**Clinical impacts of copy number variations in B-cell differentiation and cell cycle control genes in pediatric B-cell acute lymphoblastic leukemia: a single centre experience**

Klementina Crepinsek¹², Gasper Marinsek¹, Marko Kavcic²³, Tomaž Prelog³, Lidija Kitanovski³, Janez Jazbec²³, Marusa Debeljak¹²

¹ Clinical Institute for Special Laboratory Diagnostics, University Children’s Hospital, University Medical Centre Ljubljana, Ljubljana, Slovenia
² Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia
³ Department of Oncology and Haematology, University Children’s Hospital, University Medical Centre Ljubljana, Ljubljana, Slovenia

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Correspondence to: Assist. Prof. Maruša Debeljak, Ph.D., Clinical Institute for Special Laboratory Diagnostics, University Children’s Hospital, University Medical Centre Ljubljana, Vrazov trg 1, 1000 Ljubljana, Slovenia. E-mail: marusa.debeljak@kclj.si

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**Background.** IKZF1 gene deletions have been identified as a poor prognostic factor in pediatric B-cell acute lymphoblastic leukemia (B-ALL), especially in the presence of co-occurring deletions (IKZF1plus profile). This study aimed to determine the frequency of IKZF1 deletions and deletions in other B-cell differentiation and cell cycle control genes, and their prognostic impact in Slovenian pediatric B-ALL patients.

**Patients and methods.** We studied a cohort of 99 patients diagnosed with B-ALL from January 2012 to December 2020 and treated according to the ALL IC-BFM 2009 protocol. Eighty-eight bone marrow or peripheral blood samples were analysed for copy number variations (CNVs) using the SALSA MLPA P335 ALL-IKZF1 probemix.

**Results.** At least one CNV was detected in more than 65% of analysed samples. The most frequently altered genes were PAX5 and CDKN2A/B (30.7%, 26.1%, and 25.0%, respectively). Deletions in IKZF1 were present in 18.2% of analysed samples and were associated with an inferior 5-year event-free survival (EFS; 54.8% vs 85.9%, p = 0.016). The IKZF1plus profile was identified in 12.5% of the analysed samples, and these patients had an inferior 5-year EFS than those with deletions in IKZF1 only and those without deletions (50.8% vs 75.0% vs 85.9%, respectively, p = 0.049). Overall survival (OS) was also worse in patients with the IKZF1plus profile than those with deletions in IKZF1 only and those without deletions (5-year OS 76.2% vs 100% vs 93.0%, respectively). However, the difference between the groups was not statistically significant.

**Conclusions.** Our results are in concordance with the results obtained in larger cooperative clinical trials. Copy number variations analysis using the SALSA MLPA kit is a reliable tool for initial diagnostic approach in children with B-ALL, even in smaller institutions in low- and middle-income countries.

Key words: B-acute lymphoblastic leukemia; IKZF1 deletions; IKZF1plus; MLPA; pediatric; copy number variations (CNVs)

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**Introduction**

Improvements in risk stratification and new therapeutic approaches have dramatically improved treatment outcomes in pediatric B-cell acute lymphoblastic leukemia (B-ALL). In developed countries, the overall survival for these patients is approaching 90%. Nevertheless, some genetic
subtypes still imply poor outcomes, and 10–20% of patients experience a relapse that is often accompanied by treatment resistance and failure.\textsuperscript{3,4} Therefore, the need for new diagnostic and prognostic markers remains of paramount importance.

