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Original Article:

# *IKZF1* Alteration in Pediatric B-Cell Acute Lymphoblastic Leukemia: A Single-Center Report on the Frequency of *IKZF1* Deletions and Its Subtypes

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**Abstract**

**Introduction:** The most prevalent malignancy during childhood is B-cell acute lymphoblastic leukemia (B-ALL). Many genetic variations are the causes of B-ALL. IKZF1 alterations are prevalent in childhood B-ALL cases, which are associated with a poor prognosis. This study examined seventy-two pediatric B-ALL patients for the frequency of IKZF1 alteration and types of IKZF1 deletions.

**Materials and Methods:** In this study, bone marrow aspirate specimens at the stage of diagnosis in pediatric B–ALL patients were used. The diagnosis of B-ALL was performed following cytomorphology, cytochemistry, and immunophenotyping based on the 2016 World Health Organization (WHO) guidelines. ALL translocations, including TCF3-PBX1 fusion, ETV6-RUNX1 fusion, BCR-ABL1 fusion, and KMT2A-AFF1 fusion, were performed on DNA specimens of all patients. IKZF1 status was checked with the SALSA MLPA P335 ALL-IKZF1 probemix Kit.

**Results:** The common-B ALL subtype was detected in 64/72 patients (88.9%). CD2 and CD13 aberrant expressions were found in 5/72 (6.9%) and 7/72 patients (9.7%), respectively. Molecular analysis for translocation revealed the frequency of ETV6-RUNX1 in 12/72 patients (16.7%) and BCR-ABL1 in 3/72 (4.2%). IKZF1 alterations were found in 13/72 patients (18%), of whom 10 (13.9%) had IKZF1 deletions. Three common types of IKZF1 deletions were found.

**Conclusion:** The frequency of IKZF1 deletion in this study is similar to the results already obtained in larger studies. The type of IKZF1 deletion related to poor outcomes has a higher frequency in this study. Because of the relatively high prevalence of IKZF1 deletion, its determination is important for better risk stratification and prognosis in pediatric B-ALL patients.

**Keywords:** Acute leukemia, B-ALL, Deletion, IKZF1.

## 1. Introduction

Almost 20% of all pediatric malignancies are acute lymphoblastic leukemia (ALL). In other words, ALL is an aggressive hematological disease that affects patients under the age of 15 [1]. The outcome for children with B-cell ALL (B-ALL) has considerably improved during the past 50 years with a 90% improvement in affluent countries, while 20-25% children still continue to

experience relapse and death in low-income countries. [2,3].

Over the years, B-ALL has had several poor prognosis contributors, including hypodiploid, *KMT2A*-rearranged such as, t(4;11); *KMT2A-AFF1*), t(1;19); (*TCF3-PBX1*), t(9;22); *BCR-ABL1* positive; *BCR-ABL1*-like ALL, and intrachromosomal amplification of chromosome 21 (iAMP21) [4]. *IKZF1* gene alterations were first detected in the 1990s and have frequently been shown to be important for prognosis in B-ALL. [5].

The *IKZF1* gene encodes IKAROS, which is a transcription factor consisting of 519 amino acids. IKAROS is a member of the family of zinc-finger (ZF) proteins binding to DNA. Other members of this family include HELIOS, AIOLOS, EOS, and PEGASUS, which are also called *IKZF2*, *IKZF3*, *IKZF4*, and *IKZF5*, respectively. The *IKZF1* gene is situated on the short arm of chromosome 7 at 7p12.2 and contains 8 exons. The protein-coding areas are exons 2 to 8. Exons 4, 5, and 6 all encode four DNA-attached ZFs, whereas exon 8 provides the 2 C-terminal domain ZFs essential for hetero-or homodimerization. (Figure 1)[6].

IKAROS was identified as one of the necessary and primary lymphoid lineage regulators developments (especially in the differentiation of B-cells progenitor), chromatin remodeler and terminal B-cell maturation. IKAROS modulates genes that are responsible for cell differentiation and cell survival, where the loss of functionality in this gene is a significant factor to suppress the differentiation of cells and the shift to acute B-cell leukemia. [7].

