

# *IKZF1* deletions predict a poor prognosis in children with B-cell progenitor acute lymphoblastic leukemia: A multicenter analysis in Taiwan

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Despite current risk-directed therapy, approximately 15–20% of pediatric patients with acute lymphoblastic leukemia (ALL) have relapses. Recent genome-wide analyses have identified that an alteration of *IKZF1* is associated with very poor outcomes in B-cell progenitor ALL. In this study, we determined the prognostic significance of *IKZF1* deletions in patients with childhood ALL. This study analyzed 242 pediatric B-cell progenitor ALL patients in Taiwan. We developed a simple yet sensitive multiplex quantitative PCR coupled with capillary electrophoresis to accurately determine the allele dose of *IKZF1*, and high resolution melting was used for mutation screening for all coding exons of *IKZF1*. Twenty-six (10.7%) pediatric B-cell progenitor ALL patients were found to harbor these deletions. Most of the deletions were broader deletions that encompassed exon 3 to exon 6, consistent with previous reports. Genomic sequencing of *IKZF1* was carried out in all cases and no point mutations were identified. Patients with *IKZF1* deletions had inferior event-free survival ( $P < 0.001$ ), and overall survival ( $P = 0.0016$ ). The association between *IKZF1* deletions and event-free survival was independent of age, leukocyte count at presentation, and cytogenetic subtype by multivariate Cox analysis ( $P = 0.003$ , hazard ratio = 2.45). This study indicates that detection of *IKZF1* deletions upon diagnosis of B-cell progenitor ALL may help to identify patients at risk of treatment failure. *IKZF1* deletions could be incorporated as a new high-risk prognostic factor in future treatment protocols. To the best of our knowledge, this is the first study to examine the poor prognosis of *IKZF1* deletions in an Asian population. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.02031.x, 2011)

The cure rate of childhood acute lymphoblastic leukemia (ALL) is >80% in most advanced countries, and is expected to reach 90% in the near future.<sup>(1,2)</sup> Treatment success depends on the progressive intensification of standard chemotherapy and the development of detailed risk classification schemes that tailor the intensity of therapy to the predicted risk of relapse. Risk stratification in B-cell progenitor ALL is based on a number of recurring chromosomal abnormalities, including hyperdiploidy, hypodiploidy, translocations t(12;21)(*ETV6-RUNX1*), t(9;22)(*BCR-ABL1*), and t(1;19)(*TCF3-PBX1*), rearrangement of the mixed-lineage leukemia (*MLL*) gene, and an early response to chemotherapy (minimal residual disease).<sup>(1)</sup> Nonetheless, 10–20% of patients will experience a relapse. Relapsed ALL ranks as the fourth most common childhood malignancy, with an overall survival rate of only 30%.<sup>(3)</sup>

The heterogeneity of childhood ALL likely depends on the activation of different leukemogenic pathways defining suscepti-

bility to current treatment protocols. Childhood ALL can be grouped by genome-wide expression profiling, and the gene expression profiles also have close relationships with recurrent chromosomal abnormalities, immuno-phenotypes, treatment outcomes, drug resistance, and minimal residual disease. Gene expression signatures have been constructed and proposed as new prognostic markers in treatment protocols.<sup>(4–10)</sup> Even though RNA-based risk markers may be useful in the future, quantification of mRNA in clinical samples is technically demanding and is strongly dependent on pre-analytic handling of the bone marrow. In principle, DNA-based markers are more robust and may be more applicable in practice.

The recent application of high-resolution genomic profiling has extended the understanding of genetic lesions underlying childhood ALL. Using a single-nucleotide polymorphism (SNP) array, Mullighan *et al.* identified an average of six copy number alterations per patient of childhood ALL. Mutations of genes encoding transcriptional regulators of B lymphoid development, including *PAX5*, *EBF1*, and *IKZF1*, occur in more than 40% of patients with B-cell progenitor ALL.<sup>(11–14)</sup> Deletions of *IKZF1*, which encodes lymphoid transcription factor *IKAROS*, are very frequent in *BCR-ABL1* positive ALL and in the progression of CML to lymphoid blast crisis.<sup>(12)</sup> A comprehensive analysis of high-risk precursor B cell ALL carried out by Mullighan *et al.*<sup>(15)</sup> revealed that genetic alterations of *IKZF1* were associated with a high risk of relapse. This strong association between *IKZF1* and adverse outcomes was confirmed in a Dutch DCOG-ALL9 cohort. Martinelli *et al.*<sup>(16)</sup> also showed that *IKZF1* deletions were likely to be genomic alterations that significantly affect the prognosis of *BCR-ABL1* positive ALL in adults. These studies suggest that deletions of *IKZF1* may be a new prognostic marker to identify high-risk ALL patients who will fail initial chemotherapy.

Multiplex quantitative PCR is a new approach to detect gene deletions, duplications, and rearrangements. In recent years, several new techniques have been developed for the quantitative assay of PCR products. Capillary electrophoresis is a simple, high-performance, reliable, high-resolution, time-saving, and low labor-intensive technique that has promise as a sensitive and specific tool for separating biomolecules and detecting variations in DNA.<sup>(17)</sup> High resolution melting (HRM) is a method for large-scale mutation analysis.<sup>(18)</sup> This study applied these methods to carry out a comprehensive analysis to identify the

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genetic alterations of *IKZF1* in childhood ALL, and investigate the prognostic impact of *IKZF1* deletions in Taiwan.

