evolved from a minor clone (subclone), and not from the major clone, as seen at diagnosis. The remaining two (12%) cases lacked some of the imbalances present at diagnosis and also exhibited novel aberrations at the time of relapse, suggesting the presence of ancestral clones from which the relapses evolved (Supplementary Tables S3 and S4).

Recurrent gene targets/genomic imbalances in diagnostic samples from patients who subsequently had relapse/IF

Fifty-eight diagnostic samples were informative in the SNP array analyses (Supplementary Figure S1 and Supplementary Tables S2 and S4). A total of 14 recurrent focal gene targets and 10 recurrent genomic imbalances were identified (Supplementary Table S5). Among these, the following were significantly more common in the relapse/IF group compared with the 170 successfully analyzed cases without such events: deletions/UPDs of *ADD3* (chr10: 111,775,607-111,830,275), *ATP10A* (chr15:26,033,764-26,093,401), *IKZF1* (chr7:50,418,242-50,422,230), *NR3C1* (chr5:142,781,168-143,058,402), *PAN3* (chr13:28,691,097-28,806,503), *SPRED1* (chr15: 38,403,504-38,558,017), del(6)(p22.2p22.2) (chr6:26,153,335-26, 167,951), del(16)(p13.3p13.3) (chr16:15,257,80-16,706,91), del(16) (q22.1q22.1) (chr16:67,473,040-67,879,400) and del(17)(p11. 2p13.2) (chr17:47,523,93-16,232,477). Eleven of the 58 patients who subsequently had relapse/IF harbored $\geqslant 2$ of these deletions.

The recurrent gene targets/imbalances differed between the T-ALL and BCP ALL cases. In T-ALL, the only recurrent changes were deletions/pUPDs of *CDKN2A* (chr9:21,970,901-21,970,989), *PAX5* (chr9:36,927,364-37,010,622) and del(13)(q14.2q14.2) (chr13: 49,015,957-49,070,345). The frequencies of these changes did not vary significantly between T-ALL patients with or without relapse/IF (Supplementary Table S6).

Among the BCP ALL cases with relapse/IF, the following changes were recurrent as well as significantly more frequent than in those without such events: deletions/UPDs of *ADD3*, *ATP10A*, *EBF1* (chr5:158,366,263-158,502,378), *IKZF1*, *NR3C1*, *PAN3*, *SPRED1* and *TBL1XR1* (chr3:176,911,471-177,336,402) and deletion of the pseudoautosomal region 1 (PAR1) (chrX:14,011,68-15,666,46), del(6)(p22.2p22.2), del(16)(p13.3p13.3) and del(17)(p11.2p13.2). When focusing on only the 38 patients who relapsed, 8 target genes and 1 genomic imbalance were significantly more common than in those who did not relapse (Table 2).

The recurrent gene targets and genomic imbalances in cases who subsequently had relapse/IF also varied among the different BCP ALL cytogenetic subgroups (Supplementary Tables S7 and S8). None of the recurrent aberrations found in the HeH, near-haploid, t(9;22), der(11)(q23)/*MLL* and t(12;21) groups was more common in cases who subsequently relapsed. However, among the remaining BCP ALL cases, comprising those with NK, cytogenetic failure or other cytogenetic abnormalities, with relapse/IF a total of six different gene targets and two imbalances were recurrent: deletions/pUPDs of *ATP10A*, *CDKN2A*, *ETV6*, *IKZF1*, *PAX5* and *SPRED1* and deletion of PAR1 and del(6)(p22.2p22.2). Of these, deletions/pUPDs of *ATP10A*, *IKZF1*, *SPRED1* and PAR1

Table 2. Frequencies of recurrent gene targets/imbalances in the diagnostic BCP ALL samples from patients with relapse compared with patients without such an event

