

# ***IKZF1* deletion is an independent predictor of outcome in pediatric acute lymphoblastic leukemia treated according to the ALL-BFM 2000 protocol**

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## ABSTRACT

*IKZF1* gene deletions have been associated with a poor outcome in pediatric precursor B-cell acute lymphoblastic leukemia. To assess the prognostic relevance of *IKZF1* deletions for patients treated on Berlin-Frankfurt-Münster Study Group trial ALL-BFM 2000, we screened 694 diagnostic acute lymphoblastic leukemia samples by Multiplex Ligation-dependent Probe Amplification. Patients whose leukemic cells bore *IKZF1* deletions had a lower 5-year event-free survival ( $0.69 \pm 0.05$  vs.  $0.85 \pm 0.01$ ;  $P < 0.0001$ ) compared to those without, mainly due to a higher cumulative incidence of relapses ( $0.21 \pm 0.04$  vs.  $0.10 \pm 0.01$ ;  $P = 0.001$ ). Although *IKZF1* deletions were significantly associated with the *P2RY8-CRLF2* rearrangement, their prognostic value was found to be independent from this association. Thus, *IKZF1* deletion is an independent predictor of treatment outcome and a strong candidate marker for integration in future treatment stratification strategies on ALL-BFM protocols.

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## Introduction

Approximately 20% of children with acute lymphoblastic leukemia (ALL) still suffer from relapse and may benefit from improved risk stratification and adapted treatment strategies. In this context, the influence of *IKZF1* gene aberrations on therapeutic outcome has received much attention in recent investigations. *IKZF1* encodes for Ikaros, a zinc-finger transcription factor family member, which functions sequence-specific in transcriptional regulation and chromatin remodeling and is required for the development of all lymphoid lineages.<sup>1-3</sup> Somatic aberrations of *IKZF1* have recurrently been observed in precursor B-cell ALL (pB-ALL), most frequently in those carrying a *BCR/ABL1* rearrangement, and have been shown to confer a poor treatment outcome.<sup>4-15</sup> However, aberrations of *IKZF1* were also found to associate with additional genetic alterations recurrently observed in childhood ALL, such as *JAK* mutations and translocations involving the chemokine receptor like factor 2 (*CRLF2*) gene.<sup>4,7,14-18</sup>

Juxtaposition of *CRLF2* to the *IgH@* enhancer or the *P2RY8* promoter leads to elevated expression of wild-type *CRLF2* in ALL and has been shown to exert a negative prognostic impact on outcome.<sup>17,18</sup> However, the prognostic relevance seems to be treatment-dependent and it is not clear yet whether the prognostic impact of *IKZF1* is independent of these concurrent lesions.<sup>19</sup> In the present study, we assessed

the prognostic role of *IKZF1* deletions in a large cohort of 694 pediatric ALL patients treated according to the ALL-BFM 2000 protocol.

## Design and Methods

### Patients

In accordance with institutional review board regulations, clinical samples were obtained from children with ALL before treatment. The study was approved by the institutional review board of the Hannover Medical School, Hannover, Germany, and informed consent obtained from patients and/or their legal guardians in accordance with the Declaration of Helsinki. Diagnostics, risk group assignment, and treatment were performed according to the ALL-BFM 2000 protocol.<sup>20-22</sup> Risk group stratification (standard risk, SR; intermediate risk, IR; high risk, HR) was mainly based on minimal residual disease (MRD) analysis after induction (time point 1, TP1) and consolidation therapy (TP2).<sup>20</sup> SR patients were MRD-negative on both TP and HR patients had MRD levels of  $10^{-3}$  or over at TP2. MRD-IR patients had positive MRD detection at either one or both time points but at a level of less than  $10^{-3}$  at TP2. Patients with prednisone poor-response (PR;  $\geq 1000$  leukemic blood blasts/ $\mu$ L on treatment Day 8) or induction failure ( $\geq 5\%$  leukemic blasts in the bone marrow, BM) or positivity for chromosomal translocations  $t(9;22)(q34;q11)$  or  $t(4;11)(q21;q23)$  or their molecular equivalents (*BCR/ABL1* or *MLL/AF4* rearrangement) were stratified into the HR group, independent of MRD.