## Patients and Methods

**Patients and protocols.** Viable diagnostic bone marrow (BM) or peripheral blood was obtained from 242 children with B-precursor ALL from May 1995 to August 2009 at the National Taiwan University Hospital, China Medical University Hospital, Chang Gung Memorial Hospital–Kaohsiung, and National Cheng Kung University Hospital (Taiwan). Seventy-seven patients were treated with TPOG-ALL-93 protocols, 27 with TPOG-97-VHR, and 138 with TPOG-ALL-2002 protocols. The diagnosis of ALL was based on morphologic findings of BM aspirates and immuno-phenotype analyses of leukemic cells by flow cytometry. Conventional cytogenetic analyses were done as part of the routine work-up.

The treatment protocols were previously described.<sup>(19–21)</sup> Patients were considered to have standard-risk (SR, low risk in other studies) ALL if they were between 1 and 9 years of age presenting with a leukocyte count of  $<10 \times 10^9$  cells/L, or were between 2 and 7 years of age presenting with a leukocyte count between  $10 \times 10^9$  and  $50 \times 10^9$  cells/L. Patients were considered to have high-risk (HR, intermediate risk in other studies) ALL if they were between 1 and 9 years of age presenting with a leukocyte count between  $50 \times 10^9$  and  $100 \times 10^9$  cells/L, or between 1 and 2 or 7 and 10 years of age presenting with a leukocyte count between  $10 \times 10^9$  and  $50 \times 10^9$  cells/L. In addition, those with central nervous system leukemia or cranial nerve palsy at diagnosis, or expression of myeloid antigens for TPOG-ALL-93 and those with central nervous system leukemia (cerebrospinal fluid white blood cells with blasts), cranial nerve palsy, testicular leukemia, or pre-B ALL with *TCF-PBX1* fusion for TPOG-ALL-2002 were also considered to be at high risk. Patients with at least one of the following were assigned to the very high-risk (VHR, high risk in other studies) group: age below 1 year, initial leukocyte count  $>100 \times 10^9$  cells/L, lymphoblastic lymphoma with more than 25% lymphoblasts in the bone marrow, hypodiploidy, HR patients with poor treatment response, and the presence of *BCR-ABL1*, *MLL-AF4*, or other *MLL* rearrangements in pre-B ALL.

The patients were prospectively assigned to one of three risk groups (standard, high, and very high) based on their presenting clinical features and the biological features of their leukemic cells. The risk-directed Taiwan Pediatric Oncology Group (TPOG) protocols consist of multiple chemotherapeutic agents of different intensities. The treatment protocol was upgraded if complete remission was not achieved after initial induction therapy. Events were defined as any relapse, death, or secondary malignancy. The Institutional Review Board of National Taiwan University Hospital approved the study and all of the participants provided written informed consent in accordance with the Declaration of Helsinki. Details of the protocols and risk group assignment have been published elsewhere.<sup>(19–21)</sup>

**Determination of *IKZF1* deletions by multiplex PCR with capillary electrophoresis.** As almost all *IKZF1* deletions involve exon 1 to exon 6,<sup>(12,15)</sup> two multiplex quantitative PCR reactions were designed to amplify these exons. *FGFR2* and *FBN1* were used as internal controls to determine the relative allele dosage of the gene tested. Two multiplex quantitative PCR amplifications (multiplex I included the *FGFR2* and *FBN1* genes, and exons 1, 3, and 5 of the *IKZF1* gene; multiplex II included the *FGFR2* and *FBN1* genes, and exons 2, 4, and 6 of the *IKZF1* gene) were designed in a final volume of 25  $\mu$ L containing 100 ng genomic DNA, 0.28  $\mu$ M each of *FGFR2* and *FBN1* primers, 0.12–0.8  $\mu$ M each primer of the exon of interest, 200  $\mu$ M dNTPs, 2 mM MgCl<sub>2</sub>, and 0.5 units of AmpliTaq Gold enzyme (Applied Biosystems, Foster City, CA, USA) in 1 $\times$  buf-

fer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl) as provided by the manufacturer. The sequencing of these designed primers is listed in Table S1.

Amplification was carried out using an MBS thermocycler (ThermoHybaid, Ashford, UK) with an initial denaturation step at 95°C for 10 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 45 s, extension at 72°C for 45 s, and a final extension step at 72°C for 10 min.

For rapid DNA separation and detection, a high-performance DNA analysis capillary electrophoresis system with a CK-5000 disposable cartridge (eGene, Irvine, CA, USA) was used according to the manufacturer's instructions. The gel-matrix in the gel cartridge consisted of a proprietary linear polymer with ethidium bromide dye. Briefly, 2  $\mu$ L unpurified multiplex quantitative PCR products was directly diluted 10-fold with 18  $\mu$ L deionized H<sub>2</sub>O. The samples were placed in the instrument sample tray, automatically injected into the capillary channel, and subjected to electrophoresis by selecting the OM500.mtd method from the BioCalculator software (eGene). The sample injection voltage was 5 kV with a sample injection time of 20 s followed by separation voltage of 5 kV and separation time of 500 s. Quantification of DNA fragments was based on the integrated peak area automatically determined by the BioCalculator software.