The activity of the IKAROS in the hematopoietic stem cell-multipotent progenitors (HSC–MPP) is recognized to have the potential to be transformed into lymphoid lineages. However, IKAROS activity at the final stages of lymphocyte maturation mediates normal differentiation and development to a non-proliferative phase [8]. Many studies have reported that B-ALL patients with *IKZF1* gene

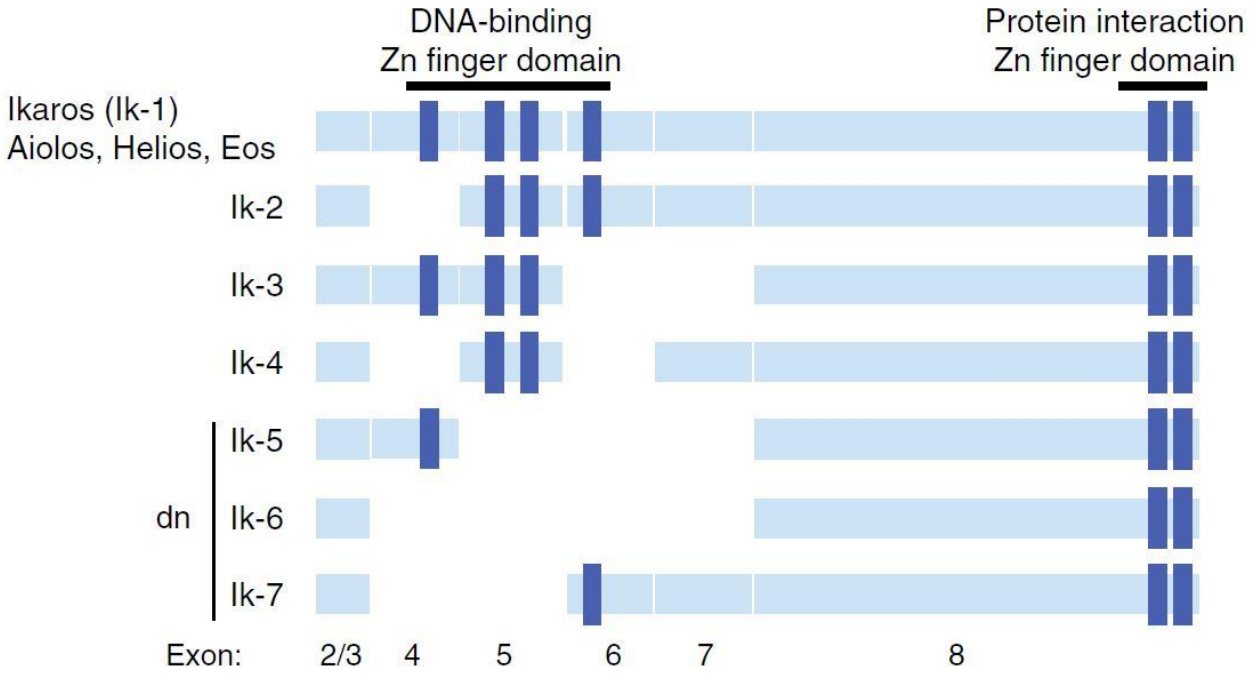


**Figure 1.** IKAROS isoforms are depicted schematically. Light blue boxes show exons. Zinc fingers (ZFs) are indicated by dark blue bars. By either alternative splicing or intragenic deletion, at least sixteen distinct isoforms can be produced; each isoform performs a particular function at different phases of lymphocyte development. The length of these isoforms varies, particularly in the number of N-terminal ZFs, which affects their ability to bind to DNA and other functional characteristics. Importantly, DNA-binding requires at least 3 N-terminal ZFs. IK1 and IK3, which are located in the nuclear, are the only isoforms that have this necessary component for DNA-binding. Two N-terminal ZF motifs can be found in IK4 and only one N-terminal ZF is present in IK5, while the 4 N-terminal ZFs are absent from IK6. The isoforms with non-DNA-binding have a dominant-negative (D-N) effect and have a cytoplasmic mis-localization as opposed to a nuclear one. [6]. Image courtesy of Yoshida et al. [8].