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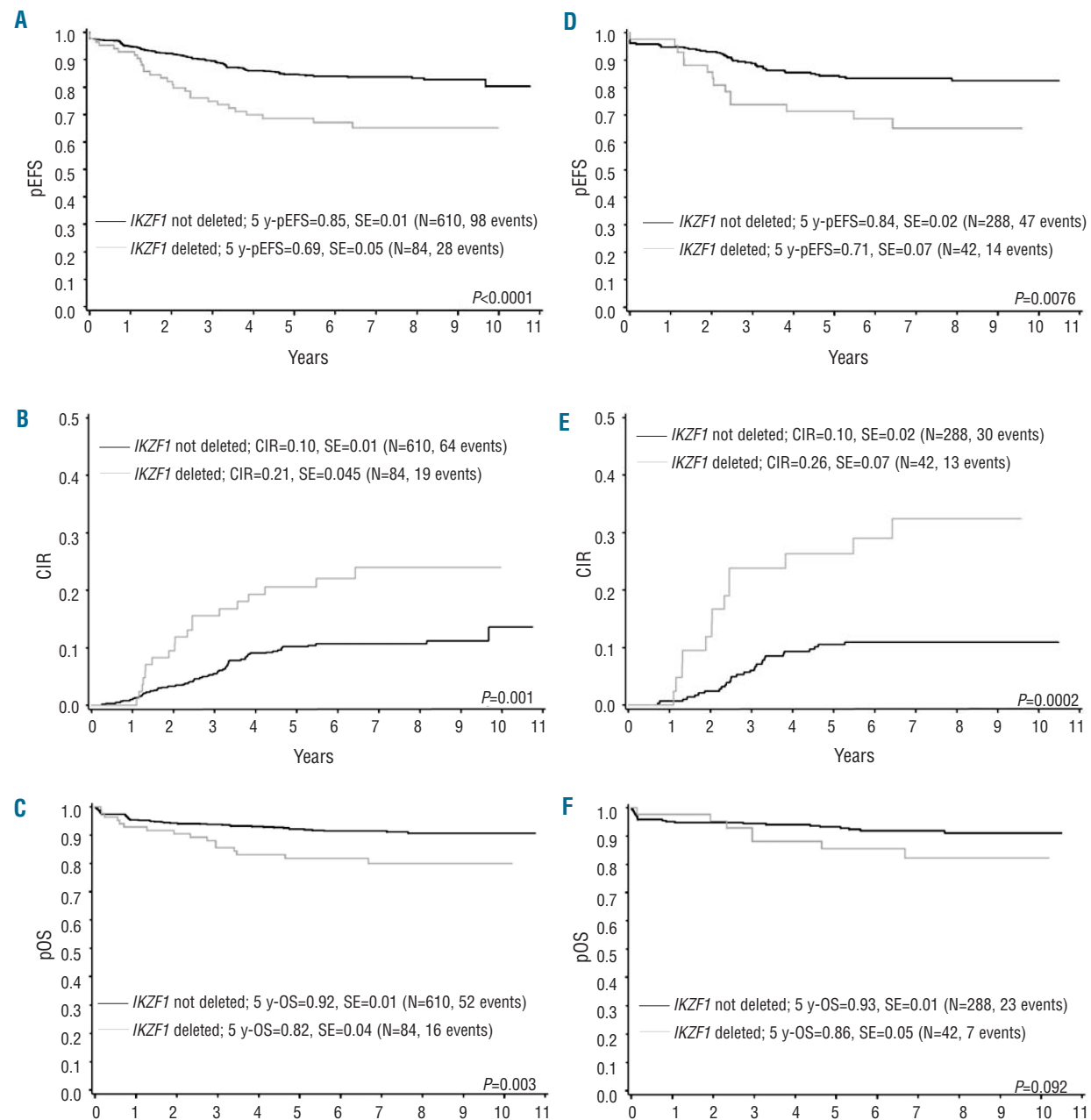
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Consecutively enrolled patients from the ALL-BFM 2000 study population with sufficient spare leukemic DNA available were included in our present study (*Online Supplementary Table S1*). BM or peripheral blood specimens had to contain more than 80% blasts, as assessed morphologically before gradient centrifugation.

