RAS pathway mutation is an added-value biomarker in pediatric Philadelphia-negative B-cell acute lymphoblastic leukemia with IKZF1 deletions

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Abbreviations: B-ALL, B-cell acute lymphoblastic leukemia; EFS, event-free survival; OS, overall survival; Ph (-), Philadelphia negative; Ph (+), Philadelphia positive; TPOG, Taiwan Pediatric Oncology Group.

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Abstract

Background: IKZF1 deletion is an unfavorable factor in Philadelphia negative (Ph-) B-cell acute lymphoblastic leukemia. However, the effects of IKZF1 deletions co-existing genetic alterations in Ph(-) ALL have not been extensively studied.

Methods: Bone marrow samples from 368 children with Ph(-) ALL were analyzed by using multiplex ligation-dependent probe amplification kit for detection of gene deletions and Sanger sequencing for mutational analysis of RAS pathway genes. The outcome was analyzed on 215 patients treated with Taiwan Pediatric Oncology Group-ALL-2002 protocol.

Results: IKZF1 deletions were present in 12.8% and IKZF1plus in 6.3% of patients. Mutations of RAS pathway genes were detected in 25.0% of IKZF1-deleted patients. The 10-year event-free survival (EFS) of IKZF1-undeleted patients was significantly better compared with IKZF1-deleted patients (80.0% vs. 47.8%, p = 0.001). Compared with outcome of patients harboring IKZF1 deletion alone, no difference in EFS was observed in patients with IKZF1plus, whereas three patients carried both IKZF1 and ERG deletions had a superior 10-year EFS (100%). The 10-year EFS of patients with any gene mutation of RAS pathway was worse than that of patients with wild-type genes (79.1% vs. 61.6%, p = 0.033). In multivariate analysis, RAS pathway mutations and IKZF1 deletion were independent predictors of inferior EFS. Co-existence of IKZF1 deletion with RAS pathway mutations had a worst 10-year EFS (11.1 ± 10.5%) and 10-year OS (53.3 ± 17.6%).

Conclusions: Our results showed that RAS pathway mutation is an added-value biomarker in pediatric IKZF1-deleted Ph(-) ALL patients.

KEYWORDS
IKZF1 deletion, pediatric, Philadelphia-negative B-ALL, RAS pathway gene mutation

1 INTRODUCTION

The IKZF1 gene encodes Ikaros, a zinc-finger transcription factor required for the development of all lymphoid lineages.1 Somatic deletions of IKZF1 were present at an overall frequency of 15% in pediatric B-cell acute lymphoblastic leukemia (B-ALL) and up to 70% of patients with BCR-ABL1 positive (Ph+) ALL.2,3 The clinical relevance of IKZF1 deletion in pediatric ALL has been reported to be a negative prognostic factor in both Philadelphia positive (Ph+) and Philadelphia negative (Ph-) ALL.2,4,5

IKZF1 deletion co-occurring with deletions of ERG attenuated its negative prognostic impact.6 Recently, IKZF1 deletions co-occurring with deletions of CDKN2A, CDKN2B, PAX5, or PAR1 region in the absence of ERG deletions were termed IKZF1plus and associated with very poor outcome.7 However, differences in the usage of therapeutic agents and risk classification system between different treatment protocols might lead to the inconsistent effects of IKZF1 deletion on outcome.8–12 Therefore, comparing the prognostic relevance of IKZF1-deletion status with or without co-existing gene deletions in the same treatment protocol could help to clarify their role in the impact on the outcomes.

Mutations of RAS pathway, which activate the RAF–MEK–ERK kinase axis and are sensitive to MEK inhibitors,13 have been linked to relapse and chemotherapy resistance in pediatric B-ALL,14 and a higher frequency of KRAS mutations was reported in Taiwanese pediatric patients with B-ALL compared with that of Children’s Oncology Group (COG) (10.2 vs. 5.7%).15,16 It is of worth to study the impact of RAS pathway mutations on the outcomes of IKZF1-deleted pediatric patients with B-ALL.

