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Original Article

High CRLF2 Expression Could Identify Acute Lymphoblastic Leukemia Patients with Poor Outcome but Not IKZF1

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Abstract

Background: Overexpression of cytokine receptor-like factor 2 (CRLF2) caused by different genetic aberrations has been observed in acute lymphoblastic leukemia (ALL) and correlated with poor outcome. Most patients with high CRLF2 expression are clustered in the Philadelphia-like (Ph-like) ALL subgroup. Ph-like ALL is reported to be associated with alterations in IKZF1 gene, encoding the transcription factor Ikaros.

Aim: To identify CRLF2 and IKZF1 alterations in Egyptian patients with ALL and to determine their prognostic significance.

Methods: Peripheral blood samples from 34 newly diagnosed ALL patients treated at an Egyptian tertiary oncology center and 14 controls were assessed for CRLF2 and IKZF1 mRNA expression using real-time polymerase chain reaction.

Results: CRLF2 was significantly overexpressed in ALL patients compared to controls (p = 0.038). The response to treatment was significantly better in patients with low CRLF2 expression (p = 0.029). The rate of remission, relapse and induction death was 82%, 12% and 6% in the low CRLF2 expression group and 41%, 18% and 41% in the high expression one. Overall survival was significantly shorter among ALL with high CRLF2 (p = 0.034). IKZF1 expression level did not differ significantly between patients and controls. Patients with low IKZF1 exhibited significantly higher leucocytic count and lower platelet count (p = 0.038 and 0.044, respectively). IKZF1 overexpression did not correlate significantly with response to treatment or survival.

Conclusion: High CRLF2 expression was associated with poor outcome among ALL patients. Further research is needed to improve the diagnostic and therapeutic approaches in ALL patients with poor prognosis.

Keywords: Acute lymphoblastic leukemia, Cytokine receptor-like factor 2, IKZF 1, qRT-PCR.
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Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous disorder characterized by complex molecular changes such as fusion proteins, copy number alterations, as well as gene mutations [1]. In B-cell ALL (B-ALL), frequent genetic abnormalities in key pathways and chromosomal translocations have been involved in risk stratification for therapy selection [2]. Conversely, in T-cell (T-ALL), although many genomic aberrations have been identified, only few were proved to be of prognostic value, and none has been incorporated in therapeutic approaches [3].

Significant progress in leukemia genomics has led to the recognition of genes and pathways undergoing dysregulation in ALL. BCR-ABL1-like ALL

was recognized depending on a gene expression signature of leukemic cells that mimics that of BCR-ABL1–positive ALL, although such leukemic cells do not have a BCR-ABL1 translocation [4]. These cases have a highly diverse range of genetic changes that activate tyrosine kinase signaling [5].

Philadelphia (Ph)-like ALL was identified as a provisional entity in the 2016 World Health Organization classification of myeloid tumors and acute leukemias [6] and several researches have reported numerous genetic events underlying Ph-like ALL. The frequency of Ph-like ALL in various ages and its related clinical criteria and outcomes have resulted in the identification of new opportunities for precision medicine treatments [7]. The Ph-like ALL prevalence varies according to age, sex, race and ethnicity. Ph-like ALL is more common by three folds than Ph-positive ALL among children and peaks among young adults (up to 30%), with a plateau of 20-24% at the age of 40-86 years [8, 9].

High cytokine receptor-like factor 2 (CRLF2) expression was reported in about half of Ph-like ALL patients [10]. In one study, high CRLF2 expression level was found in 15% of T-ALL patients and was associated with worse prognosis, defining a subset of high-risk T-ALL cases who might benefit from treatments which hinder JAK/STAT5 signaling pathway activation [11]. Several other studies confirmed the unfavorable prognosis accompanying CRLF2 rearrangements including a study on Egyptian ALL patients [12, 13]. Deletions of the IKZF1 gene were detected in approximately 28% of BCR-ABL1 negative ALL children and in ≥ 80% of adult BCR-ABL1 positive patients [14]. These deletions correlated significantly with high relapse rate and unfavorable outcomes in children and adult cases [15]. A lack of correlation between IKZF1 deletion and the expected deregulated IKZF1 gene expression was reported [16,17].

These observations prompted us to investigate the expression of CRLF2 and IKZF1 in a group of Egyptian ALL patients and their relation to treatment outcomes.