In the previous years, deletions in the $IKZF1$ gene have been identified as an important predictor of relapse in B-ALL.\textsuperscript{5-9} $IKZF1$ gene is located on chromosome 7p12.2 and consists of 8 exons, and of those, exons 2–8 are protein-coding. The gene codes for the transcription factor IKAROS, which regulates the expression of genes that control cell cycle progression and cell survival. It is involved in the development of all lymphoid lineages, especially in the differentiation of B-progenitor cells. Exons 4–6 encode four zinc finger DNA-binding domains that are essential for the tumour-suppressive function of IKAROS, and exon 8 encodes two zinc fingers that are responsible for the homo- or heterodimerization of IKAROS.\textsuperscript{10,11} Genomic deletions in $IKZF1$ occur in around 15% of pediatric B-ALL cases.\textsuperscript{5,6,8,12-14} Their occurrence is exceptionally high in $BCR$-$ABL1$-positive (70%)\textsuperscript{15,16} and $BCR$-$ABL1$-like (40%)\textsuperscript{13,17} B-ALL, and have been associated with poor treatment response and an increased risk of relapse.\textsuperscript{5-9} Deletions are most common in exons 4–7 or affect the whole gene, however, other less common lesions may also be present (e.g., deletions in exons 2–8, 2–7), and they are all associated with an unfavourable outcome in pediatric B-ALL.\textsuperscript{6,18}

Recently, another, minimal residual disease (MRD) dependent prognostic profile $IKZF1_{\text{plus}}$ with an immensely poor prognostic value was identified. This profile is defined by additional deletions in genes involved in cell differentiation and cell cycle regulation. $IKZF1$ deletions that co-occur with deletions in $CDKN2A$, $CDKN2B$, $PAX5$, or the PAR1 region (deletions of $CSF2RA$ and $IL3RA$, but not $CRLF2$) in the absence of $ERG$ deletions are associated with the worst event-free and overall survival.\textsuperscript{21} This profile is already being used in the current AIEOP-BFM ALL 2017 trial as a high-risk criterion.\textsuperscript{39} Copy number variations (CNVs) in some aforementioned genes ($PAX5$, $CDKN2A$, $CDKN2B$) also seem to be independently associated with poor prognosis, however, the results remain conflicting.\textsuperscript{19,22-25} The inclusion of the CNV status of these genes may significantly improve risk stratification in B-ALL, but more studies are needed to elucidate their true prognostic effect.

Deletions in $IKZF1$ are mostly observed in high-risk pediatric ALL subtypes. Many studies have confirmed the association of $IKZF1$ deletions and $IKZF1_{\text{plus}}$ profile with poor treatment outcomes. In Slovenia, no studies have been done yet to determine the frequency of $IKZF1$ deletions and CNVs in other cell differentiation and cell cycle regulation genes in pediatric B-ALL patients and treatment outcomes for these patients. Due to the importance of these alterations in the prognosis and choosing the best treatment approach, it is of great importance to determine their presence. Therefore, the study aimed to analyze bone marrow samples from Slovenian pediatric patients diagnosed with B-ALL from January 2012 to December 2020 for the presence of these CNVs and to determine their prognostic value.

Patients and methods

Patients and samples

In total, 99 children with B-ALL that were treated at the University Children’s Hospital, University Medical Centre Ljubljana between January 2012 and December 2020 according to the ALL IC-BFM 2009 protocol were included in this study. Diagnoses were established following standard clinical, cytomorphological, and immunological criteria.

We obtained bone marrow samples for 92 patients as part of the diagnostic procedure before starting treatment. For 7 patients, bone marrow samples were not available, therefore, peripheral blood samples were obtained for the analysis. For four patients, there was no sufficient material available to perform the multiplex ligation-dependent probe amplification (MLPA) assay, 3 samples contained less than 40% of blasts and were excluded from analysis, and for an additional four, the assay failed due to poor sample quality. Therefore, data analysis was performed on 88 patient samples (82 bone marrow and 6 peripheral blood). The bone marrow samples contained $77.4 \pm 16.7\%$ of blast in average, and for the peripheral blood samples this value was $77.8 \pm 16.0\%$. For survival analysis, patient samples from the year 2020 were excluded, due to the short follow-up period. Therefore, the survival analysis was carried out on 72 patients diagnosed between January 2012 and December 2019.

Informed consent was obtained from all subjects involved in the study, or their parents. The
study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Republic of Slovenia (reference number KME 51/03/11).

DNA extraction

Genomic DNA was extracted from bone marrow or peripheral blood samples using the FlexiGene DNA Kit 250 (QIAGEN®, Hilden, Germany) according to the manufacturer’s instructions. All samples were quantified using DS-11 FX+ Spectrophotometer (DeNovix, Wilmington, USA), and stored at 4°C. Before analysis, the DNA concentration was established at 25 ± 1 ng/μL for the MLPA assay.