### Multiplex ligation-dependent probe amplification (MLPA) analysis

Recently, it was shown that MLPA is ideal for rapid high-throughput testing of large cohorts with a view to establish incidence and prognostic significance of certain gene aberrations.<sup>23</sup> MLPA analysis was performed using the MLPA SALSA kit P335-

A3 ALL-*IKZF1* (MRC-Holland, Amsterdam, The Netherlands; [www.mlpa.com](http://www.mlpa.com)) according to the manufacturer's protocol. The assay includes probes for each of the eight exons of the *IKZF1* gene and is able to detect deletions of the whole gene as well as all types of focal intragenic deletions. Selected exons of the genes *EBF1*, *CDKN2A/B*, *PAX5*, *ETV6*, *BTG1*, *RB1* and the *PAR1* region (approx. 230 kbp downstream of *SHOX*, *CRLF2*, *CSF2RA* and *IL3RA*) are also covered. The *P2RY8-CRLF2* fusion can be identified through assessment of *CSFRA* and *IL3RA* deletions. Fragment analyses were performed on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA) and data analyzed using Peak Scanner v1.0 and Coffalyser v9.4 software.



**Figure 1.** Treatment outcome of patients with pediatric ALL comparing patients with and without *IKZF1* deletions. Kaplan-Meier estimates for the whole cohort of 694 patients: (A) event-free survival (EFS) at 5 years, (B) cumulative incidence of relapse (CIR) at 5 years, and (C) overall survival (OS) at 5 years. For the intermediate risk group: (D) EFS, (E) CIR, and (F) OS.

Relative copy number was calculated after intra-sample normalization against control fragments and inter-sample normalization against control samples (healthy blood donor DNA). A ratio of  $1 \pm 0.3$  represents a normal copy number of 2, ratios of less than 0.7 and less than 0.3 represent heterozygous and homozygous deletions, respectively, while ratios of more than 1.3 indicate amplifications. Ratios for all fragments were visually inspected for plausibility and outliers included/excluded where appropriate. In a separate cohort of 25 patients (23 with *IKZF1* deletion), we compared the results from Affymetrix SNP 6.0 arrays with MLPA kit P335 (Online Supplementary Figure S1). All 23 deletions that were known from SNP 6.0 arrays were also detected by MLPA, in 19 cases with exact overlap concerning the deleted exons. In 3 cases, not all exons were called correctly by MLPA, probably due to subclonality of the deletions. In one additional case, the deletion that was found in exon 3 by MLPA was detected in intron 3 on the SNP 6.0 array due to the location of SNP markers on the array. Comparison of the data obtained by the MLPA kit P335-A3 and the MLPA kit P202-A1 could be undertaken in 78 out of the 84 cases with *IKZF1* deletions and only in one case was the deletion not detected using the MLPA kit P202, due to subclonality. Visual inspection of the results did not allow reliable detection of the lesion in P202 due to a higher background noise. (Online Supplementary Figure S2).

### Statistical analysis

Event-free survival (EFS) was calculated from the date of diagnosis to last follow up or to the first event (no complete remission [CR] as event on Day 0, relapse, secondary malignancy, or death of any cause). Overall survival (OS) was calculated from the date of diagnosis to date of death by any cause. Rates were calculated according to Kaplan-Meier and compared by log rank test. Cumulative incidence of relapse (CIR) functions were constructed by the method of Kalbfleisch and Prentice and compared by the Gray test. Cox's regression was used to calculate the hazard ratio for an event mediated by *IKZF1* deletions. Proportional differences between patient groups were analyzed by  $\chi^2$  or Fisher's exact tests. Statistical analyses were carried out using the SAS statistical program (Version 9.1, Cary, NC, USA) and two-sided *P* values below 0.05 were considered statistically significant.