**Determination of *IKZF1* mutations by HRM for mutation analysis.** We designed several PCR reactions for point mutation analysis in all coding regions of *IKZF1*. Samples with abnormal peaks (Fig. S1, exon 5 as an example) were selected for sequencing. Details of the primers and PCR conditions for HRM analysis are listed in Table S2.

**Statistical analysis.** Fisher's exact test was used to compare baseline clinical variables across groups for categorical data, and the non-parametric Mann–Whitney *U*-test was applied for continuous variables. A *P*-value  $<0.05$  (two-sided) was considered significant.

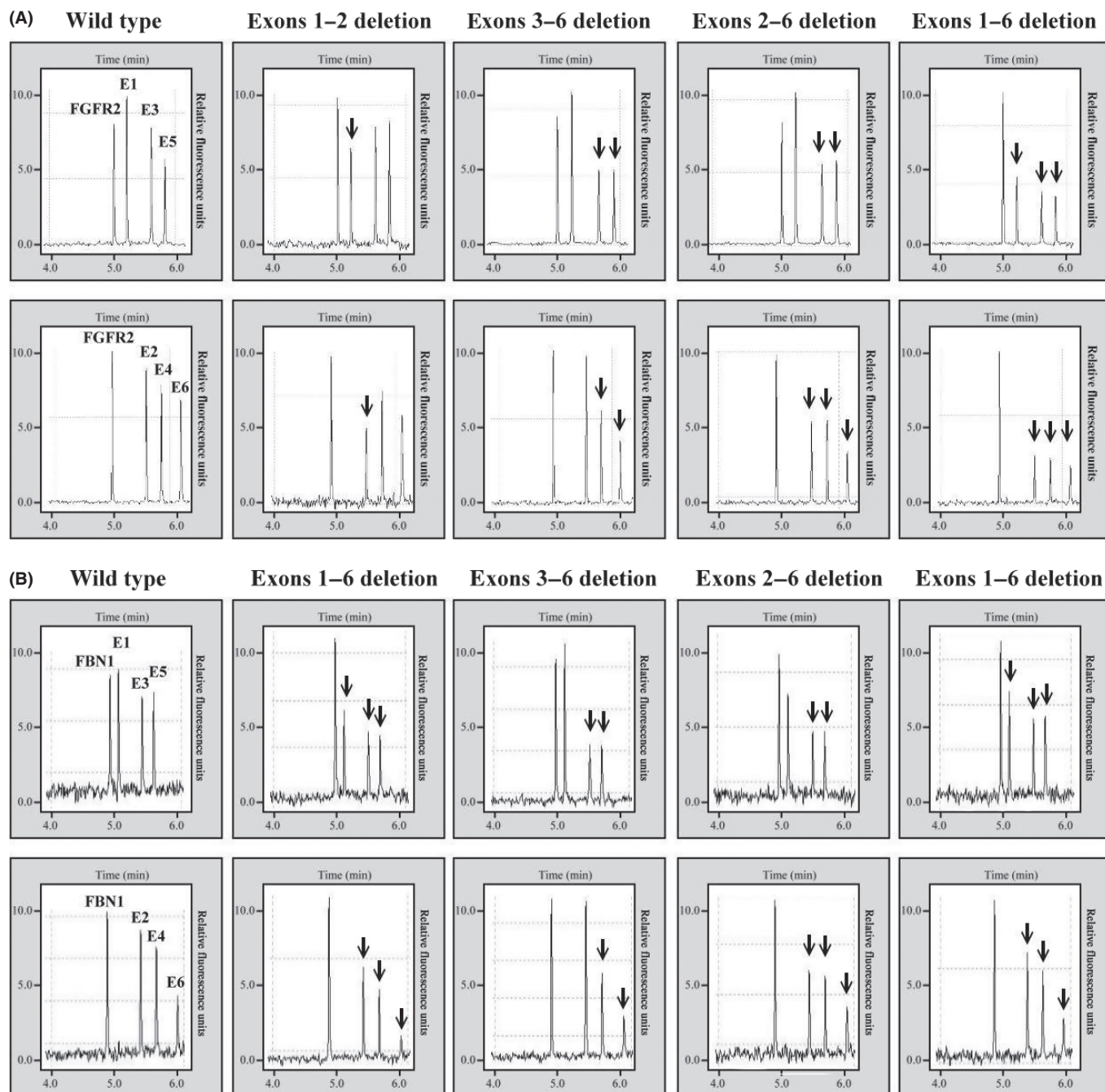
The overall survival (OS) was calculated using the Kaplan–Meier method and the log–rank test was used to compare differences between survival curves. The OS was measured from the protocol commencement date until the date of death regardless of cause, excluding patients who were alive at the last follow-up. Event-free survival (EFS) was defined only for patients who achieved complete remission, and was measured from the date of attaining complete remission until the date of relapse. Patients with no reports of relapse by the end of follow-up were censored on the date of last follow-up.