TABLE 1. The demographic and clinical characteristics of Slovenian B-ALL patients included in the study

| Characteristic                           | Nr. of patients |
|-----------------------------------------|-----------------|
| Nr. of patients                         | 99              |
| Sex                                     |                 |
| Male                                    | 54 (54.5%)      |
| Female                                  | 45 (45.5%)      |
| Primary genetic abnormalities           |                 |
| ETV6-RUNX1                               | 28 (28.3%)      |
| BCR-ABL1                                 | 7 (7.1%)        |
| KMT2A rearrangements                     | 4 (4.0%)        |
| TCF3-PBX1                                | 4 (4.0%)        |
| Hyperdiploidy                            | 27 (27.3%)      |
| Hypodiploidy                             | 3 (3.0%)        |
| iAMP21                                   | 2 (2.0%)        |
| No recurrent abnormalities               | 24 (24.2%)      |
| Age at diagnosis                         |                 |
| < 1                                      | 3 (3.0%)        |
| 1–5                                      | 57 (57.6%)      |
| ≥ 6                                      | 39 (39.4%)      |
| Risk group                               |                 |
| Standard risk                            | 17 (17.2%)      |
| Intermediate risk                        | 59 (59.6%)      |
| High risk                                | 23 (23.2%)      |
| FC- minimal residual disease             |                 |
| Day 15                                   |                 |
| < 0.1%                                   | 35 (35.4%)      |
| 0.1–10%                                  | 48 (48.5%)      |
| > 10%                                    | 13 (13.1%)      |
| Unknown                                  | 3 (3.0%)        |
| Day 33                                   |                 |
| < 0.01%                                  | 73 (73.7%)      |
| 0.01–1%                                  | 20 (20.2%)      |
| > 1%                                     | 3 (3.0%)        |
| Unknown                                  | 3 (3.0%)        |

DNA was analysed for copy number alterations using the SALSA MLPA P335 ALL-IKZF1 probemix, according to the manufacturer’s instructions (MRC Holland, Amsterdam, the Netherlands). The P335 probemix allows for the detection of deletions and duplications in B-cell differentiation and cell cycle control genes (IKZF1, CDKN2A/B, PAX5, EBF1, ETV6, BTG1, and RB1), as well as in genes from the X/Y PAR1 region (CRLF2, CSF2RA, SHOX, IL3RA, and P2RY8). It also contains 13 reference probes that function as internal controls. Additionally, analysis for copy number alterations for the determination of ERG status was carried out on samples that carried deletions in IKZF1 and at least one additional gene (namely CDKN2A, CDKN2B, PAX5, CSF2RA, and IL3RA) with the SALSA MLPA P327 iAMP21-ERG probemix. The P327 probemix is used for the detection of deletions, duplications or amplifications of specific sequences on chromosome 21, including intragenic deletions of ERG. It contains 59 MLPA probes that bind to several regions on chromosome 21, including the ERG gene, and 13 reference probes. DNA samples from healthy donors were used as controls.

MLPA reactions were carried out on a 96-well PCR thermocycler SimpliAmp Thermal Cycler (Applied Biosystems, Thermo Fisher, Massachusetts, USA), and the products were separated by capillary electrophoresis on an ABI-3500 genetic analyser (Applied Biosystems, Thermo Fisher, Massachusetts, USA). The resulting peak intensities were analysed using Coffalyser software (MRC-Holland) which performed the intrasample and intersample normalization of the peaks with the manufacturer’s reference probes and normal control DNA, respectively. Values above 1.3 were considered as gain, between 1.3 and 0.75 normal, between 0.75 and 0.25 heterozygous loss, and below 0.25 homozygous loss.