Methods

Patients and controls

The current work included 34 newly diagnosed children and adult ALL cases attending a tertiary oncology center in Egypt. The diagnosis of ALL was based on standard cytomorphology in addition to immunophenotypic criteria. Peripheral blood samples were obtained from the 34 ALL cases and 14 healthy controls.

Mononuclear cells isolation and RNA extraction

Mononuclear cells underwent isolation via density gradient centrifugation by lymphocyte separation medium (Lonza, Walkersville, MD). Isolation of RNA from mononuclear cells was performed by miRNeasy Mini kits (Qiagen, Germantown, MD). NanoDrop was utilized to determine the purity and concentration of RNA.

Quantitative real-time polymerase chain reaction

High capacity reverse transcription kit (Applied Biosystems) was used to synthesize cDNA from 2 µg RNA. Preparation of 20 µL reaction was performed as follow: 2 µL 10x RT buffer, 0.8 µL 25x dntps 100 mM, 2µl 10x random primers, 1 µL Multiscribe reverse transcriptase enzyme (50U/µl), 1µl RNase inhibitor and 13.2 µL nuclease free water along with the extracted RNA. This was followed by incubation of sample wells in thermal cycler at 25°C for ten minutes, 37°C for 120 minutes, 85°C for five minutes then 4°C hold.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was undergone on the StepOne™ utilizing TaqMan gene expression assays for CRLF2 and IKZF1 (Life Technologies, Grand Island, NY). Real-time PCR was performed using Applied Biosystems TaqMan Gene Expression Assays (CRLF2 assay ID Hs_00845692, IKZF1 assay ID Hs_00958474). House-keeping gene GAPDH was utilized as an internal control. The relative gene expression concentration was calculated as ($2^{-\Delta\Delta Ct}$). The median value was used to divide patients into low and high expression groups.

Statistical analysis

Statistical analyses were done utilizing GraphPad Prism software, La Jolla, CA. Continuous variables were tested for the normality of distribution using the Shapiro-Wilk test. Those with abnormal distribution were expressed as median + interquartile range (IQR) and analyzed using Mann-Whitney test. Categorical variables were expressed as number + percentage and analyzed using chi-square test. Overall survival (OS) was considered from the

date of diagnosis to the date of mortality and analyzed using the Kaplan–Meier method. A p value < 0.05 was considered significant.

Ethical considerations

The study was approved by the Institution Review Board of the Faculty of Medicine, Mansoura University (approval # R/17.9.39). All samples were obtained in accordance with the Declaration of Helsinki, with informed consent from the adult patients or parents/guardians of pediatric patients.

Results

The characteristics of included patients are shown in Table 1. The median age of the 14 healthy controls was 17 years (IQR= 5-16.25).

Table 1: Characteristics of 34 ALL patients

Characteristic	**Description**	
Age (years)	Median (IQR)	7.5 (5-16.25)
Gender		
Male	*n* (%)	21 (62)
Female	*n* (%)	13 (38)
Adult/Child		
Adult	*n* (%)	7 (21)
Child	*n* (%)	27 (79)
WBC count (*10^3/uL)	Median (IQR)	11.7(5.4-39.3)
Haemoglobin (g/dL)	Median (IQR)	9 (7.5-11)
Platelets count (*10^3/uL)	Median (IQR)	53.5 (28-99.5)
ALL immunophenotype		
B-ALL	*n* (%)	19 (56)
T-ALL	*n* (%)	15 (44)
Blast % in BM	Median (IQR)	90 (72-90)
LDH (U/L)	Median (IQR)	865 (490-1678)
Last documented status		
Alive	*n* (%)	23 (68)
Dead	*n* (%)	11 (32)

ALL: Acute lymphoblastic leukemia**; BM**: Bone marrow, **IQR**: Interquartile range, **LDH**: Lactate dehydrogenase**, WBC**: White blood cells

CRLF2 and IKZF1 gene expression levels among acute lymphoblastic leukemia patients

CRLF2 mRNA level was significantly higher in ALL patient than in healthy controls (median [IQR] = 4.16 [0.7-23.94] and 1.9 [0.21-4.27], respectively; p = 0.038) (Figure 1a). The median delta Ct of T-ALL and B-ALL cases was comparable (4.9 [IQR: 1.38-31.75] and 6 [IQR: 0.74-30.97], respectively). CRLF2 mRNA level was significantly higher in B-ALL and T-ALL cases than in controls (p = 0.042 and 0.016, respectively).