mutation/deletion experience a greater rate of relapse [5,9–11]. Moreover, many investigations have also demonstrated that *BCR-ABL1*-positive B-ALL mainly exhibits *IKZF1* mutations/deletions. These mutations/deletions account for 59%, 65%, and 69% of cases reported by Iacobucci et al., Mullighan et al., and Dupuis et al., respectively [12–14]. *IKZF1* mutations and deletions usually fall into 3 types: (a) deletions in exons 2 to 7 (Δ2-7); (b) deletions in exons 4 to 7 (Δ4–7); and (c) monoallelic or biallelic deletions of the whole gene, in which coding exons are lost ("whole deletions" is the term used below and the second most common type of deletion) [15]. The most prevalent deletion is Δ4-7 (~33%), which results in the production of the Ikaros-6 (Ik6) dominant-negative (D-N) isoform. Ik6 is produced in large amounts and is localized in the cytoplasm because of the absence of the DNA-binding domain and nuclear localization sequences. The third prevalent deletion is Δ2-7, which results in haploinsufficiency by deleting the start codon. Generally, Δ4–7 and whole gene deletions make up the majority (~65%) of the total *IKZF1* deletion in pediatric B-ALL in cohort studies. These single lesions were demonstrated to have prognostic significance [5,9] and according to some research, the type of *IKZF1* deletion may have varied outcomes [6,11].

The ability to determine *IKZF1* status at the diagnosis in B-ALL patients may improve with more accurate risk group stratification and the most efficient treatment approaches [16]. For this reason, the status of *IKZF1* should be investigated. Therefore, this study identified *IKZF1* gene status and types of *IKZF1* deletion in pediatric B-ALL patients who were referred to Payvand Clinical and Specialty laboratory for diagnosing their malignancies.

## 2. Materials and Methods

### Patients

Between January 2019 and December 2021, bone marrow aspirate (BMA) specimens from seventy-two pediatric patients at the stage of diagnosis as B–ALL were taken and deployed in this study. The diagnosis of malignancy as the B-ALL was established following cytomorphology, cytochemistry, and immunophenotyping based on the 2016 World Health Organization (WHO) guidelines [17].

### Immunophenotyping and translocation-associated fusion genes

Bone marrow samples were adjusted to a cellular concentration of 10,000 cells/µL. Subsequently, 50µL of sample per tube was added and then were the surface markers stained with the following monoclonal antibodies incubated in the dark: CD2, CD3, CD7, CD9, CD10, CD13, CD15, CD19, CD20, CD22, CD33, CD34, CD38, CD45, CD66c, CD117, and HLA-DR. For intracellular markers such as TDT, iCD3, iCD79a, iIgM, and iMPO, the Fix&Perm reagent kit (Invitrogen, USA) was used according to the manufacturer's protocols. After incubation, RBC was lysed with Lysis Buffer (AP-RAD, IRAN). Subsequently, they were washed with phosphate buffered saline (PBS) and resuspended cells in 400-µL PBS for analysis. Cells were acquired with the FACSLyric flow cytometer (BD, USA) and the data were analyzed using the FACSuite software v 0.1 (BD, USA).

RNAs were isolated using the Qiagen RNeasy Mini Kit (QIAGEN, Germany) and cDNA was synthetized by a standard procedure (Invitrogen, USA) according to the manufacturer's protocols. The presence of ALL translocations, including *TCF3-PBX1* fusion, *BCR-ABL1* fusion, *ETV6-RUNX1* fusion, and *KMT2A-AFF1* fusion, was detected by RT-PCR (Roche Applied Science, Germany).

### DNA extraction

Genomic DNA was extracted using the FlexiGene DNA Kit (QIAGEN, Germany) as per the manufacturer's protocol. All specimens were quantified using a BioPhotometer (Eppendorf, Germany). The DNA concentration for the MPLA assay was set at 20 ± 1 ng/µL.