Gene targets/imbalances	*38 with R* N *(%)*	*146 without R* N *(%)*	P-*value*[a]
Gene targets[b]			
ADD3 (10q25.1)[c]	5 (13)	2 (1.4)	**0.005**
ATP10A (15q12)[c]	5 (13)	1 (0.7)	**0.002**
CDKN2A (9p21.3)[c]	11 (29)	36 (25)	0.677
EBF1 (5q33.3)[c]	6 (16)	4 (2.7)	**0.006**
ETV6 (12p13.2)[c]	8 (21)	33 (23)	1.000
IKZF1 (7p12.2)[c]	15 (39)	15 (10)	**<0.001**
PAN3 (13q12.2)[c]	4 (11)	3 (2.1)	**0.034**
PAX5 (9p13.2)[c]	13 (34)	36 (25)	0.232
RAG1 (11p12)[c]	5 (13)	5 (3.4)	**0.033**
SPRED1 (15q14)[c]	5 (13)	1 (0.7)	**0.002**
TBL1XR1 (3q26.31)[c]	5 (13)	5 (3.4)	**0.027**
Imbalances			
del(3)(q13.2q13.2)[d]	6 (16)	10 (6.8)	0.104
del(6)(p22.2p22.2)[d]	6 (16)	6 (4.1)	**0.015**
del(6)(q14.1q25.3)[d]	7 (18)	12 (8.2)	0.077
dup(8)(q24.21q24.21)[d]	3 (7.9)	3 (2.1)	0.104
del(13)(q14.2q14.2)[d]	5 (13)	10 (6.8)	0.199
PAR1 deletion[d]	5 (13)	6 (4.1)	0.051

Abbreviations: BCP ALL, B-cell precursor acute lymphoblastic leukemia; *N*, number; PAR1, pseudoautosomal region 1 on Xp/Yp; R, relapse. [a]*P*-values as ascertained by Fisher's exact test. Significant *P*-values are indicated in bold. [b]Focal and large deletions, whole chromosome losses (rare) and partial and whole chromosome uniparental isodisomies combined. [c]Smallest overlap: *ADD3* (chr10:111,775,607-111,830,275); *ATP10A* (chr15:26,033,764-26,093,401); *CDKN2A* (chr9:21,970,901-21,970,989); *EBF1* (chr5:158,366,263-158,502,378); *ETV6* (chr12:11,943,969-12,015,706); *IKZF1* (chr7:50,418,242-50,422,230); *PAN3* (chr13:28,691,097-28,806,503); *PAX5* (chr9:36,927,364-37,010,622); *RAG1* (chr11:36,481,900-36,608,273); *SPRED1* (chr15:38,403,504-38,558,017); and *TBL1XR1* (chr3:176,911,471-177,336,402). [d]Smallest overlap: chr3:112,069,766-112,208,750; chr6:26,153,335-26,167,951; chr6: 107,146,121-109,614,226; chr8:130,572,304-130,626,097; chr13:49,015, 957-49,070,345; and chrX:14,011,68-15,666,46.

Table 3. Frequencies of recurrent gene targets/imbalances in the diagnostic BCP ALL samples with normal karyotypes, cytogenetic failures or other cytogenetic abnormalities from patients with relapse compared with patients without such an event

Gene targets/imbalances	*10 with R* N *(%)*	*48 without R* N *(%)*	P-*value*[a]
Gene targets[b]			
CDKN2A (9p21.3)[c]	6 (60)	15 (31)	0.089
IKZF1 (7p12.2)[c]	7 (70)	7 (15)	**0.001**
PAX5 (9p13.2)[c]	6 (60)	16 (33)	0.112
Imbalances			
PAR1 deletion[d]	3 (30)	5 (10)	0.131

Abbreviations: BCP ALL, B-cell precursor acute lymphoblastic leukemia; *N*, number; PAR1, pseudoautosomal region 1 on Xp/Yp; R, relapse. [a]*P*-values as ascertained by Fisher's exact test. Significant *P*-values are indicated in bold. [b]Focal and large deletions, whole chromosome losses (rare) and partial uniparental isodisomies combined. [c]Smallest overlap: *CDKN2A* (chr9:21,970,901-21,970,989); *IKZF1* (chr7:50,418,242-50,422,230); and *PAX5* (chr9:36,927,364-37,010,622). [d]Smallest overlap: chrX:14,011,68-15,666,46.

were significantly more common than in cases without relapse/IF (data not shown). When considering only samples from patients who relapsed, three gene targets and PAR1 deletions were recurrent, but only *IKZF1* aberrations were significantly associated with relapse (Table 3).

When comparing specific gene aberrations between diagnostic and relapse/IF samples, *MSH6* deletions (chr2:47,857,914-48,035, 137) were only recurrent among the relapse/IF samples, *IKZF1* deletions at diagnosis were always preserved at relapse/IF and deletions of *BTG1* (chr12:92,278,448-92,537,956) and *NR3C1* were enriched at relapse/IF (as exemplified in Supplementary Figure S3).

Survival in relation to clinical and genetic features

Neither clinical variables (age, WBC count and gender) nor recurrent genetic abnormalities were significantly associated with pEFS or pOS among the 23 T-ALL patients treated according to the 1992 and 2000 NOPHO protocols.