Statistical analysis

All statistical analyses were performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA).
RESULTS

Study group

The cohort included 54 males and 45 females (N = 99), with median age 4 years (range from 1 day to 23 years). Of these, 75 harboured recurrent genetic abnormalities (28 ETV6-RUNX1, 27 hyperdiploid karyotype, 7 BCR-ABL1, 4 KMT2A rearrangements, 4 TCF3-PBX1, 3 hypodiploid karyotype and 2 iAMP21), while no genetic alterations were identified in 24 patients (Figure 1). Based on age, white blood cell (WBC) count at diagnosis, blast cell counts on day 8, genetic abnormalities, and MRD at days 15 and 33, 17 patients were classified as standard risk (SR), 59 as intermediate risk (IR), and 23 as high risk (HR). The main patient characteristics are summed up in Table 1.

Detection and analysis of IKZF1 deletions by MLPA

Altogether, 92 patient samples and 5 controls were analysed by MLPA using the SALSA MLPA P335 ALL-IKZF1 probemix. Four patient samples failed the MLPA analysis. Out of the remaining 88 samples, IKZF1 deletions were found in 16 (18.2%). The most common was the deletion of the whole gene, which was observed in eight patients (50%). Others were focal deletions. The second most common deletion was the deletion of exons 4–8, which was found in four patients (25%). This deletion was described as rare in other studies. Other deletions that were also detected in our cohort were the deletion of exons 2–8 (two patients; 12.5%), 4–7 and 5 (both found in one patient; 6.3%). Eleven patients with IKZF1 deletion had additional deletions present, which put them in the IKZF1 plus group. Of these, six carried additional deletions in CDKN2A, CDKN2B and PAX5, three in CDKN2A and CDKN2B, one in only CDKN2B, and one had deletions in the PAR1 region. ERG deletions were not found in any of these 11 samples.

The comparison of patient characteristics depending on IKZF1 deletion is summarized in...
Table 2. Of the 16 patients that harboured IKZF1 deletions, five patients had no recurrent genetic alterations. The presence of primary recurrent genetic abnormalities found in patients with IKZF1 deletions is shown in Figure 2. IKZF1 deletions were significantly more common in Ph+ patients than in Ph− patients (6/7, 85.7% vs. 10/81, 12.3%, p = 0.0001), and so was the presence of the IKZF1plus profile (4/7, 57.1% vs. 7/81, 8.6%, p = 0.004).

The IKZF1 deletions were significantly more common in males than in females (p = 0.0045), however, the presence of the IKZF1plus profile did not significantly differ between the sexes (p = 0.0607).

Patients with IKZF1 deletions were also older at diagnosis than those without deletions (median age 6 years, interquartile range (IQR) = 8 years vs. median age 4 years, IQR = 3.25 years, p = 0.052), and so were the patients with IKZF1plus profile in comparison to those who did not have this profile (median age 6 years, IQR = 8 years vs. median age 4 years, IQR = 4, p = 0.136), however the differences were not statistically significant. Patients with IKZF1 deletions showed higher blast count values on the 8th day of chemotherapy treatment (median blast count 252 blasts/μL, IQR = 2018.5 blasts/μL vs. median blast cell count 17 blasts/μL, IQR = 192,5 blasts/μL, p = 0.0005), higher values of MRD on day 15 (median MRD15 6.15% IQR = 30.67% vs. median MRD15 0.184%, IQR = 1.24%, p = 0.0005), and 33 of treatment (median MRD33 0.001%, IQR = 0.15% vs. median MRD33 0.000%, IQR = 0.01%, p = 0.033) compared to those without deletions. Similarly, blast cell count and MRD15 values of patients with the IKZF1plus profile were higher compared to patients without the profile (median blast cell counts 224 blasts/μL, IQR = 2112 blasts/μL vs. median blast cell count 24 blasts/μL, IQR = 220 blasts/μL, p = 0.006; median MRD15 3.8%, IQR = 17.6% vs. median MRD15 0.24%, IQR = 2.23%, p = 0.030), while the difference was not significant for MRD33. The deletions were also more common in the HR group than in the IR (10/23, 43.5% vs. 6/52, 11.5%, p = 0.0045). Patients in the HR group also exhibited the IKZF1plus profile more often than those in the IR (7/23, 30.4% vs. 4/52, 7.7%, p = 0.0160) and the SR group (7/23, 30.4% vs. 0/13, 0%, p = 0.0294).