## Results and Discussion

Genetic aberrations in *IKZF1* were screened in diagnostic specimens of 694 ALL patients and *IKZF1* deletions were detected in 84 out of 694 samples (12%) (Table 1). This frequency is within the range of abundances reported

**Table 1.** MLPA analysis of *IKZF1* deletions in 694 patients with pediatric ALL.

<i>IKZF1</i> status	N. of patients	%
Not-deleted	610	88
Deleted <sup>1</sup>	84	12
Whole gene	29	35
Focal	55	65
$\Delta$ 4-7 deletion	23	42
$\Delta$ 2-7 deletion	4	7
Other focal deletion	28	51

<sup>1</sup>Deletions are divided into whole gene and focal deletions while focal deletions are divided into three subgroups.  $\Delta$ 4-7 and  $\Delta$ 2-7 deletions represent deletions of exons 4-7 and exons 2-7 respectively, following the nomenclature of NCBI. All deletions are visualized in Online Supplementary Table S3.

in other previously reported cohorts.<sup>7,9,13</sup> Twenty-nine of these deletions covered the whole gene (35%), while 55 were focal deletions (65%). As described previously, focal lesions detected were mainly  $\Delta$ 4-7 (23 cases, 42%) and  $\Delta$ 2-7 (4 cases, 7%) deletions (Table 1 and Online Supplementary Figure S2).<sup>6,8,9,14,23</sup>  $\Delta$ 4-7 and  $\Delta$ 2-7 deletions represent deletions of exons 4-7 and exons 2-7, respectively. All deletions are visualized in Online Supplementary Figure S2.

Comparing leukemic and clinical characteristics of patients with and without *IKZF1* deletion, no significant differences were observed concerning gender, age or presence of an *MLL/AF4* rearrangement (Table 2). There was a

**Table 2.** Patients' characteristics and response to treatment according to *IKZF1* deletion status in 694 pediatric patients with ALL.

Feature	<i>IKZF1</i> not deleted n (%)	<i>IKZF1</i> deleted n (%)	<i>P</i> <sup>1</sup>
Number of patients	610 (100)	84 (100)	
Sex			0.91
Male	353 (57.9)	48 (57.1)	
Female	257 (42.1)	36 (42.9)	
Age at diagnosis (years)			0.42
1–9	460 (75.4)	60 (71.4)	
$\pm$ 10	150 (24.6)	24 (28.6)	
Presenting WBC count (x10 <sup>9</sup> /L)			0.01
< 1	237 (38.9)	19 (22.6)	
1–4.99	222 (36.4)	35 (41.7)	
5–9.99	83 (13.6)	14 (16.7)	
$\geq$ 10	68 (11.1)	16 (19.0)	
Prednisone response <sup>2</sup>			0.99
Good	529 (86.7)	71 (84.5)	
Poor	78 (12.8)	10 (11.9)	
No information	3 (0.5)	3 (3.6)	
MRD <sup>3</sup>			<0.01
MRD-SR	226 (37.0)	23 (27.4)	
MRD-IR	241 (39.5)	35 (41.7)	
MRD-HR	32 (5.2)	15 (17.9)	
No information	111 (18.3)	11 (13.0)	
Immunology			0.01
Non-T-ALL	496 (81.3)	76 (90.5)	
T-ALL	110 (18.0)	6 (7.1)	
No information	4 (0.7)	2 (2.4)	
<i>ETV6/RUNX1</i>			<0.01
Negative	423 (69.3)	76 (90.5)	
Positive	141 (23.1)	4 (4.75)	
No information	46 (7.6)	4 (4.75)	
<i>BCR/ABL1</i>			0.03
Negative	590 (96.7)	78 (92.9)	
Positive	10 (1.6)	5 (5.9)	
No information	10 (1.6)	1 (1.2)	
<i>MLL/AF4</i>			0.99
Negative	570 (93.4)	81 (96.4)	
Positive	1 (0.2)	0	
No information	39 (6.4)	3 (3.6)	
Final risk group			0.06
SR	219 (35.9)	21 (25.0)	
IR	288 (47.2)	42 (50.0)	
HR	103 (16.9)	21 (25.0)	