On the other hand, there was no significant difference in the relative expression level of IKZF1 mRNA between ALL cases and healthy controls (median [IQR] = 30.69 [6.9-94.6] and 17.43 [9.43-42.49], respectively; p = 0.346) (Figure 1b). The median delta Ct of T-ALL and B-ALL cases was comparable (35.2 [IQR: 7-90.9] and 25.6 [IQR: 6.8-103], respectively; p = 0.78). IKZF1 mRNA level was not significantly higher in B-ALL and T-ALL cases than in controls (p = 0.47 and 0.34, respectively).

Correlation between CRLF2 and IKZF1 expression levels and patients' characteristics

The correlation between CRLF2 and IKZF1 expression levels and the clinical characteristics of patients is summarized in Table 2. No significant correlation was found with age, sex, hemoglobin level or the percentage of blasts in bone marrow. Patients with high CRLF2 expression showed a non-significant trend toward higher white blood cells count, lower hemoglobin level, and lower platelet count when compared to those with low expression.

Patients with low IKZF1 expression exhibited a significantly higher white blood cell count than those with high expression, and a significantly lower platelets count (Table 2). None of the other variables correlated significantly with IKZF1 expression level.

The relation between CRLF2 and IKZF1 expression levels and treatment outcome

The treatment outcome correlated significantly with the level of expression of CRLF2 in favour of low expression (p = 0.029). The response to treatment in patients with low CRLF2 expression was remission in 14 (82%), relapse in 2 (12%) and induction death in 1 (6%); while in patients with high expression it was remission in 7 (41%), relapse in 3 (18%) and induction death in 7 (41%) (Figure 2a).

The treatment outcome did not differ significantly according to the level of IKZF1 expression (p = 0.39). The remission, relapse and induction death rates were 76%, 6% and 18% in the low IKZF1 expression group and 53%, 18% and 24% in the high expression one (Figure 2b).

Table 2: Correlation between patients' characteristics and the level of expression of CRLF2 and IKZF1 among acute lymphoblastic leukemia patients

		CRLF			IKZF1		
		Low (*n*=17)	**High** (*n*=17)	***p* value**	**Low** (*n*=17)	**High** (*n*=17)	***p* value**
Age (years)	Median (IQR)	6 (3-14)	11 (6-18)	0.11	6 (3.5-14)	11 (5.5-33.5)	0.2
Gender							
Male	*n* (%)	11 (64.7)	10 (58.8)	0.9	10 (58.8)	11 (64.7)	0.9
Female	*n* (%)	6 (35.3)	7 (41.2)		7 (41.2)	6 (35.3)	
ALL type							
B-ALL	n (%)	11 (64.7)	8 (47.1)	0.48	10 (58.8)	9 (52.9)	0.9
T-ALL	n (%)	6 (35.3)	9 (52.9)		7 (41.2)	8 (47.1)	
WBC count (*103 /µL)	Median (IQR)	11.1 (4.9-32.2)	25.7 (5.5-70.4)	0.43	25.7 (9.4-81.6)	6.9 (4.9-26.5)	0.038
Hemoglobin (g/dL)	Median (IQR)	9.6 (7.7-11.3)	8.4 (7.2-10.8)	0.45	8.5 (7.7-10.6)	9.9 (6.8-11.2)	0.77
Platelets count (*103 /µL)	Median (IQR)	59 (50.4-90.5)	50.6 (18.3-137.3)	0.44	50.8 (19.3-83)	63.5 (36.3-172)	0.044
Blast % in BM	Median (IQR)	90 (70-90)	90 (80-90)	0.89	85 (70-90)	90 (82.5-92.5)	0.09
LDH (U/L)	Median (IQR)	984 (508-1899)	692 (383-1060)	0.13	984 (614-1883)	558 (292-1447)	0.13

ALL: Acute lymphoblastic leukemia, **BM**: Bone marrow, **IQR**: Interquartile range, **LDH**: Lactate dehydrogenase, **WBC**: White blood cells

CRLF2 and IKZF11 expression levels and overall survival

It was found that patients with low CRLF2 expression have significantly better OS compared to those with high expression (HR = 0.27 [95% Confidence Interval: 0.092 to 0.897]; $p = 0.034$) (Figure 3a). There was no significant difference in OS between cases with low and high IKZF1expression (HR = 0.56 [95% Confidence Interval: 0.178 to 1.738], $p = 0.31$) (Figure 3b).