### Analysis of *IKZF1* status

DNA was analyzed for *IKZF1* status using the SALSA multiplex ligation-dependent probe amplification (MLPA) P335 C1 ALL-IKZF1 probemix Kit (MRC Holland, Netherlands). This kit included 8 probes for 8 exons in the *IKZF1* hotspot region (Table 1). In a nutshell, according to the manufacturer's instructions, MLPA was performed in 5 steps as following: 1) Denaturation of the DNA phase: a 5 µl DNA sample is heated (total quantity of 100 ng of DNA) for 5 mins at 98°C. 2) Hybridization of probes to DNA; Add 3µl hybridization master mix, incubate for one min at 95°C and hybridize for at least 16 h at 60°C. 3) Ligation of hybridized probes; Reduce thermocycler temp to 54°C, add 32 µl Ligase-65 master mix, incubate for 15 mins at 54°C, and increase temp for inactivating the ligase enzyme for 5 mins at 98°C. 4) Ligated probes amplify by PCR; Cool down to room temp, add 10µl polymerase master mix at room temp, then start PCR (35 cycles {95°C 30 secs, 60°C 30 secs, 72°C 60 secs}, 72°C 20 mins), and 5) Separation of fragments using capillary

**Table 1.** P335 probes for *IKZF1* investigation.

| Exon | Length | Partial sequence |
|---|---|---|
| Ex1 | 269 | TCTTGGCCCCAA-AGCGCGACGCAC |
| Ex2 | 208 | AGACATGTCCCA-AGTTTCAGGTGA |
| Ex3 | 379 | GGGAGGACAGCA-AAGCTCCAAGAG |
| Ex4 | 264 | TACGAATGCTTG-ATGCCTCGGGAG |
| Ex5 | 142 | TGCGGGGCCTCA-TTCACCCAGAAG |
| Ex6 | 470 | TTTTCTGCAGTT-GGTAAACCTCAC |
| Ex7 | 343 | CAAGATAGGATC-AGAGAGATCTCT |
| Ex8 | 288 | GGTGTGCCGCCA-CCCAAGTGCCAA |

electrophoresis. Four healthy controls were used as the reference controls. Intensities of peak results were analyzed using Gene Runner software, which performed the peak ratio in the patient sample/control sample. The gain was defined as cut-off values greater than 1.3, normal values ranging from 1.2 to 0.8, heterozygous deletion 0.4 to 0.7, and homozygous deletion less than 0.25.

### Statistical analysis

The statistical software SPSS 18.0 (SPSS, USA) was used to conduct data analyses. Relationships between variables were estimated using chi-square. Other quantitative results were presented as the median. Statistical significance was considered at *P* values < 0.05.

## 3. Results

### Patients

The study included 72 BMA samples from B-ALL patients with 42 males and 30 females. The male/female ratio was 1.4 and the median age was 4 (ranging from 1 to 14 years). Cytomorphology analysis of all bone marrow smears with wright-giemsa stain indicated 20-98 percent blast cells with a median of 86 percent; also, Myeloperoxidase (MPO) stain as a cytochemistry analysis of blast cells showed that all patients were MPO negative. General characteristics of the patients are presented in Table 2.

The immunophenotype subtypes of common-B, pro-B, and pre-B-ALL were detected in 64/72 (88.9%), 6/72 (8.3%), and 2/72 (2.8%) patients, respectively. Five patients (6.9%) showed CD2 aberrant expression (T-lineage-related marker) and all were found in the common B-ALL subtype. This percentage was reported in a previous study by Eddy et al. [18]. CD13 aberrant was expressed (Myeloid related marker) in 7 patients (9.7%) with common B-ALL subtype, and one of these patients CD13 co-expressed with CD33. CD15 was expressed in one patient (1.4%) with pro B-ALL subtype. Molecular analysis for common translocation revealed t(12;21); *ETV6-RUNX1* in 12/72 patients (16.7%), t(1;19);*TCF3-PBX1* in 1/72 patients (1.4%), t(9;22);*BCR-ABL1* in 3/72 patients (4.2%), and 2/72 patients (2.8%) positive for t(4;11) *KMT2A-AFF1* the genetic abnormality. The frequency of *ETV6-RUNX1*, *TCF3-PBX1*, *BCR-ABL1*, and *KMT2A-AFF1* in B-ALL children was reported in many cohort studies that included 20-28%, 5-6%, 3-4%, and 1-2%, respectively [4,19].