In our cohort, 13 patients (14.8%) experienced an event (either relapse or death). Among these, four patients had no recurrent genetic alterations, three patients carried the ET6-RUNX1 fusion gene, two the BCR-ABL1 fusion gene, two were hyperdiploid, one had a KMT2A rearrangement and one carried the TCF3-PBX1 fusion gene. One patient was classified as SR, eight as IR and four as HR.

In this group, five patients (5/13, 38.5%) carried an IKZF1 deletion, and of those, four had additional deletions, but lacked the ERG deletions, which met the criteria for the IKZF1plus profile. The presence of IKZF1 deletions and the IKZF1plus profile was...
higher in the group of patients who experienced an event in comparison to those who did not, however the difference did not reach statistical significance (5/13 vs. 11/75, p = 0.055 and 4/13 vs. 7/75, p = 0.053, respectively).

Detection and analysis of other gene deletions and duplications by MLPA

The SALSA MLPA P335 ALL-IKZF1 probemix can detect deletions or duplications in the following B-cell differentiation and cell cycle control genes: IKZF1, EBF1, CDKN2A/B, PAX5, ETV6, BTG1, RBL, and in the PAR1 region (SHOX area, CRLF2, CSF2RA, IL3RA and P2RY8 genes). At least one CNV was detected in 60 patient samples (68.2%). Of those, 60% carried three or more CNVs, 28.3% carried only one CNV, and 11.7% carried two CNVs (Figure 3).

The most common CNVs were those in the PAX5 gene that were present in 30.7% of analysed samples, followed by CDKN2A and CDKN2B that were altered in 26.1% and 25.0% of analysed samples, respectively. In these genes, deletions were more common than amplifications. CNVs were also very common in the PAR1 region, 22.7% of analysed samples had at least one CNV present in this region, and in these genes, amplifications were observed more often than deletions. Detailed information about CNVs in all analysed genes are shown in Figure 4.

Prognostic significance of IKZF1 deletions

First, the patients were divided into two groups, a group with and a group without IKZF1 deletions. The 5-year EFS was significantly worse for patients harbouring IKZF1 deletions, compared to those without the deletions (54.8% vs. 85.9%, p = 0.016) (Figure 5A). The 5-year OS was slightly worse as well for these patients, although the difference was not statistically significant (81.5% vs. 93.0%, p = 0.295) (Figure 5B).

The 5-year EFS and OS were also compared between groups with no IKZF1 deletions, with deletions in the IKZF1 gene only and with the IKZF1plus profile. The difference in EFS between groups was statistically significant (p = 0.049). Pairwise comparison showed that patients in group IKZF1plus had significantly poorer EFS in comparison to those in group with no IKZF1 deletions (5-year EFS 50.8% vs. 85.9%, p = 0.016), while the difference was not significant between other groups (Figure 6A).

The OS analysis between groups showed no differences between the groups (Figure 6B).

A multivariate Cox regression model was applied to this data to see, whether after adjusting for other relevant risk factors, IKZF1 deletions profile retained a prognostic impact on event-free survival. We included the following variables in the model: sex, age at diagnosis, risk group (HR vs. nonHR) and the presence of IKZF1 deletions. The overall model showed borderline significance (p = 0.05). When each separate variable was inspected, it was observed that both sex and the presence of the IKZF1 deletions showed a certain trend (males having poorer survival than females (HR = 1.44), and those with the IKZF1 deletions having poorer survival than those without the deletions (HR = 1.15)), however, they did not reach significance (p = 0.072 and p = 0.078, respectively). Similar results were obtained when this analysis was applied for the IKZF1plus profile. This can most likely be attributed to the small sample size of our sample for survival analysis.