Among the 209 BCP ALL patients, age <10 years and WBC counts $<100\times10^9$/l were significantly associated with superior 10-yr pEFS (Figures 1a and b), whereas only age <10 years was associated with favorable 10-yr pOS ($P=0.004$). Gender had no impact on pEFS or pOS (data not shown). Modal chromosome numbers showed no significant associations with pEFS or pOS, and there were no pEFS and pOS differences between the cases with ($n=169$) or without ($n=40$) known modal numbers (data not shown). Among the cytogenetic subgroups, cases with t(9;22), *MLL* rearrangements and near-haploidy had an inferior 10-yr pEFS compared with those with HeH, t(12;21), t(1;19), NK or other cytogenetic abnormalities (Figure 1c). However, this did not translate into differences with regard to pOS (data not shown). Eighty-two (39%) BCP ALL cases were grouped as standard risk, 83 (40%) as intermediate risk and 44 (21%) as high risk; these groups differed significantly with regard to 10-yr pEFS (Figure 1d) but not pOS ($P=0.059$).

SNP array analyses were successfully performed on 145 (69%) of the 209 BCP cases treated according to the 1992 and 2000 NOPHO protocols; there were no differences in the 10-yr pEFS (73 vs 77%, $P=0.724$) or the 10-yr pOS (85 vs 87%, $P=0.797$) between those analyzed or not. Among the gene targets/imbalances recurrent in patients who relapsed (Table 2), deletions of *ATP10A*, *IKZF1*, *SPRED1* and PAR1 were significantly associated with decreased pEFS rates (Figure 2). Deletions of *IKZF1* and *SPRED1* were also significantly associated with poor pOS (Figure 3).

IKZF1 deletions are an independent risk factor for decreased EFS irrespective of risk group assignment

Multivariate Cox regression analyses revealed that deletions of *IKZF1* were the strongest independent risk factor for inferior pEFS in BCP ALL when taken into consideration age, WBC counts, the cytogenetic subgroups NK, HeH, t(1;19), der(11)(q23)/*MLL*, t(12;21) and cytogenetic failures/other cytogenetic abnormalities, risk group assignment and PAR1 deletions (Table 4). *IKZF1* was still an independent risk factor when including t(9;22), near-haploidy and deletions of *ATP10A* and *SPRED1* (significantly associated with EFS in univariate analyses; Figure 2).

IKZF1 deletions were more common in the high-risk group (Supplementary Table S9; $P=0.001$), but *IKZF1* status was also the strongest risk factor for pEFS among the standard- and intermediate-risk groups. In contrast, the *IKZF1* status was not associated with pOS; the only parameter associated with pOS in the multivariate analysis was age >10 years (data not shown).

DISCUSSION

The present large-scale SNP array analysis of a uniformly treated pediatric ALL patient cohort was undertaken for four main reasons: (i) to identify gene targets and genomic imbalances of importance for the leukemogenic process; (ii) to analyze the clonal relationship between diagnostic and relapse samples; (iii) to ascertain genetic changes that are more prevalent at diagnosis in patients with subsequent relapse/IF and hence possibly important for treatment failure; and (iv) to pinpoint genetic aberrations that confer a significant prognostic impact in unselected pediatric ALL patients. Although such issues have been addressed previously, the current study provides additional and novel data pertaining to the four above-mentioned goals. First, we investigated a population-based series, not focusing solely on high-risk ALL,[4] T-ALL,[15] specific cytogenetic subgroups in BCP ALL[16,17] or excluding some subgroups.[13] Also, none of our patients was lost to follow-up in contrast to several previous studies, and for some of our cases the observation time was close to 20 years with a median follow-up of 10 years, whereas the maximum follow-up time has been $\leqslant10$ years in earlier studies,[14,17,18,20,21] (Figures 1 and 2). Furthermore, the SNP arrays used provide higher resolution (>1 M SNPs) compared with previous SNP array-based ALL studies (250 K–500 K),[4,13–18] making it possible to delineate imbalances in greater detail and to identify previously unknown gene targets, as exemplified below.