Discussion

New molecular markers which could predict poor outcome, were recently discovered among ALL patients who do not exhibit known poor-prognostic criteria and thus they have potential clinical value. Among these is BCR-ABL1-like gene expression signature, which has frequent deletions of IKZF1 [4, 5, 18-20].

Identification of BCR/ABL1-like cases is still challenging. However, Herold et al [15] reported that most (58%) patients with high CRLF2 expression clustered in this Ph-like ALL subgroup. Those cases with CRLF2 overexpression were found to correlate with high-risk ALL markers, and poor OS in both T and B-ALL subtypes without CRLF2 rearrangement detected [21]. So, it was important to find out a user-friendly and economically diagnostic tool to recognize and identify these cases at presentation. Recently, one group produced an easy, fast and reproducible assay, depending on qRT-PCR analysis, to recognize and predict BCR/ ABL1-like ALL patients [22]. Although, they proposed that CRLF2 overexpression alone was insufficient for induction of a BCR/ABL1-like profile. But CRLF2 expression was included in common algorithms utilized for identification of BCR/ABL1-like patients [5, 15, 20]. Therefore, we investigated the expression levels of CRLF2 and IKZF1 in a group of B- and T-ALL patients.

The prevalence of Ph-like ALL varies as regards age, sex, race and ethnicity. Its prevalence is approximately 21% in adolescents (aged 16-20 years) and 20 - 24% among adults above 40 years, with a peak (27%) among young adults 21 - 39 years of age [9, 23].

In our study, the prevalence of high CRLF2 expression level was more of less similar to that reported in previous studies. The differences in prevalence may be due to the heterogenicity in patients' age groups between studies [23, 24]. Ethnicity may be a contributing factor as well, as Hispanic individuals were found to exhibit a greater Ph-like

ALL prevalence [20, 23]. This was explained by the greater frequency of germline Ph-like ALL risk variant in GATA3 (rs3824662) [25]. Interestingly, this risk allele was also observed in a dominant inheritance risk model in an Egyptian study of ALL [26].

Our results demonstrated that OS was significantly lower in ALL patients with elevated CRLF2. This is consistent with the results of previously reported studies that showed an association between elevated CRLF2 expression values and the relatively unfavorable prognosis among B-ALL patients [19, 20].