### Immunophenotype and translocation results

Five of 12 patients (41.6%) with *ETV6-RUNX1* fusion expressed CD13, and data showed a correlation between *ETV6-RUNX1* fusion and CD13 expression (5/12, 41.6% vs 2/60, 3.3%, P < .001). These data were observed in previous studies [18] and other fusions were not associated with CD2 aberrant expression.

**Table 2.** Demographic characteristics of B-ALL patients

| Nr. Of Patients | 72 |
|---|---|
| Age | |
| Years, median (range) | 4 (1-14) |
| Sex, n (%) | |
| Male | 42 (58.3%) |
| Female | 30 (41.7%) |
| Immunophenotype, n (%) | |
| Pro-B | 6 (8.3%) |
| Common-B | 64 (88.9%) |
| Pre-B | 2 (2.8%) |
| Genetic abnormality | |
| *ETV6-RUNX1* | 12 (16.7%) |
| *BCR-ABL1* | 3 (4.2%) |
| *TCF3-PBX1* | 1 (1.4%) |
| *KMT2A-AFF1* | 2 (2.8%) |
| Blasts %, median (range) | 86 (20-98) |

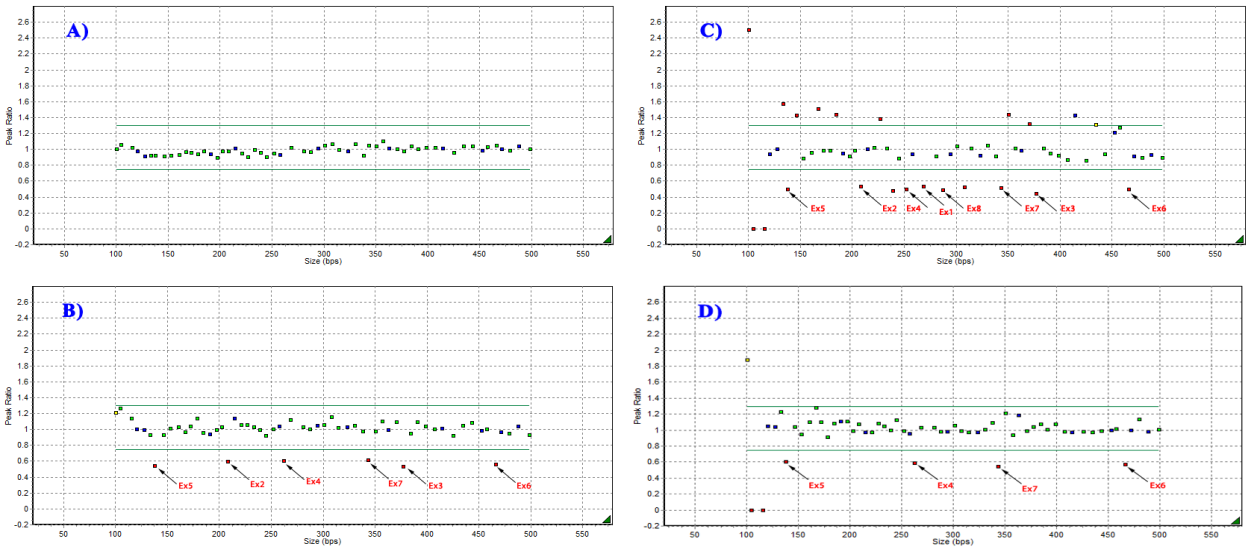


**Figure 2.** Analysis of the data from four different patients with the MPLA P335-IKZF1 kit. This kit contains 57 MLPA probes with amplification products between 120 and 504 nt and is designed for detecting of alteration in 9 different genes. Eight probes were used for detecting alteration in 8 exons of *IKZF1* genes (Table 1); deletion of each of the 8 exons is shown with the black pointer. A) Patient without any deletion in *IKZF1* gene. B) Patient with a deletion in Δ2-7. C) Patient with the whole deletion of *IKZF1* gene. D) Patient with a deletion in Δ4-7. Green, blue and red dots show the normal, reference and abnormal gene probe, respectively. Ex: exon.