Among the recurrent gene targets (Table 2), focal deletions of *SPRED1* have never been reported before. The reason for this may partly be due to the smaller number of samples analyzed in most of the previous studies, because when reviewing the Supplementary Data from published SNP array analyses of ALL,[4,13,14,16] we identified only one relapse sample, reported by Mullighan *et al.*,[4] with a deletion including *SPRED1* among other genes; this is the only previous study comprising a similar number of patients as our study. Furthermore, it is possible that the higher resolution of our SNP arrays could be an additional reason, as two of our *SPRED1* deletions were small focal deletions that could have escaped detection in previous studies. SPRED1 is highly expressed in hematopoietic cells and acts as a negative regulator of RAS/RAF/MAPK (mitogen-activated protein kinase). Germline loss-of-function mutations of the *SPRED1* result in increased RAS-MAPK signal transduction and cause Legius syndrome, a disorder that displays a 'mild' neurofibromatosis type 1 phenotype and that may be associated with acute myeloid leukemia.[22,23] *SPRED1* has previously not been implicated in ALL but mutations of other RTK-RAS genes, such as *FLT3*, *KRAS*, *NRAS* and *PTPN11*, have been reported to be enriched at ALL relapses.[16,24]

The only genomic imbalance significantly associated with relapse (Table 2) was del(6)(p22.2p22.2), involving the histone