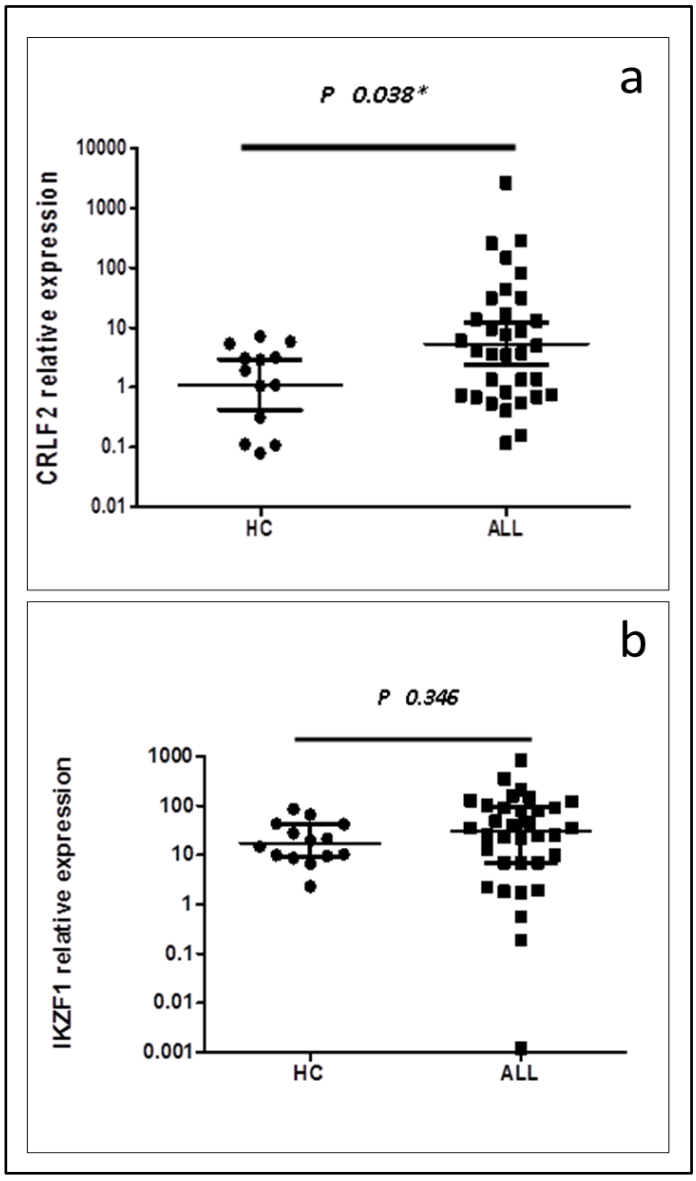

Figure 1: Comparing CRLF2 mRNA (a) and IKZF1 mRNA (b) expression in acute lymphoblastic leukemia (ALL) patients to that in healthy controls (HC) by qRT-PCR

Palmi et al [10] demonstrated high CRLF2 expression level in T- ALL and reported to be associated with worse prognosis defining a subset of high risk ALL patients. In both children and adults, Ph-like ALL was reported to be associated with high rate of treatment failure, and poor survival compared to non-Ph-like ALL patients [10, 19, 20]. Similarly, in our study, the outcome of high CRLF2 patient group is particularly poor as regard relapse rate, induction death compared to low CRLF2 expressing group (p = 0.029).

The clinical significance of IKZF1 deletions in ALL is still controversial. Mullighan et al [27] described IKZF1 deletions as a significant predictor of unfavorable outcome in high-risk B-ALL cases. On the other hand, Chen et al [28] failed to confirm that IKZF1 deletions were independent prognostic marker for high-risk B-ALL patients.

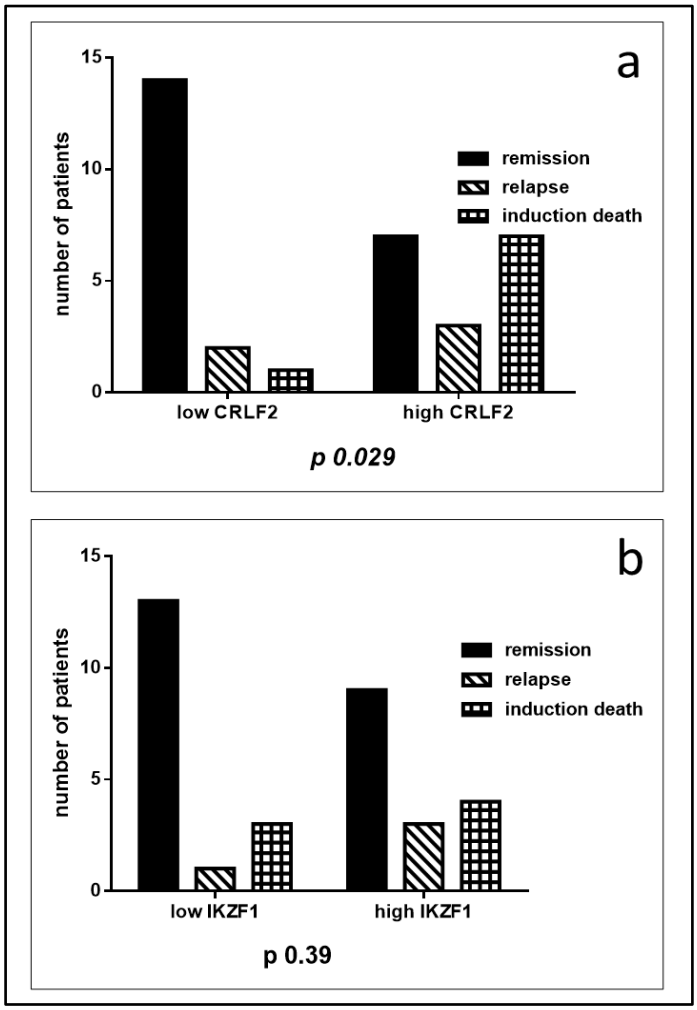

Figure 2: Correlation between CRLF2 (a) and IKZF1 (b) expression and treatment outcome in acute lymphoblastic leukemia patients