Cox proportional hazard models were constructed for EFS and OS, with covariates that included sex, white blood cell (WBC) count ( $<10\,000/\mu$ L, 10 000–50 000/ $\mu$ L, 50 000–100 000/ $\mu$ L, and  $>100\,000/\mu$ L), age ( $<1$  year, between 1 and 10 years, and  $>10$  years), the status of *IKZF1* deletions, and genetic subtypes. Stepwise backward selection was carried out. All calculations were done using the SAS software package, version 9 (SAS Institute, Cary, NC, USA).

## Results

**Multiplex PCR with capillary electrophoresis detected recurrent deletions.** Of the 242 patients analyzed, 26 (10.7%) harbored *IKZF1* deletions (Fig. 1). The incidence of *IKZF1* deletions was consistent with a previous report on unselected childhood B-progenitor ALL patients,<sup>(11)</sup> but was lower than that reported for high-risk patients.<sup>(15)</sup> All of the 26 deletions were hemizygous, and exons 3–6 were most involved (Table 1), similar to previous studies.<sup>(12,15)</sup> High resolution melting was used to screen the point mutations in all of the coding exons of *IKZF1*, however no patient was detected in this cohort.

**Patient characteristics.** The clinical characteristics of the patients at the time of diagnosis are shown in Table 2. There were 126 boys and 116 girls with a median age of 4.99 years



**Fig. 1.** Separation of amplified DNA fragments by capillary electrophoresis on the high-performance DNA analysis (HDA) system. (A) Multiplex PCR I with *FGFR2*, exons 1, 3, and 5 of *IKZF1* gene. (B) multiplex PCR II with *FGFR2*, exons 2, 4, and 6 of *IKZF1* gene. Arrows indicate the deletion of the target exon.

(range, 0–18 years). Eighty-six patients were at SR, 95 at HR, and 61 at VHR. The TPOG revised the chemotherapy from TPOG-ALL-93 (for SR and HR patients) and TPOG-97-VHR (for VHR patients) to TPOG-ALL-2002 in 2002. The TPOG-ALL-2002 protocol is still in use for treatment of childhood ALL in Taiwan. The clinical features of these patients according to the different protocol periods are detailed in Table 2.

***IKZF1* deletions associated with very poor clinical outcomes.** The EFS and OS were analyzed for patients with and without *IKZF1* deletions. Patients with *IKZF1* deletions had a trend of high initial WBC count and received the VHR protocols. Patients with *IKZF1* deletions had inferior 5- and 10-year

EFS than those without ( $15.42 \pm 8.0\%$  vs  $77.89 \pm 3.0\%$  and  $15.42 \pm 8.0\%$  vs  $76.35 \pm 3.2\%$ , respectively,  $P < 0.0001$ ), and inferior 5- and 10-year OS ( $47.58 \pm 11.1\%$  vs  $78.21 \pm 3.0\%$  and  $38.07 \pm 12.3\%$  vs  $77.47 \pm 3.1\%$ , respectively,  $P = 0.0016$ ) (Fig. 2).

Excluding SR patients, the EFS and OS were analyzed for HR and VHR patients. Those with *IKZF1* deletions still had inferior 5-year EFS and OS than those without ( $6.56 \pm 6.3\%$  vs  $66.88 \pm 44\%$  and  $28.24 \pm 11.8\%$  vs  $68.32 \pm 4.3\%$ ;  $P < 0.0001$  and  $0.0063$ , respectively) (Fig. 2).

There was no difference in 5-year EFS and OS for patients treated with TPOG-ALL-2002 or TPOG-ALL-93 and



**Table 1. Deletion patterns in patients with *IKZF1* deletions (n = 26)**

Patient	Exons involved	<i>BCR/ABL1</i> status	Hemizygous or homozygous deletions
1	1-3	Negative	Hemizygous
2	3-6	Positive	Hemizygous
3	2-6	Positive	Hemizygous
4	3-6	Positive	Hemizygous
5	1-2	Negative	Hemizygous
6	2-6	Negative	Hemizygous
7	3-6	Negative	Hemizygous
8	3-5	Negative	Hemizygous
9	3-6	Negative	Hemizygous
10	2-6	Positive	Hemizygous
11	2	Negative	Hemizygous
12	3-6	Negative	Hemizygous
13	3-6	Negative	Hemizygous
14	2-6	Negative	Hemizygous
15	2-6	Negative	Hemizygous
16	2-6	Negative	Hemizygous
17	2-6	Negative	Hemizygous
18	3-5	Positive	Hemizygous
19	2-5	Negative	Hemizygous
20	3-6	Negative	Hemizygous
21	3-6	Negative	Hemizygous
22	1-2	Negative	Hemizygous
23	1-6	Negative	Hemizygous
24	1-6	Negative	Hemizygous
25	3-6	Positive	Hemizygous
26	3-6	Positive	Hemizygous

**Table 2. Clinical characteristics of patients with childhood acute lymphoblastic leukemia who participated in this study (n = 242)**