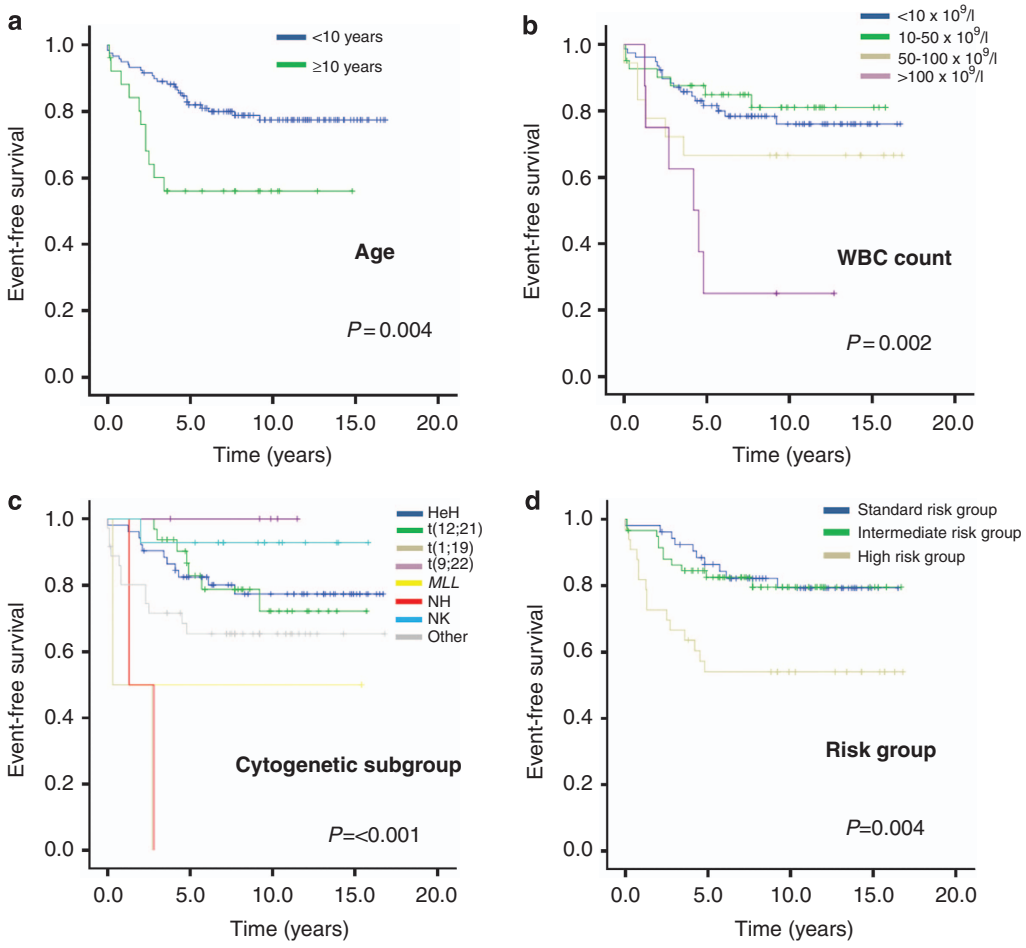

Figure 1. Kaplan–Meyer estimates of EFS of the 209 BCP ALL patients in relation to clinical and cytogenetic features. The 10-yr pEFS in the total patient cohort enrolled in the NOPHO ALL 1992 and 2000 treatment protocols was 0.75 (s.e. 0.03) (not shown in the plots). (**a**) Age <10 years: 10-yr pEFS 0.78 (s.e. 0.04) vs age $\geqslant 10$ years: 0.56 (0.10). (**b**) WBC count $<10 \times 10^9$/l: 10-yr pEFS 0.76 (0.05) vs 10–50 $\times 10^9$/l: 0.81 (0.07) vs 51–100 $\times 10^9$/l: 0.67 (0.11) vs $>100 \times 10^9$/l: 0.25 (0.15). (**c**) Cytogenetic subgroups HeH: 10-yr pEFS 0.77 (0.06) vs t(12;21): 0.72 (0.10) vs t(9;22): 0.00 (0.00) vs t(1;19): 1.00 vs *MLL* rearrangement: 0.50 (0.35) vs near-haploidy (NH): 0.00 (0.00) vs NK: 0.93 (0.07) vs other (comprising other cytogenetic abnormalities and cytogenetic failures): 0.65 (0.08). (**d**) Risk groups standard: 10-yr pEFS 0.79 (0.06) vs intermediate: 0.80 (0.06) vs high: 0.54 (0.09).

genes *HIST1H2BD* and *HIST1H1E* in the smallest overlapping region. HIST1H2BD and HIST1H1E are part of the nucleosome structure of the chromosomal fiber and essential for cytokinesis.[25] Although these genes have not been reported to be deleted in ALL or otherwise associated with this disease, the observed 6p imbalances overlap to some extent with 6p deletions reported in Down syndrome-associated ALL and other histone clusters on 6p have been shown be deleted in pediatric ALL, with methylation arrays suggesting that histone deletions are associated with methylation alterations.[26,27] Thus, *HIST1H2BD* and *HIST1H1E* may be added to the growing list of acute leukemia-associated genes, for example *DNMT3*, *EZH2*, *HOX* family and *MLL*, that contribute to the leukemogenic process through deregulated CpG methylation or histone modification.[28–31]

Among the other recurrent gene targets, most—*CDKN2A*, *EBF1*, *ETV6*, *IKZF1*, *PAX5*, *RAG1* and *TBL1XR1*—have been thoroughly discussed previously.[17,19,27,32,33] However, three of the presently identified gene targets, *ADD3*, *ATP10A* and *PAN3*, have been less emphasized in previous studies. ADD3 has an important role in the skeletal organization of the cell membrane in erythrocytes,[34] ATP10A is an aminophospholipid translocase responsible for transporting amphipathic molecules,[35] and PAN3 is involved in degradation of poly(A) tails in cytoplasmic mRNA.[36] Both *ADD3* and *PAN3* have been reported to have a role in ALL—*ADD3* is a *NUP98* partner in T-ALL[37] and significantly associated with a gene expression cluster group with poor outcome in high-risk BCP ALL,[38] and *PAN3* is recurrently deleted in HeH ALL.[39] Previous to this report, deletions of *ATP10A* have not been clearly associated with ALL. However, when reviewing Supplementary Data published by Mullighan *et al.*,[4] we identified two high-risk ALL cases with deletion of this gene.

It is worthy of note that *PAN3* was the only gene target specifically associated with a particular cytogenetic subgroup—all *PAN3* deletions were identified in the HeH subgroup. The other gene targets were either involved in at least two cytogenetic subgroup, such as *ADD3*, *SPRED1* and *RAG1* in HeH- and t(12;21)-positive BCP ALLs, or, as *CDKN2A* and *PAX5*, in all BCP ALL subtypes, strongly suggesting that different cytogenetic subgroups evolve through similar co-operative submicroscopic changes.

The analyses of the paired samples revealed that the relapse/IF clones, in relation to those present at diagnosis, were identical, displayed clonal evolution or reflected evolution from an ancestral clone, that is, a preleukemic clone from which both the diagnostic and the relapse clones evolve; the latter clones may hence harbor both additional and identical, as well as fewer, changes when