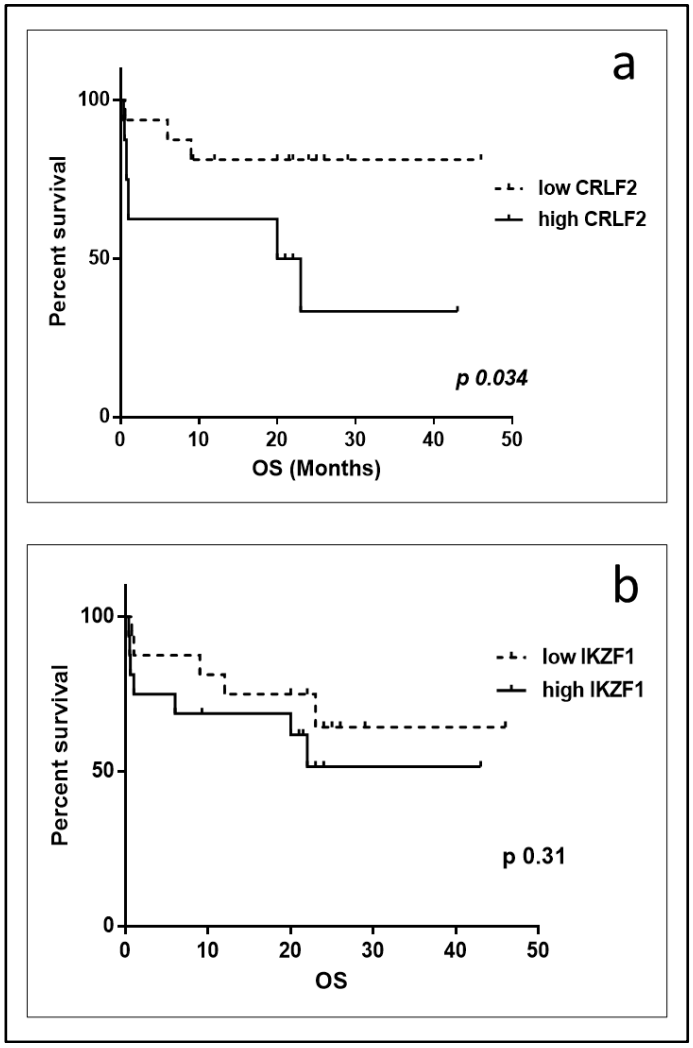

Figure 3: Correlation between CRLF2 (a) and IKZF1 (b) expression and overall survival in acute lymphoblastic leukemia patients

Our analysis demonstrated no difference in IKZF1 expression between T and B-cell ALL and healthy controls. Palmi et al [16] finding was confirmed by Qazi and Uckun [29] who demonstrated no changes in IKZF1 expression from homozygous or heterozygous IKZF1 deletions in primary leukemic cells from Ph+ or Ph- cases. Also, one study by Palmi et al [16] supported the absence of association between IKZF1 deletions and deregulated IKZF1 gene expression.

Despite these findings, we found a significantly higher white blood cells count and lower platelets count in the low IKZF1 expression patients' group when compared to those with high IKZF1 expression. This was explained by Palmi et al [16] who advocated that the unfavorable outcome of IKZF1 deletion might related to genetic instability rather than deletions.

Study limitations

The small number of patients prohibited the assessment of the studied abnormalities as an independent prognostic indicator in ALL. This would necessitate prospective assessment for CRLF2 and IKZF1 in a larger cohort of both B and T ALL cases and using extensive genetic profile to identify this high-risk patient group.

Conclusion

Our study showed that CRLF2 overexpression was linked to poor treatment outcome and worse survival among ALL patients. These results suggest that it may be considered in risk stratification-based therapeutic policy. More research is required for the determination of prognostic significance of IKZF1 in ALL patients.

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The results of the current study were presented as a poster at the 8th Annual Meeting of the Society of Hematologic Oncology (SOHO); Houston, Texas, USA; 9-12 September 2020 (virtual event).

Authors' contribution

Conception or design: LMS and ShA; Acquisition, analysis or interpretation of data: LMS, SuA and MWF; Drafting the manuscript or revising it: All authors; Approval of the manuscript version to be published: All authors; Agreement to be accountable for all aspects of the work: All authors.

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

Data availability

Deidentified individual participant data used to produce the results of this study are available from the corresponding author (LMS) upon request.

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Study registration

None.

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