Variable	TPOG-ALL-93 + TPOG-97-VHR (n = 104)	TPOG-ALL-2002 (n = 138)	All patients (n = 242)
Gender			
Male	57 (54.8)	69 (50.0)	126 (52.1)
Female	47 (45.2)	69 (50.0)	116 (47.9)
Age			
Mean ± SD	5.79 ± 3.84	6.58 ± 4.47	6.24 ± 4.22
<1	7 (6.7)	4 (2.9)	11 (4.6)
1-9	80 (76.9)	105 (76.1)	185 (76.5)
≥10	17 (16.4)	29 (21.0)	46 (19.0)
WBC			
<10	46 (44.2)	54 (39.1)	100 (41.3)
10-49	26 (25.0)	48 (34.8)	74 (30.6)
50-99	11 (10.6)	11 (8.0)	22 (9.1)
>100	21 (20.2)	25 (18.1)	46 (19.0)
Risk group			
SR	34 (32.7)	52 (37.7)	86 (35.5)
HR	43 (41.4)	52 (37.7)	95 (39.3)
VHR	27 (26.0)	34 (24.6)	61 (25.2)
<i>IKZF1</i>			
Deletion	10 (9.6)	16 (11.6)	26 (10.7)
Non	94 (90.4)	122 (88.4)	216 (89.3)

Results are shown as n (%). HR, high risk; Non, wild-type; SR, standard risk; VHR, very high risk; WBC, white blood cell.

TPOG-97-VHR in this study cohort (Fig. 3A,B). For patients treated before 2002, patients with *IKZF1* deletions had inferior 5- and 10-year EFS than those without ( $20.00 \pm 12.7\%$  vs  $74.37 \pm 4.6\%$  and  $20.00 \pm 12.7\%$  vs  $72.05 \pm 4.8\%$ , respec-

tively;  $P = 0.0009$ ), and inferior 5- and 10-year OS ( $40.00 \pm 15.5\%$  vs  $70.21 \pm 4.8\%$  and  $30.00 \pm 14.5\%$  vs  $69.15 \pm 4.8\%$ , respectively;  $P = 0.0355$ ) (Fig. 3C,D). For patients treated after 2002, patients with *IKZF1* deletions had inferior 5-year EFS and OS than those without ( $11.11 \pm 10.1\%$  vs  $80.26 \pm 4.1\%$ ,  $P < 0.0001$ , and  $57.14 \pm 14.6\%$  vs  $85.21 \pm 3.7\%$ ,  $P = 0.004$ , respectively) (Fig. 3E,F).

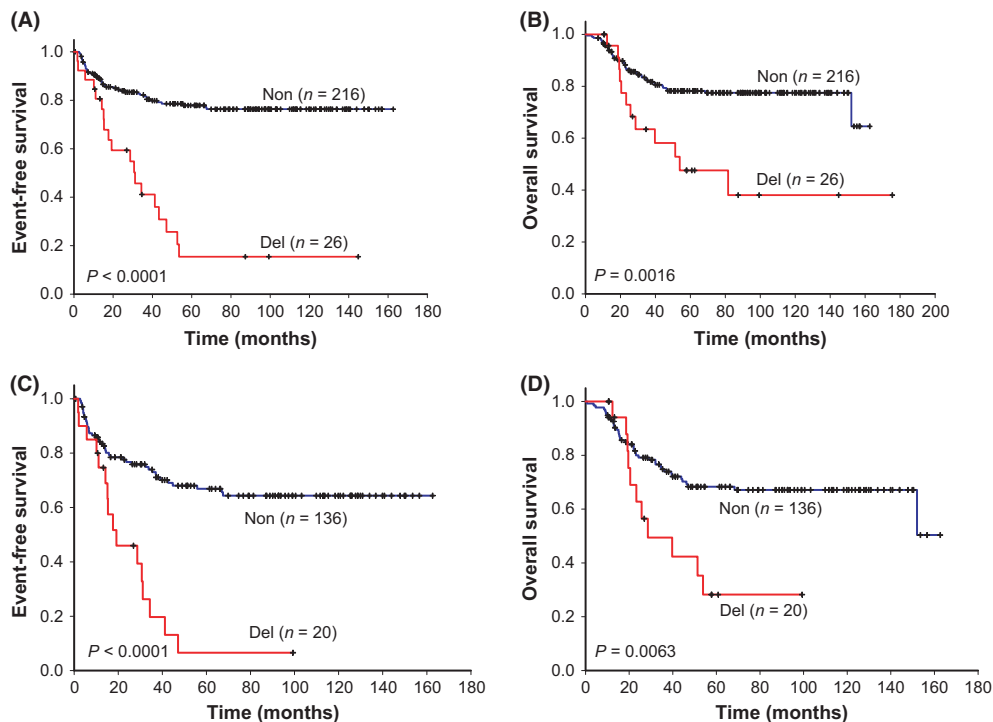
**Multivariate analysis.** In multivariate analysis, considering age, presenting leukocyte count, sex, cytogenetic changes, *IKZF1* deletions, and step-wise variable selection ( $P < 0.5$  for model entry and  $<0.25$  to remain in the mode), *IKZF1* deletions remained significantly associated with EFS at a  $P$  threshold of 0.05 (hazard ratio (HR) 2.45, 95% confidence interval (CI) 1.36-4.44,  $P = 0.0001$ ). *BCR-ABL1* (HR 4.20, CI 1.92-9.22,  $P = 0.0003$ ), *MLL* rearrangements (HR 4.55, CI 1.51-13.67,  $P = 0.007$ ), and high WBC counts (HR 1.65, CI 1.31-2.08,  $P < 0.0001$ ) were also significantly associated with the event in this model (Table 3).