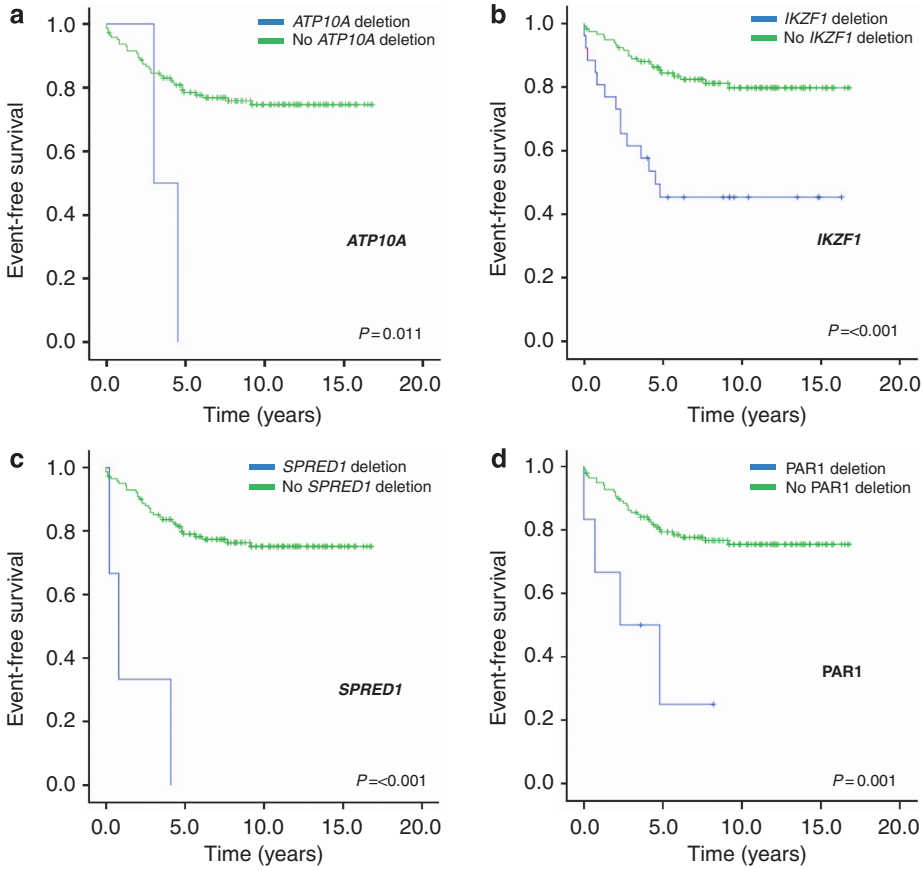

Figure 2. Kaplan–Meyer estimates of EFS of the 145 BCP ALL cases analyzed by SNP arrays. The 10-yr pEFS of the analyzed patient cohort enrolled in the NOPHO ALL 1992 and 2000 treatment protocols was 0.73 (0.04) (not shown in the plots). In each plot, the curves representing gene targets and imbalances are denoted in blue and those representing unaffected gene targets/imbalances in green. (**a**) *ATP10A*: 10-yr pEFS 0.00 (0.00) vs 0.75 (0.04). (**b**) *IKZF1*: 10-yr pEFS 0.45 (0.10) vs 0.80 (0.04). (**c**) *SPRED1*: 10-yr pEFS 0.00 (0.00) vs 0.75 (0.04). (**d**) Pseudoautosomal region 1 (PAR1) on Xp/Yp: 10-yr pEFS 0.25 (0.20) vs 0.75 (0.04).

compared (Supplementary Table S3).[4] This agrees well with previous immunogenotypic, FISH and cytogenetic studies of ALL.[7-12] However, in contrast to previous SNP array-based analyses,[4,13,14] we identified fewer clones arising from ancestral clones and more identical clones at the time of the event. This may partly be due to the fact that we also analyzed IF samples, of which 3 of the 4 showed identical clones, indicating that the time to this event is too short for evolution to occur. In fact, when considering only the cases with relapse, 4 of the 13 showed identical clones at diagnosis and relapse, well in agreement with Kawamata *et al.*[14] who reported identical clones in 4 of the 14 cases. Furthermore, the higher frequencies of evolution from ancestral clones reported by Mullighan *et al.*[4] and Yang *et al.*[13] may partly be due to the fact that they either only studied high-risk cases or included a larger number of such cases, as both high-risk cases analyzed in our diagnosis/relapse cohort displayed clonal evolution from ancestral clones. Nor did we observe any significant frequency differences with regard to genomic imbalances/UPDs between diagnostic and relapse/IF samples, as previously reported,[4,13,16] apart from a slightly increased frequency of deletions at relapse. This may be due to the fact that we investigated a consecutive series of patients as similar findings were also reported in a previous population-based study.[18] It should be stressed, however, that the present results do not refute that specific subgroups, such as HeH and t(12;21), may harbor more changes at relapse.[16,17]