Cooperation of BCR-ABL1 and IKZF1 deletion is one reason leading to inferior outcome of patients with IKZF1 deletion in Ph(+) ALL; however, whether co-occurring genetic abnormality of IKZF1 deletion associated with inferior outcome in Ph(-) ALL is still need to be clarified. In the present study, we aimed to determine the prognostic impact of IKZF1 deletion with or without co-existence of other gene deletions or RAS pathway mutations in pediatric Ph(-) B-ALL patients treated with Taiwan Pediatric Oncology Group (TPOG)-ALL-2002 protocol.
2 | MATERIALS AND METHODS

2.1 | Patients

Between 1996 and 2012, 368 patients aged ≤18 years consecu-
tively diagnosed with B-ALL excluding infant and Ph (+) cases were
enrolled in this study. Standard morphological diagnosis, immunophe-
notyping, and genetic/cytogenetic analysis were performed at initial
diagnosis as described previously. Of them, 215 patients treated
with TPOG-ALL-2002 protocol were subjected to outcome analy-
sis. The study was approved by the Institutional Review Boards of
Chang Gung Memorial Hospital-Linkou (IRB No. 13MMHIS27, 103–
6655A3, and 201701453A3) and other collaborative medical centers,
Taiwan.

2.2 | Samples

Bone marrow (BM) leukemic cells were enriched by Ficoll-Hypaque
(GE Healthcare, Uppsala, Sweden) density-gradient centrifugation and
cryopreserved until tested. Genomic DNA was extracted by using
a DNA extraction kit (Genta Puregene or QiAamp System, Min-
nneapolis, MN). Total RNA was extracted from leukemic cells by using
Trizol reagent (Invitrogen Corporation, Carlsbad, CA), and com-
plementary DNA (cDNA) was then synthesized by using the Super-
script II Rnase H2 reverse transcriptase kit (Invitrogen Corporation,
Carlsbad, CA).

2.3 | Molecular genetic analyses

Copy number alterations of IKZF1, PAX5, CDKN2A, CDKN2B, PAR1
region (CRLF2, CSF2RA, and IL3RA genes), and ERG were assessed
by multiplex ligation-dependent probe amplification (MLPA) analyses
(SALSA MLPA P335 ALL and P327 iAMP21-ERG kits; MRC-Holland,
Amsterdam, The Netherlands). Mutation analysis of RAS pathway
genes including NRAS, KRAS, and PTPN11 was detected as described
previously.

2.4 | Statistical analysis

Overall survival (OS) was measured from the time of diagnosis to the
date of death, or last follow-up, whichever came first. Event-free sur-
vival (EFS) was measured from the time of diagnosis to the date of fail-
ure in patients including relapse, or death, or last follow-up. All sta-
tistical analyses were carried out using the SPSS 17.0 for Windows
(SPSS, Chicago, IL). Categorical variables were compared using Fisher
exact test. Multivariate analysis was done by Cox proportional haz-
ard regression. Survival curves were constructed by Kaplan–Meier
estimate and differences were evaluated by log rank test. Two-tailed
p values of <0.05 were considered as statistically significant.

3 | RESULTS

3.1 | Frequency and prognostic impact of IKZF1 deletions

IKZF1 deletions were observed in 47 of 368 (12.8%) pediatric patients.
Of patients with IKZF1 deletions, 13 (27.7%) had a deletion of exons 4–
7, 14 (29.8%) of exons 1–8, and 20 (42.5%) belonged to other group,
including deletion of ex 1 (N = 1), ex 3 (N = 1), ex 5 (N = 1), ex 1–3
(N = 1), ex 2–7 (N = 4), ex 2–8 (N = 1), ex 3–7 (N = 1), ex 4–8 (N = 4),
ex 5–7 (N = 1), ex 5–8 (N = 1), ex 5/7 (N = 2), ex 1/5/7 (N = 1), and
ex 1/2/4/5/6/7/8 (N = 1). IKZF1 deletion was associated with an infe-
tor 10-year EFS compared to those with no IKZF1 deletion (47.8 ±
10.6% vs. 80.0 ± 3.1%, p = 0.001; Figure 1A). There was no difference
in 10-year OS between patients with and without IKZF1 deletion (81.5
± 7.9% vs. 85.0 ± 2.8%, p = 0.805, Figure 1B). We further analyzed
the outcome of patients carrying different IKZF1 deletion subtypes.
Patients carried DEL 4–7 had a worst 10-year EFS (22.7 ± 17.7%) and
10-year OS (66.3 ± 16.3%) compared with patients harboring DEL 1–8
(10-year EFS: 53.3 ± 17.3%; 10-year OS: 77.8 ± 13.9%) and other IKZF1
deletion types (10-year EFS: 67.1 ± 13.5%; 10-year OS: 100%) but it did
not reach statistical significance (EFS: p = 0.320; OS: p = 0.194) (Fig-
ure 1C and D).