If the analysis was restricted to the HR and VHR patients, similar results were obtained and *IKZF1* deletions still had a poor prognostic impact (HR 2.07, CI 1.07-3.98,  $P = 0.030$ ) (Table 3). In addition, for patients treated with the current protocol (TPOG-ALL-2002), the *IKZF1* deletions remained poor prognostic factors (HR 2.92, CI 1.27-6.72,  $P = 0.012$ ) (Table 3).

## Discussion

In this comprehensive analysis of the *IKZF1* gene, *IKZF1* deletions were identified as an independent poor prognostic marker in B-cell progenitor childhood ALL in Taiwan. To the best of our knowledge, this is the first report of *IKZF1* deletions and the poor prognostic value in an Asian population. Although the SNP array is the most comprehensive method for detecting deletions or other genetic changes, a capillary system coupled with multiplex PCR to type the *IKZF1* deletions is also feasible in routine clinical practice.

Precise assessment of the relapse hazard for individual patients with childhood ALL followed by adjustment of treatment intensity is central to the successful management of childhood ALL. In most protocols, risk stratification is based primarily on the patient's clinical features such as age, WBC counts, and genetic backgrounds of the leukemic cells.<sup>(22)</sup> For many years, cytogenetics had a major prognostic value in childhood ALL. Patients with *BCR-ABL1* and *MLL* gene rearrangements have poor outcomes, but these genetic changes cannot account for all relapsed patients.<sup>(22)</sup> Genome-wide, gene-expression profiling offers a powerful new approach to the study of leukemia cell biology, and potentially provides a new molecular classification of leukemia. Searching for genetic changes related to treatment response across different regimens may provide clues about the general mechanisms that regulate drug sensitivity in leukemic cells and refine the current risk-classification system. *IKZF1* alteration was identified in 10% of childhood ALL in an unselected cohort in the first, large genome-wide study of childhood ALL.<sup>(11)</sup> However, *IKZF1* was deleted in 83.7% of *BCR-ABL1* patients.<sup>(12)</sup> *BCR-ABL1* is notorious for its poor treatment response. The same group also identified the poor prognostic implications of *IKZF1* deletions in children with B-cell progenitor ALL. The *IKZF1* deletions correlated with increased incidence of relapse and resistance to chemotherapy. The poor prognostic impact of *IKZF1* alterations was independent of *BCR-ABL1* translocations and other known risk factors. This strong association between *IKZF1* and adverse outcomes was confirmed by Kuiper *et al.* in a Dutch DCOG-ALL9 cohort.<sup>(23)</sup> In an adult study with subgroups of Philadelphia chromosome patients, *IKZF1* was associated with a high relapse rate.<sup>(16)</sup> These findings highlight the prognostic significance of



**Fig. 2.** Survival curves of childhood acute lymphoblastic leukemia patients with and without *IKZF1* deletions. (A,B) All patients with acute lymphoblastic leukemia who participated in this study ( $n = 242$ ). (C,D) Patients from this study who were grouped as high-risk and very high-risk. Non, wild-type *IKZF1*; Del, deletions of *IKZF1*.