Gene targets overrepresented in, or even unique for, relapse samples are most likely associated with treatment resistance and hence important to identify already at the time of diagnosis—perhaps being present only in a minor diagnostic subclone[40]—in order to adjust treatment accordingly. We identified deletions of *MSH6*, *BTG1* and *NR3C1* to be enriched at relapse. MSH6 is a critical component of the DNA mismatch repair system, and it has been shown that decreased *MSH6* expression is associated with relapse and drug insensitivity in childhood BCP ALL.[13] BTG1 is antiproliferative and acts as a cofactor involved in transcriptional regulation, mRNA turnover, and histone modification.[41] *BTG1* deletions have retrospectively been found in subclones present at diagnosis that subsequently became the major clones at relapse,[42] and loss of *BTG1* expression causes glucocorticoid resistance in ALL cell lines.[43] Although requirements of glucocorticoid receptors for glucocorticoid-induced response is well known, acquired aberrations of the glucocorticoid receptor-encoding gene *NR3C1* have previously been considered a rare contributor to ALL relapse.[44] However, our findings add to a previous study showing *NR3C1* deletions to be enriched in relapse samples from BCP ALL patients.[4] Furthermore, *NR3C1* abnormalities initially observed at relapse have retrospectively been detected in subclones at diagnosis, something that most likely contributes to the glucocorticoid resistance in such cases.[40] Thus, taken together, aberrations of *MSH6*, *BTG1* and *NR3C1* may influence treatment response.

In recent years, a few genetic changes in T-ALL have been shown to correlate with outcome, such as aberrant expression of *TAL1*, *LYL1* and *TLX3*.[45] However, for the majority of aberrations identified in T-ALL, either no such impact is seen or remains to be

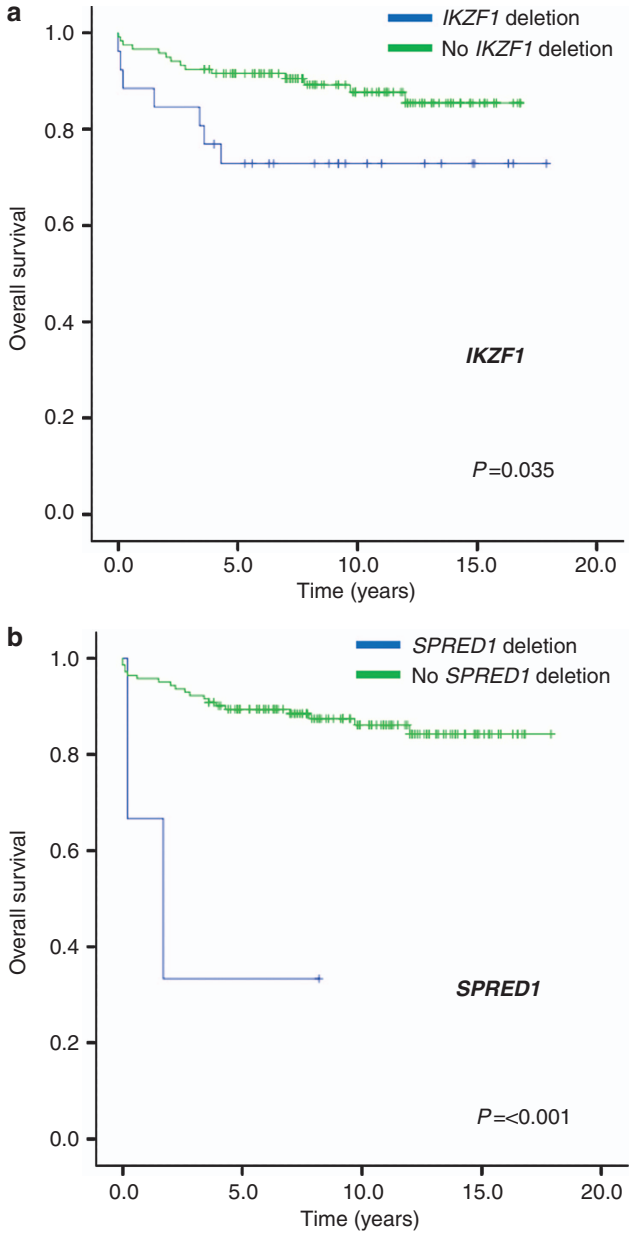

Figure 3. Kaplan–Meyer estimates of OS of the 145 BCP ALL cases analyzed by SNP arrays. The 10-yr pOS in the analyzed patient cohort enrolled in the NOPHO ALL 1992 and 2000 treatment protocols was 0.85 (0.03) (not shown in the plots). In each plot, the curves representing gene targets and imbalances are denoted in blue and those representing unaffected gene targets/imbalances in green. (**a**) *IKZF1*: 10-yr pOS 0.73 (0.09) vs 0.88 (0.03). (**b**) *SPRED1*: 10-yr pOS 0.33 (0.27) vs 0.86 (0.03).

elucidated. In fact, to the best of our knowledge, no T-ALL-associated genetic changes are at present used in clinical routine to stratify patients into different risk groups. So, perhaps not surprisingly, we were unable to pinpoint any gene targets or genomic imbalances that could be used for prognostication and thus treatment stratification of T-ALL in general.