3.2 | Prognostic impact of IKZF1 deletion with co-existed gene deletions

Among the 47 pediatric IKZF1-deleted patients, 16 (34.0%), 14 (29.8%),
11 (23.4%), and 1 (2.1%) patients had co-existed deletions of
CDKN2B, CDKN2A, PAX5, and PAR1, respectively. Four (9.5%) of 42
IKZF1-deleted patients, which had available samples for analysis of
ERG deletions, had co-existed ERG deletions. IKZF1plus composed of 23
of 368 (6.3%) patients who had all the genes examined. As shown in
Figure 2A and B, there was a trend that patients with IKZF1plus had an
inferior 10-year EFS but it did not reach statistical significance (37.9
± 13.3% vs. 60.9 ± 12.5%, p = 0.392) and a comparable 10-year OS
(82.3 ± 11.2% vs. 79.5 ± 10.7%, p = 0.635) compared with that of
patients with IKZF1 deletion alone. No significant difference in out-
come was also observed between patients with IKZF1 deletion alone
and IKZF1plus patients with more than two additional unfavorable dele-
tion in CDKN2A, CDKN2B, PAX5, or the PAR1 region (10-year EFS: 60.9
± 12.5% vs. 29.5 ± 13.3%, p = 0.209; 10-year OS: 79.5 ± 10.7% vs. 79.6
± 13.6%, p = 0.785) (Figure S1). In contrast, three patients carrying
both IKZF1 and ERG deletions had 100% EFS and OS as of last follow-up
(Figure 2C and D).
3.3 | **IKZF1 deletion with co-existed RAS pathway mutations conferred inferior outcome**

RAS pathway mutations including PTPN11, NRAS, and KRAS were detected in 4.2% (n = 14), 12.8% (n = 43), and 8.0% (n = 27) of 336 patients, respectively. Of the IKZF1-deleted patients, co-existed mutations of RAS pathway genes were detected in 2.3% for PTPN11, 15.9% for NRAS, and 11.4% for KRAS. Totally, 25.0% (n = 11) of 44 IKZF1-deleted patients, of which 93.6% had available samples for analysis of RAS pathway mutations, carried RAS pathway mutations. The outcomes of patients with or without any of the three RAS pathway gene mutations were analyzed in 215 patients treated with TPOG-ALL-2002. As shown in Figure 3, the 10-year EFS (61.6 ± 7.7% vs. 79.1 ± 3.4%, p = 0.033) and 10-year OS (72.4 ± 7.0% vs. 88.2 ± 2.7%, p = 0.020) of patients with any of RAS pathway gene mutations were worse than that of patients with wild-type RAS pathway genes.

In multivariate analysis (Table 1), white blood cell count > 50 × 10^9/L, KMT2A-rearranged, IKZF1 deletion (hazard ratio [HR] = 2.827, p = 0.002), and RAS pathway mutations (HR = 2.360, p = 0.005) were independent predictors of inferior EFS. Among the four genetic predictors of inferior EFS, KMT2A-rearranged (HR = 3.455, p = 0.032) and RAS pathway mutations (HR = 1.982, p = 0.0026) were also independent predictors of inferior OS. Coexistence of IKZF1 deletion with RAS pathway mutations had a worst 10-year EFS (11.1 ± 10.5%) compared with patients with IKZF1 deletion alone (61.4 ± 13.8%), RAS pathway mutations alone (74.2 ± 7.9%), or wild-type of both genetic alterations (81.5 ± 3.4%) (p < 0.0001, Figure 4A). Patients co-existing IKZF1 deletion with RAS pathway mutations also had a worst 10-year OS (53.3 ± 17.6%) compared to patients with IKZF1 deletion alone (95.8 ± 4.1%), RAS pathway mutations alone (78.2 ± 6.9%), or wild-type of both genetic alterations (87.0 ± 3.0%) (p = 0.017, Figure 4B).
FIGURE 2  Outcomes of pediatric Ph (-) B-ALL patients treated with TPOG-ALL-2002 according to \textit{IKZF1} deletion or \textit{IKZF1}^{plus} groups (A and B) and according to \textit{IKZF1} deletion with co-existed \textit{ERG} deletions (C and D).