<sup>1</sup> $\chi^2$  test comparing *IKZF1* normal and deleted groups, patients with no information excluded from test; <sup>2</sup>good: less than 1000 leukemic blood blasts/ $\mu$ L on treatment Day 8, poor: more than 1000/ $\mu$ L; <sup>3</sup>MRD risk groups<sup>17</sup>: MRD-SR: TP1+2 negative, MRD-IR: TP1 and/or TP2 <10<sup>-3</sup>, MRD-HR: TP2  $\geq$ 10<sup>-3</sup>.

positive association of *IKZF1* deletion with higher white blood cell counts (WBC) at diagnosis ( $P=0.01$ ), pB-ALL immunophenotype ( $P=0.01$ ), positivity for a *BCR/ABL* rearrangement ( $P=0.03$ ), as well as negativity for an *ETV6/RUNX1* rearrangement ( $P<0.01$ ). Of interest, only 5 out of 15 *BCR/ABL1*-positive cases harbored a deletion, which contrasts with previously published data describing the presence of *IKZF1* deletions in approximately 80% of *BCR/ABL1*-positive ALL.<sup>5,6,8</sup>

Most studies published so far focused on pB-ALL, and we also see a significant association of *IKZF1* deletions with the pB-ALL phenotype. However, our cohort was made up of a total of 116 T-ALL patients, 6 of whom showed an *IKZF1* deletion (5.2%). Out of the 6 T-ALL *IKZF1*-deleted patients, one was treated in the SR group while the others were HR patients. The SR patient is in CR, as are 2 of the HR patients, while the other 3 died (only one after a relapse).

Whereas no significant association of *IKZF1* status with PR was observed, the presence of *IKZF1* deletions was significantly more frequent in MRD-HR patients as compared to MRD-IR and MRD-SR patients ( $P<0.01$ ). Five-year EFS was significantly lower in patients with an *IKZF1* deletion compared to those without ( $0.69\pm 0.05$  vs.  $0.85\pm 0.01$ ;  $P<0.0001$ ) (Figure 1A). This difference was mainly attributable to a significantly higher CIR in *IKZF1*-deleted patients ( $0.21\pm 0.04$  vs.  $0.10\pm 0.01$ ;  $P=0.001$ ) (Figure 1B). This effect was most pronounced in the IR group, where the 5-year EFS was  $0.71\pm 0.07$  in *IKZF1*-deleted patients compared to  $0.84\pm 0.02$  in those without ( $P=0.008$ ). The corresponding CIR was  $0.26\pm 0.07$  and  $0.10\pm 0.02$ , respectively ( $P=0.0002$ ) (Figures 1D and E). Fifty percent of all events and 13 of 19 relapses (68%) occurred in the IR group. No significant differences in EFS were seen in SR patients (deletion-positive vs. negative patients: 5-year EFS  $0.85\pm 0.08$  vs.  $0.92\pm 0.02$ ,  $P=0.42$ ; CIR  $0.08\pm 0.02$  vs.  $0.05\pm 0.05$ ,  $P=0.54$ ). While in HR-patients, EFS was  $0.46\pm 0.11$  in the *IKZF1*-deleted versus  $0.69\pm 0.05$  in the non-deleted group ( $P=0.03$ ), there was no significant difference in CIR ( $0.25\pm 0.10$  vs.  $0.15\pm 0.04$ ,  $P=0.28$ ).