## Analysis of the type of IKZF1 deletions by MLPA

Seventy-two patient samples and four normal samples were analyzed by MLPA P335 ALL-IKZF1 probemix. *IKZF1* deletions were found in 10 patients (13.9%). All 10 patients had heterozygous deletions in the *IKZF1* gene (Figure 2).

The most common type of *IKZF1* deletion was the whole gene deletion, which was observed in six patients (60%). The subsequent types of deletions identified were the deletion of Δ2–7 (two patients; 20%) and deletion of Δ4–7 (found in two patients; 20%) predicted to result in the D-N isoform (IK6) (Table 3). In patients with *IKZF1* deletions, two patients had *BCR-ABL1*-positive and one had *KMT2A-AFF1*-positive. Furthermore, three patients were found with whole exon *IKZF1* heterozygous duplications, none of whom had any gene fusions. The frequency of *IKZF1* deletion in *BCR-ABL1*-positive patients was 66.7% (2/3 patients), and the frequency of *IKZF1* deletion in *BCR-ABL1*-negative patients was 11.6% (8/69 patients). Two patients with *BCR-ABL1*-positive showed *IKZF1* deletions with whole gene deletion in one and a deletion in exon 4–7 in the other one.

**Table 3.** Type of *IKZF1*deletion

| Patient | Sex/age | Exon 1 | Exon 2 | Exon 3 | Exon 4 | Exon 5 | Exon 6 | Exon 7 | Exon 8 | Deletion Pattern* | Genetic abnormality |
|---|---|---|---|---|---|---|---|---|---|---|---|
| P1 | M/12 | Del | Del | Del | Del | Del | Del | Del | Del | Whole | - |
| P2 | F/13 | Normal | Del | Del | Del | Del | Del | Del | Normal | Δ2-7 | - |
| P3 | F/13 | Del | Del | Del | Del | Del | Del | Del | Del | Whole | - |
| P4 | F/3 | Del | Del | Del | Del | Del | Del | Del | Del | Whole | - |
| P5 | M/3 | Normal | Del | Del | Del | Del | Del | Del | Normal | Δ2-7 | - |
| P6 | F/7 | Normal | Normal | Normal | Del | Del | Del | Del | Normal | Δ4-7 | - |
| P7 | M/7 | Del | Del | Del | Del | Del | Nor | Del | Del | Whole | *KMT2A-AFF1* |
| P8 | M/1 | Del | Del | Del | Del | Del | Del | Del | Del | Whole | - |
| P9 | M/3 | Del | Del | Del | Del | Del | Del | Del | Del | Whole | *BCR-ABL1* |
| P10 | F/11 | Normal | Normal | Normal | Del | Del | Del | Del | Normal | Δ4-7 | *BCR-ABL1* |

* Exon 2 and whole gene deletions consider loss-of-function/haploinsufficiency and deletion in exon 4-7 consider dominant-negative deletions. M: Male, F: Female, Del: Deletion.

## 4. Discussion

As mentioned, *IKZF1* deletions significantly correlated with the *BCR-ABL1* fusion oncogene. In fact, *IKZF1* deletions are frequently found in B-ALL and lymphoid blast crisis CML [5], indicating that IKAROS deficiency plays a key role in regulating the oncogenic potential of the *BCR-ABL* kinase. As a consequence, IKAROS's regulation of the G1-S transition is lost, which promotes hyperproliferation and the emergence of leukemia. [5]. *IKZF1* deletions and mutations are linked to a poor outcome in terms of overall survival and a high relapse rate. Most research on this has focused on pediatric B-ALL, although it also seems to apply to adult B-ALL. [5,10,20–24]. In children, the deletion of *IKZF1* is a more powerful predictor of relapse than other genetic defects (e.g., *EBF1* or *PAX5* deletions) or the standard classification according to the clinical and cytogenetic criteria [5,21]. The clinical implications for patients with *IKZF1* deletion who are non-high-risk group may be stratified into a high-risk arm. Identification of *IKZF1* deletions may be used as a specific target for a therapeutic approach in the future. [25].