FIGURE 4. Frequency of copy number variations: (A) Gene deletions in the cohort. (B) Gene amplifications in the cohort.

| Genes       | Frequency (%) |
|-------------|--------------|
| BTG1        | 17.0         |
| CDKN2A      | 17.0         |
| CDKN2B      | 17.0         |
| CRLF2       | 17.0         |
| CSF2RA      | 17.0         |
| IL3RA       | 17.0         |
| IKZF1       | 17.0         |
| JAK2        | 17.0         |
| IL3RA       | 17.0         |
| P2RY8       | 17.0         |
| SHOX        | 17.0         |
| RB1         | 17.0         |

A B

N=88
We further looked at the group of patients, classified as non-high risk (either SR or IR). In this group, six patients carried the \textit{IKZF1} deletions, and of those, two patients experienced an event (2/6, 33.3%), and among the patients without the deletions, seven experienced an event (7/59, 11.9%) (p = 0.1907). The 5-year EFS for those without the deletions was 83.6%, while it was only 50% for those with the deletion. Once again, there is a certain trend to be seen, however, the difference was not statistically significant (p = 0.114). We also examined patients with the \textit{IKZF1} plus profile in the non-HR group. The frequency of events was higher amongst the patients with the profile compared to those without it (2/4, 50% vs. 7/61, 11.5%, p = 0.0890), and their EFS was poorer, although not significantly (50.0% vs. 83.7%, p = 0.087).
Discussion

Various alterations in genes involved in cell differentiation and cell cycle regulation are a hallmark of B-ALL. The role of deletions in the IKZF1 gene in B-ALL has previously been described as very prognostically revealing and predictive for relapse.6,21 Due to their association with poorer treatment outcomes, it is important to detect them to adjust the treatment protocol accordingly. Moreover, alterations in other genes, such as PAX5 and CDKN2A/2B, are also often present. However, information regarding their prognostic impact is still rather ambiguous19,22,23,26, therefore, more studies need to be carried out to define their true value for patient risk stratification.

Our study was carried out on a smaller (88 patients), yet consecutive, unselected, and well-controlled population of pediatric patients with B-ALL. This is the first report about CNVs in cell differentiation and cell cycle regulation genes in Slovenian pediatric B-ALL patients. In our cohort, IKZF1 deletions were identified in 18.2% of analysed samples. This is in concordance with reports of IKZF1 deletions ranging from 10.7 to 15.9% in other studies that analysed unselected cohorts.6,8,12-14,21 The IKZF1 deletions were more common in the HR group when compared to the IR and SR groups, as was also shown in the study done by Dörge et al.6 Six out of seven (86%) patients with the BCR-ABL1 translocation also carried deletions in the IKZF1 gene, four of those had the IKZF1plus profile. As it was previously described, these deletions are very common, however, not exclusively, present in BCR-ABL1 ALL.12,15,16,21 Some studies excluded patients with the BCR-ABL1 translocation and determined the IKZF1 status only in the BCR-ABL1-negative patients. These studies reported frequencies of IKZF1 deletions from 9.4 to 16%.7,27-30 Our results show that 12.3% of patients without BCR-ABL1 in this cohort carried IKZF1 deletions, which is again similar to previously published results.

The most common IKZF1 deletion in our cohort was, as was also reported by other studies, the deletion of the whole gene (50%). Interestingly, however, the second most common deletion was that of exons 4–8 (25%), whereas this deletion was described as rare in other studies. The second most common deletion reported by other groups was that of exons 4–76,8,18,31 which results in a dominant-negative isofom (IK6). This deletion occurred in only one patient in our cohort. Nevertheless, all IKZF1 deletions, including the rare ones, have an important prognostic impact.15 Therefore, all should be considered when selecting the appropriate treatment.

In our cohort, IKZF1 deletions and the IKZF1plus profile were more common amongst males and were associated with higher blast values on day 8 of chemotherapy, higher values of MRD15 and MRD33, and were more often found in the HR group. The presence of IKZF1 deletions and the IKZF1plus profile was higher amongst the patients who experienced an event (relapse or death) than those who did not, albeit this difference was not significant. Two of the patients who carried these deletions and experience a relapse also had the BCR-ABL1 fusion gene, which placed them in the HR group. However, three others with these deletions and an event did not exhibit genetic alterations that would predict a poorer outcome – one patient carried the ETV6-RUNX1 fusion, one was hyperdiploid and one had no recurrent genetic abnormalities. This shows that alterations in cell differentiation and cell cycle regulation genes may play a crucial role in disease development even in patients with primary genetic alterations that are thought to be favourable, which is in concordance with reports of these deletions being an independent prognostic factor.6