FIGURE 3  EFS (A) or OS (B) of pediatric Ph (-) B-ALL treated with TPOG-ALL-2002 according to the status of RAS pathway genes.
**FIGURE 4** Impact of RAS pathway mutations in EFS (A) or OS (B) according to the IKZF1 status in pediatric Ph (-) B-ALL treated with TPOG-ALL-2002

4 | DISCUSSION

IKZF1 deletions and IKZF1\textsuperscript{plus} were detected in 12.8% and 6.3%, respectively, of Ph (-) B-ALL in the current study, which was comparable to the frequency of 14.6% of Ph (-) B-ALL and 6.0% of all pediatric B-ALL cases in the previous studies of other investigators. Cumulated evidences suggested that IKZF1 deletion should be considered to be a candidate of unfavorable factor for risk stratification within treatment protocols. However, comparison among studies were difficult due to differences in patient population and treatment protocols. In the present study, we made a unique comparison to assess the impact of IKZF1 deletion and co-existed other genetic alterations on the outcomes of Ph (-) ALL patients treated with same protocol.

The present results were consistent with the EORTC Children’s Leukemia Group study 58951 trial showing inferior EFS but comparable OS in patients with IKZF1 deletion in Ph (-) ALL. In addition, no significant difference in EFS and OS was observed in patients with different IKZF1-deleted subtypes, supporting that all IKZF1 deletion subtypes were associated with an unfavorable prognosis in pediatric B-ALL.

The prognostic effects of IKZF1 deletion in combination with other gene deletions was also analyzed in the present study. We found that ERG deletion was a negative effector of outcome of IKZF1-deleted patients, which was in line with the previous study of Clappier et al. Although we observed an inferior EFS in patients with IKZF1 deletion in Ph (-) ALL. In addition, no significant difference in EFS and OS was observed in patients with different IKZF1-deleted subtypes, supporting that all IKZF1 deletion subtypes were associated with an unfavorable prognosis in pediatric B-ALL.