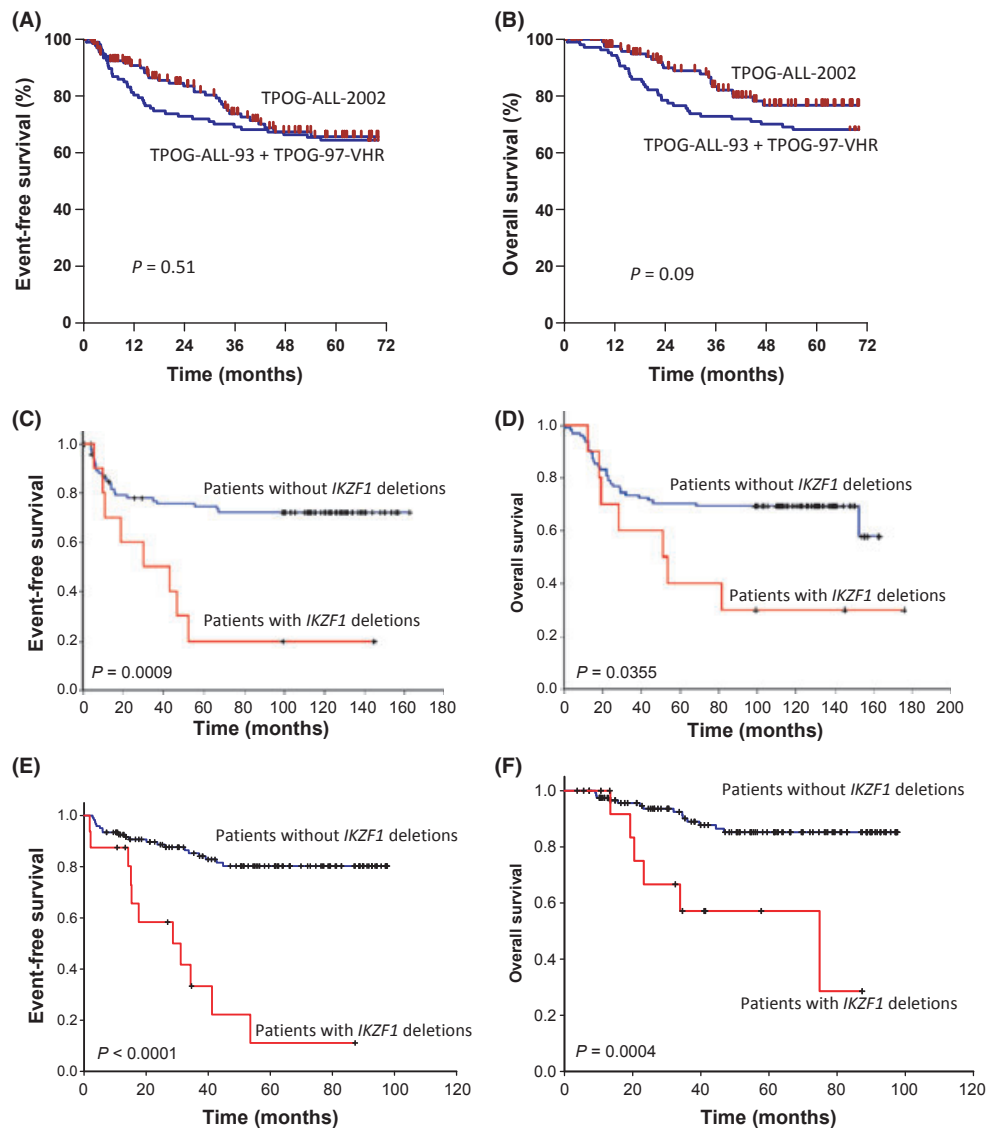
*IKZF1* deletions in ALL. In the current study, 19 of 26 patients with *IKZF1* deletions had subsequent relapses. Six patients with *BCR-ABL1* also had these deletions and all suffered relapses. Deletions of *IKZF1* were associated with poor EFS and OS in our cohort of Taiwanese patients with childhood ALL. Even selecting only HR and VHR patients for analysis, the EFS and OS of patients with deletions remained poor. In addition, most patients with *IKZF1* deletions under the TPOG-ALL-2002 suffered from relapses and only had  $11.11 \pm 10.1\%$  of 5-year EFS in this study. The result hinted that patients with *IKZF1* deletions cannot be cured under the current protocol in Taiwan. New therapeutic strategies, such as hematopoietic stem cell transplantation, may be considered for patients with *IKZF1* deletions in future clinical trials in Taiwan. One limitation of this study is the unknown status of MRD of these patients, so we could not analyze the relationship of MRD and *IKZF1* deletions.

Although patients with the Philadelphia chromosome have a high incidence of *IKZF1* deletions, *IKZF1* deletions are not restricted to this subtype of patients. In the current study, only six of 26 patients with this deletion had *BCR-ABL1*. Several recent reports have also indicated that *IKZF1* deletions are not restricted to the subtype of ALL with *BCR-ABL1*, and such deletions appear in normal cytogenetic patients.<sup>(15,24,25)</sup> There is a HR subgroup of *BCR-ABL1*-negative ALL that is characterized by *IKZF1* deletions, and it has a genetic profile similar to cases with *BCR-ABL1* fusion. These findings have important clinical implications. Currently, few tests can rapidly identify patients who lack known chromosomal alterations and have a high risk of relapse. The data here validates that detection of *IKZF1* alterations should be considered upon diagnosis of childhood ALL, particularly in those with clinical HR features (e.g. high leukocyte count). However, not all patients with *IKZF1* deletions succumb to relapse. Wannders *et al.* used MRD and *IKZF1* alterations to predict a 79% relapse rate in childhood

ALL in the DCOG-ALL9 protocol<sup>(26)</sup>. Furthermore, Harvey *et al.* carried out gene expression profiles and copy-number profiles in HR patients, and the HR patients could be separated into eight groups. *IKZF1* deletions were found in several cluster groups. Patients with *IKZF1* deletions had extremely good outcomes in cluster R6, in contrast with poor outcomes in cluster R8.<sup>(27)</sup> Gene expression cluster, MRD, and *IKZF1* alterations may be able to refine the current classification system of B-cell ALL.