Our study was conducted on pediatric patients diagnosed with B-ALL. In our study, *IKZF1* deletions were detected in 13.9% of all B-ALL patients, similar to those reported by Moorman et al. [21]. Many studies have reported a different range, from 13 to 30.3%, in B-ALL pediatrics [7,26–29]. All the detected deletions were heterozygous or monoallelic. The frequency of IKZF1 deletion in BCR-ABL1-positive patients was 66.6%, a frequency confirmed by similar reports ranging from 56.8 to 70.6% [30–33]. Moreover, in the current study, 11.6% of patients with BCR-ABL1-negative disease had IKZF1 deletions; similar results were observed by Hinze et al. in Germany (12.2%) and Gupta et al. in India (13%) [34,35]; different frequencies were reported ranging from 7 to 16%. [10,29,36–39]. Van der veer et al. reported that *BCR-ABL1*-poitive pediatric B-ALL with *IKZF1* deletions was related to unfavorable outcome, indicating the need for alternative therapy despite of Imatinib exposure [31].

There are two functional subgroups established from the various *IKZF1* deletion types: 1) loss-of-function or haploinsufficiency (deletions in exon 2 or 8 and entire gene deletions), and 2) dominant-negative deletions (Δ4-7) [12]. We found 6/10 patients (60%) with haploinsufficiency and 2/10 patients (20%) with loss-of-function, and 2/10 patients (20%) with D-N deletions. In this study, among the *BCR-ABL1*-negative patients, 87.5 and 12.5% (7 and 1 patient) of *IKZF1* deletions were haploinsufficiency and D-N deletions, respectively. In previous studies, haploinsufficiency (57-79%) and dominant-negative deletions (21-46%) in *BCR-ABL1*-negative patients [36,40,41], and the type of *IKZF1* deletion in *BCR-ABL1*-positive patients, each haploinsufficiency and D-N deletions included 50% of patients. This frequency was confirmed in a previous study by Van der veer et al. [31]. Boer et al. reported that the prognosis of uncommon types of *IKZF1* deletions (such as Δ2-7 and Δ2-8) was the same or poorer compared to the well-known major Δ2-7 and whole deletions. However, all types of *IKZF1* deletions have an important prognostic impact [11]. The difference in the results of the type of *IKZF1* deletion is probably due to the difference in the investigation method. Mitchell et al. [42] reported that between the MLPA and PCR methods, there was disagreement in 21% of patients with *IKZF1* deletions. For this reason, the simultaneous use of both methods helps to identify *IKZF1* mutations and deletions more accurately.

## 5. Conclusion

In this study, 13.9% of pediatric B-ALL patients had *IKZF1* deletions. The most frequent type of *IKZF1* deletion was the loss-of-function/haploinsufficiency deletion (80%). Because of the relatively high prevalence of *IKZF1* deletion, its determination is important for better risk stratification and prognosis in pediatric B-ALL patients. It seems that the use of the MLPA method with the PCR method helps to identify more *IKZF1* deletions.

## Ethical Considerations

### Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Tarbiat Modares University [IR.MODARES.REC. 1401.081].

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### Author's contributions

Conceptualization and Supervision: Behzad Poopak and Masoud Soleimani; Methodology: Mahdi Ghorbani and Hamed Baghdadi; Investigation, Writing – original draft, and Writing – review & editing: All authors; Data collection: Hamed Baghdadi, and Mehdi Shakouri; Data analysis:

Mahmoud Vahidi.

## Conflict of interest

The authors declare that they have no conflict of interest.

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