Previously published studies have shown a poorer event-free and overall survival of patients with IKZF1 deletions in comparison to those without such aberrations6,8,12,14,16,30, and Stanulla et al. discovered that patients with additional mutations in certain genes that define the IKZF1plus profile have even more dismal outcomes.21 Our study similarly confirmed the inferior event-free survival for patients with IKZF1 deletions and the IKZF1plus profile, however, the overall survival did not significantly differ between the groups. After adjusting for confounding factors, our data did not confirm the independent prognostic role of the IKZF1 deletions, as well as IKZF1plus, on EFS. However, the trend of poorer EFS in patients with the IKZF1 deletions and the IKZF1plus profile was observed. These discrepancies, as well as the high proportion of males presenting with the IKZF1 mutations, are most likely the result our small sample size and short follow-up duration. When inspecting non-high-risk patients with the IKZF1plus profile, the relative frequency of events among the patients with the profile was higher, and there was a trend of poorer EFS for these patients. The dismal outcomes for patients with the IKZF1plus profile have previously been confirmed by other studies, and this profile is currently already being used in cer-
tain treatment protocols\textsuperscript{30}, and more are likely to follow.

The CNV analysis of other genes showed that the most common alterations were in the PAX5 gene (30.7%), CDKN2A, and CDKN2B (26.1% and 25.0%, respectively). However, deletions in CDKN2A/2B were more common than deletions in PAX5 which was also observed by Öfverholm et al.\textsuperscript{31} and Mullighan et al.\textsuperscript{9} Deletions in PAX5 have previously been reported as much more common than intragenic amplifications in this gene, and the latter have mostly been reported only in isolated cases.\textsuperscript{31,34} However, more recently, Schwab et al.\textsuperscript{23} showed that PAX5 amplifications occur in around 1% of B-ALL cases, out of which 40% experienced a relapse, suggesting that these alterations may play an important role in leukemogenesis. Interestingly, in our cohort, the amplifications were even more common, as they occurred in 10.2% of all B-ALL cases. Out of 27 patients with CNVs in PAX5, 9 (33.3%) carried amplifications. As already suggested by Schwab et al.\textsuperscript{23}, more studies need to be conducted to evaluate the prognostic impact of PAX5 amplifications. The PAX5 deletions were also more frequent in our cohort (20.5%) than previously described (10%).\textsuperscript{35,36} Amongst the samples with PAX5 deletion, a sizable amount (61.1%) also carried the CDKN2A/2B deletions. The frequent co-occurrence of these deletions has previously been described by Kim et al.\textsuperscript{37}

In our cohort, amplifications in the PAR1 region were observed quite frequently. Altogether, 17 patients (19.3%) had at least one gene amplification in this region. This is due to the fact that our cohort is unselected, and therefore also includes patients with hyperdiploidy. In hyperdiploidy, gains in the X chromosome are very common (present in 70% of hyperdiploid childhood B-ALL cases)\textsuperscript{38}, and indeed, 14 out of our 17 patients with amplifications in PAR1 had a hyperdiploid karyotype. This karyotype is associated with a favourable outcome. However, the hyperdiploid patients in our cohort did not have a significantly better 5-year EFS, and the same was seen for the patients with amplifications in the PAR1 region that were identified with MLPA. Two patients carried deletions in this region (namely in CSF2RA, IL3RA and P2RY8 genes), which resulted in the formation of the P2RY8-CRLF2 fusion gene. This was confirmed with fluorescence in situ hybridization. While the P2RY8-CRLF2 fusion is associated with a poorer treatment response and outcomes\textsuperscript{39}, the two patients in our cohort did not experience an event.

Despite the limitations of our study due to a lower number of analyzed samples and relatively short follow-up period, it produced results that are in concordance with the results obtained in larger cooperative clinical trials. We have shown that it is possible to provide comparable results regarding the presence of certain CNVs and their prognostic value in pediatric B-ALL patients even within a single-center experience. This study is only a starting point for the more comprehensive screening of patients diagnosed with B-ALL in Slovenia that we have planned for the future and will enable us to better evaluate and treat these patients.

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