The incidence of *IKZF1* alterations in childhood ALL is different to the above reports. In unselected risk patients, *IKZF1* has been noted in approximately 9% of B-ALL.<sup>(11)</sup> In one subsequent smaller series ( $n = 40$ ), *IKZF1* deletions were detected in one case.<sup>(14)</sup> In a Japanese study analyzing 399 pediatric B-ALL patients using 250 K or 50 K arrays, *IKZF1* deletions were detected in <2% of total cases.<sup>(13)</sup> In a report by Den Boer *et al.*,<sup>(28)</sup> *IKZF1* deletions developed in 73% of patients with Philadelphia chromosome-positive ALL, and 39% of *BCR/ABL1*-like ALL. In the most recent report of Kuiper *et al.*,<sup>(27)</sup> *IKZF1* deletions were predominant in relapsed samples in paired analysis. A Japanese study showed low frequencies of *IKZF1* deletions in a Japanese population.<sup>(13)</sup> They later hypothesized that the *IKZF1* deletions may have been missed by the 50 K and 250 K SNP arrays that were used in their study.<sup>(29)</sup> In our study, the incidence was approximately 10% in an unselected patient risk group, comparable with previous reports.

The SNP array is the most comprehensive method for detecting *IKZF1* deletions. In this study, we used multiplex PCR coupled with a capillary system to screen for these deletions, which can assay several exons at the same time. Two PCR reactions, including exons 1, 3, and 5, and exons 2, 4, and 6, respectively, were designed to check the deletion status of *IKZF1*, and 26 patients were identified. All suffered from hemizygous deletions, which is consistent with a



**Fig. 3.** Survival curves of childhood acute lymphoblastic leukemia patients in this study. (A,B) Comparison of 5-year event-free survival (EFS) and overall survival (OS) for patients treated with TPOG-ALL-2002 or TPOG-ALL-93 + TPOG-97-VHR protocols. (C,D) Comparison of EFS and OS for patients with or without *IKZF1* deletions treated with TPOG-ALL-93 and TPOG-97-VHR. (E,F) Comparison of EFS and OS for patients with or without *IKZF1* deletions treated with TPOG-ALL-2002.

**Table 3. Multivariable Cox regression analysis of event-free survival for patients with childhood acute lymphoblastic leukemia who participated in this study**

Variable	Hazard ratio	95% HR	CI	P-value
All patients ( <i>n</i> = 242)				
<i>IKZF1</i>	2.45	1.36	4.44	0.0030
WBC	1.65	1.31	2.08	<0.0001
t (9;22)	4.20	1.92	9.22	0.0003
<i>MLL</i>	4.55	1.51	13.67	0.0070
Patients with high and very high risk ( <i>n</i> = 156)				
<i>IKZF1</i>	2.07	1.07	3.98	0.0300
WBC	1.49	1.16	1.91	0.0020
t (9;22)	4.06	1.83	8.99	0.0010
<i>MLL</i>	3.28	1.11	9.69	0.0320
Patients treated with TPOG-ALL-2002 ( <i>n</i> = 138)				
<i>IKZF1</i>	2.92	1.27	6.72	0.0120
WBC	1.77	1.22	2.58	0.0030
t (9;22)	4.33	1.50	12.51	0.0070

CI, confidence interval; HR, hazard ratio; WBC, white blood cell.

previous report, however, no patient had point mutations of coding exons. Nonetheless, the most important advantages of our system are the relatively low cost and simple comparison with SNP array findings.

Recently, in addition to *IKZF1* deletions, other studies have also identified a group of HR childhood ALL patients harboring a *BCR-ABL1*-like gene expression. This group of patients suffered from similar poor outcomes as patients with *BCR-ABL1*. Mullighan *et al.*<sup>(30)</sup> identified *JAK* mutations in HR childhood ALL, and the presence of *JAK* mutations was also significantly associated with alteration of *IKZF1*. Yoda *et al.* and Harvey *et al.* used different methods of approach to identify whether *CRLF2* is overexpressed in HR pediatric B-ALL patients that lack common chromosomal translocations.<sup>(24,27,31,32)</sup> *CRLF2* alterations were significantly associated with *JAK* mutations and clinical outcomes.<sup>(31)</sup> Recently, the prognostic impact of *CRLF2* alterations has been validated in the Berlin-Frankfurt-Münster (BFM) protocol. However, many patients with *JAK* mutations and *CRLF2* expression, similar to patients with *IKZF1* deletions, lack other identified known chromosomal alterations. This implies that these newly identified markers may be incorporated into future protocols as poor prognostic factors, and could be new therapeutic targets in the future.

In conclusion, multiplex PCR can be used as a cost-effective and rapid method to identify the deletion status of *IKZF1*.

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*IKZF1* abnormalities were associated with poor outcomes in the Taiwanese population in this study. To the best of our knowledge, this is the first report to validate the poor prognostic value of *IKZF1* deletions. Including alterations of *IKZF1* as a HR factor in ALL and new therapeutic strategies for this subgroup of patients should be considered.

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## Disclosure Statement

The authors have no conflict of interest to declare.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** High resolution melting plots for exon5 of *IKZF1*. Samples with abnormal peaks were picked for sequencings. All the mutations were in the intron but not the exon.

**Table S1.** Polymerase chain reaction primers and conditions used for multiplex PCR in the capillary electrophoresis assay.

**Table S2.** Primers used for PCR–high resolution melting analysis of all coding exons in the *IKZF1* gene.

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