Five-year OS was also significantly reduced in patients with *IKZF1* deletions compared to patients without ( $0.82\pm 0.04$  vs.  $0.92\pm 0.01$ ,  $P=0.003$ ). The differences were, however, not significant when analyzing the data according to the current risk groups: (SR: deletion-positive vs. negative patients: 5-year OS  $0.99\pm 0.01$  vs.  $1.00$ ,  $P=0.52$ ; IR:  $0.86\pm 0.05$  vs.  $0.93\pm 0.01$ ,  $P=0.09$ ; HR:  $0.56\pm 0.11$  vs.  $0.75\pm 0.04$ ,  $P=0.06$ ). This suggests that patients with *IKZF1* deletions who subsequently relapse are to a certain extent still treatment sensitive while in relapse.

In a Cox's regression analysis considering the additional variables gender, initial WBC, MRD, and presence of the *MLL/AF4*, *BCR/ABL1* and *ETV6/RUNX1* rearrangement, *IKZF1* deletions provided independent prognostic information (hazard ratio (HR) for an event 2.28, 95% confidence interval, CI: 1.44-3.60;  $P<0.001$ , Online Supplementary Table S2). *IKZF1* deletions have been described to be associated with rearrangements of *CRLF2* and, recently, we have shown that the *P2RY8-CRLF2* rearrangement is associated with a poor outcome in patients treated according to the ALL-BFM 2000 protocol.<sup>17</sup> In the study presented here, we confirmed a significantly higher number of *IKZF1* deletions in patients pos-

itive for the *P2RY8-CRLF2* rearrangement (13.1%) in comparison to those negative (2.6%;  $P<0.001$ ). To explore whether *IKZF1* has prognostic relevance independent of *CRLF2* status, we first excluded *CRLF2*-rearranged patients from the multivariate analysis accounting for the strong association of the two factors (*IKZF1* deletion and *P2RY8-CRLF2* rearrangement), and the resulting co-linearity in the model. In this analysis, *IKZF1* deletions still provided independent prognostic information (HR for an event 1.94; 95% CI: 1.19-3.16;  $P=0.008$ ). Cox's regression analysis including both factors resulted in a Hazard Ratio of 2.08 (95% CI: 1.32-3.28,  $P=0.002$ ) for *IKZF1*-deletions and a hazard ratio of 2.04 (95% CI: 0.94-4.41,  $P=0.07$ ) for *P2RY8-CRLF2* (Online Supplementary Tables S3 and S4).

Comparing clinical and biological characteristics of patients with whole and focal gene deletions, no significant differences for gender, WBC, immunophenotype, involvement of the central nervous system (CNS), presence of *BCR/ABL1*, *MLL/AF4* or *ETV6/RUNX1* rearrangements, PR, MRD and final risk stratification were observed (*data not shown*). Patients with focal deletions were significantly older compared to patients with whole gene deletions (>10 years: 38% vs. 10%;  $P=0.01$ ). There were no significant differences in 5-year EFS or CIR either when comparing whole and focal deletions or when comparing the different types of *IKZF1* deletions (*data not shown*).

*IKZF1* deletions were repeatedly reported to confer an increased risk of relapse and poor outcome in selected cohorts<sup>6,9</sup> and recently in an unselected cohort of 131 pB-ALL cases.<sup>12,13</sup> Here, we present data on the prognostic relevance of *IKZF1* in a large set of nearly 700 patients uniformly treated on the MRD-based ALL-BFM 2000 protocol and were able to show that *IKZF1* status exerts additive value as a prognostic factor, especially in the IR group in which still the majority of relapses occur. Therefore, the assessment of *IKZF1* status may contribute to a molecular-based stratification algorithm aiming at improving outcome for children suffering from ALL. However, more work has to be done to clarify the molecular differences between *IKZF1*-deleted leukemias of patients who relapse and those who don't. From our perspective, this is a prerequisite for an application of the *IKZF1* status in clinical decision-making.

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#### Authorship and Disclosures

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