In contrast to T-ALL, several clinical and genetic features were associated with outcome of BCP ALL, with the following significantly associated with a poor 10-yr EFS in univariate analyses: age $\geqslant$10 years, WBC counts $\geqslant 100 \times 10^9$/l and the presence of *BCR/ABL1*, *MLL* rearrangement and near-haploidy (Figure 1). Furthermore, three gene targets and one genomic

Table 4. Multivariate Cox regression analyses of EFS[a]

Risk factor	N	P-*value*[b]	*HR*	*10-yr pEFS*
WBC count ($\times 10^9$/l)		0.218	1.58	
$<$10	78			0.76
10–50	39			0.81
50–100	17			0.67
$>$100	7			0.25
Cytogenetic subgroup		0.618	1.88	
HeH	52			0.77
t(12;21)	32			0.72
t(1;19)	5			1.00
MLL	2			0.50
NK	14			0.93
Other	36			0.65
IKZF1		**0.010**	3.11	
Yes	25			0.45
No	116			0.80
PAR1 deletion		**0.036**	4.26	
Yes	6			0.25
No	135			0.75
Risk group		**0.049**	0.18	
Standard	53			0.79
Intermediate	58			0.80
High	30			0.54
Age (0–16) years		**0.017**	0.23	
$\leqslant$10	116			0.78
$>$10	25			0.56

Abbreviations: EFS, event-free survival; HeH, high hyperdiploidy (51–67 chromosomes); HR, hazard ratio; *N*, number; NK, normal karyotype; PAR1, pseudoautosomal region 1 on Xp/Yp; WBC, white blood cell. [a]Based on 141 cases (cases with t(9;22) or near-haploidy are excluded). [b]*P*-values as ascertained by Fisher's exact test. Significant *P*-values are indicated in bold.

imbalance were also significantly associated with a poor 10-yr EFS, namely *ATP10A*, *IKZF1*, *SPRED1* and PAR1 deletions (Figure 2). Deletions of *EBF1* showed a trend towards conferring decreased 10-yr EFS ($P = 0.055$). EBF1 is a transcription factor that has a central role in the development of normal B-cells, and aberrations of this gene are clearly involved in the leukemogenic process.[13,27,46]

High age and deletions of *IKZF1* and *SPRED1* were also significantly associated with a poor 10-yr OS (Figure 3). The present finding that deletions of *SPRED1* are recurrent and that they provide a negative prognostic impact in BCP ALL is clinically important, not least considering that SPRED1 is part of MAPK signaling and that inhibitors of this pathway currently are undergoing clinical trials and hence may be novel therapeutic options.[24] The multivariate analyses strongly indicated that *IKZF1* deletions were the strongest independent risk factor for poor outcome (Table 4). The transcription factor IKZF1 is essential for B-cell development, with loss of *IKZF1* leading to arrest of lymphoid differentiation.[46,47] *IKZF1* abnormalities were initially associated with an inferior outcome of high-risk BCP ALL only,[21,48] but the present data and two previous studies[18,20] clearly show that *IKZF1* aberrations also predict poor outcome in the standard- and intermediate-risk groups. In our series, deletions/pUPDs of *IKZF1* were the only genetic change significantly associated with relapse in cases without any known risk-stratifying aberrations (Table 3), as also reported in a previous study.[21] Furthermore, we and others[13,18] show that all *IKZF1* lesions at diagnosis are preserved at relapse, again supporting the poor prognostic impact of *IKZF1* aberrations.

Deletions involving the PAR1 region, associated with deregulation of *CRLF2*,[49,50] were also a significant independent risk factor (Table 4). However, all samples with PAR1 deletions from patients with subsequent relapse/IF also harbored *IKZF1* deletions, whereas

all PAR1 deletion-positive cases without *IKZF1* abnormalities remain in CR1 (Supplementary Table S10), again indicating that *IKZF1* is the most significant factor for inferior EFS. Although *SPRED1* deletions co-occurred with *IKZF1* changes (Supplementary Table S10) and the number of cases is small, it does not negate an important prognostic impact of *SPRED1* in BCP ALL, not least as all cases with *SPRED1* deletions relapsed or had IF. Thus, we suggest that analyses of *IKZF1* and *SPRED1* at diagnosis provide clinically important information and that the *IKZF1* status should be risk stratifying in future study protocols, in agreement with a recent study,[51] whereas further investigations of *SPRED1* aberrations are needed to elucidate their independent prognostic impact.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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