The outcome of patients with IKZF1 deletion combined with or without any mutations of RAS pathway genes was analyzed. Because KRAS mutations were strongly associated with poor prognosis in a relapsed pediatric B-ALL cohort. The outcome of patients with IKZF1 deletion combined with or without any mutations of RAS pathway genes was analyzed. Because KRAS mutations were strongly associated with poor prognosis in a relapsed pediatric B-ALL cohort. Moreover, Holmfeldt et al. demonstrated that IKO-ROS family, IKZF2 and IKZF3, alterations could activate RAS pathway and showed evidence of PI3K or dual PI3K/mTOR inhibitors sensitiv-
| Variable                                      | Event-free survival | Overall survival |
|-----------------------------------------------|---------------------|------------------|
|                                              | Univariate          | Multivariate     | Univariate          | Multivariate     |
|                                              | HR      | 95% CI   | p        | HR      | 95% CI   | p        | HR      | 95% CI   | p        |
| Age (>10 years vs. 1–10 years)                | 1.872   | 1.050–3.339 | 0.034   | 1.721   | 0.932–3.210 | 0.101   | 1.804   | 0.898–3.625 | 0.097   |
| Gender (male vs. female)                      | 1.231   | 0.728–2.124 | 0.312   | 1.314   | 0.542–2.328 | 0.526   |         |          |          |
| Initial WBC count (< 10^9/L) vs. 10^9/L)      | 3.880   | 2.289–6.575 | <0.0001 | 2.572   | 1.309–4.625 | 0.004   | 2.735   | 1.420–5.270 | 0.003   | 1.810   | 0.816–3.912 | 0.127   |
| Hemoglobin (>10 vs. ≤10)                      | 1.102   | 0.381–1.318 | 0.277   | 1.525   | 0.387–1.567 | 0.426   |         |          |          |
| Platelet count (< 50 × 10^9/L)                | 2.503   | 1.366–4.584 | 0.003   | 1.972   | 0.963–3.752 | 0.082   | 2.815   | 1.312–5.921 | 0.007   | 2.312   | 1.071–4.721 | 0.045   |
| ETV6-RUNX1 (Presence vs. absence)              | 0.443   | 0.190–1.033 | 0.060   |         |          |          | 0.472   | 0.217–1.512 | 0.207   |         |          |          |
| TCF3-PBX1 (Presence vs. absence)               | 0.185   | 0.026–1.338 | 0.095   |         |          |          | 0.321   | 0.021–2.121 | 0.325   |         |          |          |
| KMT2A-rearrangement (Presence vs. absence)     | 7.110   | 2.819–17.934 | <0.0001 | 5.921   | 2.056–13.215 | 0.001   | 5.569   | 1.973–15.718 | 0.001   | 3.455   | 1.112–10.732 | 0.032   |
| Hyperdiploidy (Presence vs. absence)           | 0.548   | 0.372–1.621 | 0.421   |         |          |          | 0.951   | 0.251–2.421 | 0.572   |         |          |          |
| Hypodiploidy (Presence vs. absence)            | 1.026   | 0.452–3.021 | 0.872   |         |          |          | 0.596   | 0.152–3.125 | 0.307   |         |          |          |
| IKZF1 (Deletion vs. Wt)                        | 2.557   | 1.388–4.710 | 0.003   | 2.827   | 1.452–5.351 | 0.002   | 1.098   | 0.427–2.823 | 0.847   |         |          |          |
| RAS pathway mutations (Presence vs. absence)   | 2.242   | 1.201–3.721 | 0.032   | 2.360   | 1.512–4.252 | 0.005   | 2.012   | 1.205–3.251 | 0.020   | 1.982   | 1.201–3.083 | 0.0026  |
| IKZF1^Plus (Plus vs. IKZF1 alone)              | 1.210   | 0.421–2.725 | 0.397   |         |          |          | 0.972   | 0.192–4.759 | 0.652   |         |          |          |
| ERG (Deletion vs. Wt)                         | 0.572   | 0.152–2.302 | 0.412   |         |          |          | 0.571   | 0.026–3.751 | 0.521   |         |          |          |

CI, confidence interval; HR, hazard ratio; Wt, wild type.
ity in the absence of RAS pathway mutations in high-risk ALL. Vesely et al.\textsuperscript{26} also showed that RAS pathway signaling gene sets were positively enriched in the \textit{IKZF1} dominant-negative and \textit{IKZF1}-mutant ALL cells. Thus, the possibility that activation of MEK/ERK and PI3K/mTOR caused by RAS pathway mutations in conjunction with \textit{IKZF1} deletion might confer to a more inferior outcome. In addition to provide added information on predicting inferior outcome in Ph (-) B-ALL patients with \textit{IKZF1} deletion, identification of the RAS pathway mutations might benefit \textit{IKZF1}-deleted patients by targeted therapy with MEK inhibitor.

In conclusion, we showed an added-value risk factor of RAS pathway mutations in \textit{IKZF1}-deleted patients in the TPOG-ALL-2002 cohort, which in turn would be helpful in further modification of future treatment protocol.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Lee-Yung Shih designed and supervised the study; Hsi-Che Liu, Tang-Her Jaing, Kang-Hsi Wu, Shih-Chung Wang, Hsiu-Ju Yen, Chih-Cheng Hsiao, Shih-Hsiang Chen, Pei-Chin Lin, Ting-Chi Yeh, Jiunn-Ming Sheen, Yu-Chieh Chen, Te-Kau Chang, Fang-Liang Huang, Yu-Hua Chao, Jen-Yin Hou, Chao-Ping Yang, and Lee-Yung Shih provided patients’ samples and their clinical data; Tung-Huei Lin performed statistical analysis; Lee-Yung Shih and Ying-Jung Huang developed the methodology, analyzed and interpreted the data, and wrote the manuscript. All authors have read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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