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Expression of aberrantly spliced oncogenic Ikaros isoforms coupled with clonal IKZF1 deletions and chimeric oncogenes in acute lymphoblastic leukemia

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1. Introduction

Ikaros, protein encoded by *IKZF1* gene, is a member of 'zinc-finger' family of transcription factors. It was discovered as a critical regulator of hematopoiesis, particularly lymphoid cell differentiation, proliferation and function [1,2]. Knock-out mouse experiments demonstrated crucial role of Ikaros for T- and B-cell development and revealed its role of a tumor suppressor of T-cells in mice [3–5]. The function of Ikaros is normally regulated by alternative splicing, generating a number of isoforms. At least 13 isoforms of human Ikaros have been described (Fig. 1). Long isoforms (Ik1 to 3) have at least three zinc fingers, which are able to bind DNA and, therefore, are considered to be functional. Short isoforms (Ik4 to 10) lack of two or more zinc-finger domains, so they cannot bind DNA and impair the function of Ikaros proteins in a dominant-negative (DN) manner [6,7].

Overexpression of short Ikaros isoforms, especially Ik6, was observed in some cases of acute lymphoblastic leukemia (ALL) [8,9] [10,11]. However, interest to *IKZF1* aberrations in clinical hematology arose after CGH- and SNP-arrays implementation. Genome-wide genotyping discovered high frequency of intragenic *IKZF1* deletions in leukemia [12,13]. These deletions were proved to be associated with poor prognosis of leukemia treatment [14]. To date, typical types of *IKZF1* deletions in ALL were described [15–17]. Large clinical studies validated application of *IKZF1* deletions as an independent prognostic marker [18–20]. Analysis of *IKZF1* deletions may be hampered by both biallelic and biclonal deletions [21] or deletions in minor subclones of leukemic cells [15].

Unlike *IKZF1* deletions, limited number of clinical analysis were based on expression of Ikaros isoforms in ALL [22,23]. In these studies, qualitative detection of Ik-DN PCR band in agarose gel was used. To our knowledge, quantitative analysis of the expression of various Ikaros isoforms in ALL still has not been performed. In previous studies, qualitative detection of Ik-DN PCR band in agarose gel or immunoblotting was used to discriminate different isoforms of Ikaros on RNA or protein level. The only quantitative analysis using real-time PCR was performed by Olivero et al. [24]. The authors used two sets of primers and a probe to detect separately Ik1 + 2 and Ik1 + 2 + 4 + 7 + 8 to calculate the ratio between large and short isoforms. In that and some other studies, the whole set of short isoforms Ik6, Ik9, Ik10 was not included in the analysis. Although high incidence of *IKZF1* aberrations is well known to be associated with BCR/ABL1, their relation to other chimeric oncogenes has not been investigated.

In this study, we developed and applied method of RQ-PCR estimation of different Ikaros isoform expression, as well as PCR-based and iFISH-based analyses of *IKZF1* intragenic deletions in ALL. Diagnostic cut-off of aberrant Ikaros isoforms was established by comparison of Ikaros expression between normal MNCs from healthy donors and leukemic samples. Ikaros expression profile was analyzed to detect *IKZF1* deletions and chimeric oncogenes expression. Finally, we separated a group of patients with subclonal *IKZF1* deletions and observed stability of *IKZF1* aberrations between de novo diagnosis and relapse in paired ALL cases.

2. Materials and methods

2.1. Patients and samples

270 bone marrow (BM) samples from acute lymphoblastic leukemia (ALL) patients were used in this study. All patients were diagnosed and treated at the Belarusian Centre of Pediatric Oncology, Hematology and Immunology from July 2002 to December 2015. All samples were obtained according to the local ethical guidelines. The guidelines are an accurate representation of the declaration of Helsinki and effectively applied in our Center. Ethical approval has been obtained from patients

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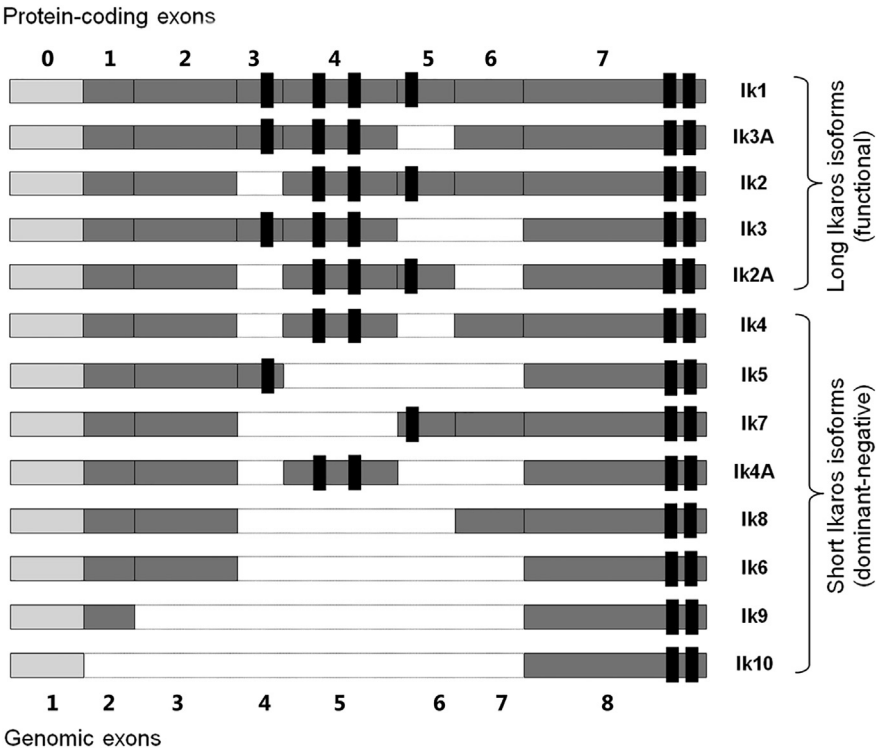

Fig. 1. Schematic representation of human Ikaros isoforms generated by alternative splicing. Zinc fingers are represented as short vertical bars.

or their parents for the use of these BM samples under local guidelines.

Morphology, immunophenotypic, molecular genetics and cytogenetic analyses were performed at first diagnosis and repeated at the first relapse. Specifically, the following translocation-associated fusion genes were investigated for 260 ALL cases by reverse transcription (RT)-PCR assay: BCR/ABL1, SIL1/TAL1, E2A/PBX, TEL/AML1, and MLL translocations (MLL1/ENL, MLL1/AFX1, MLL1/AF17, MLL1/AF-1p, MLL1/AF4, MLL1/AF6, MLL1/AF9, MLL1/AF10).

Clinical and laboratory characteristics of the patient cohort are presented in Table 1. Bone marrow blast cell count varied from 6.8 to 99.5% (median 91.25%). Liquor or excised tissue was used for *IKZF1* examination of three patients with extramedullary relapses (2 CNS and 1 testis). Bone marrow samples with < 40% of blast cells were excluded from quantitative analysis.

Both de novo and relapses of ALL were studied for *IKZF1* deletions and expression as independent samples. Patient cohort consisted of 197 de novo cases and 73 relapses of ALL, including 45 paired cases (40 patients with first relapse and 5 with two relapses).

This study was performed as retrospective, using material preserved as frozen cell pellets or cell lysates, and only partly using the latest prospective BM samples. The inclusion of patients and samples into this study was accidental and based exclusively on availability of material and quality of DNA and RNA samples. No other selection criteria were used.

For the Ikaros isoforms expression analysis fifteen control samples of peripheral blood (PB) were obtained from healthy volunteers. They were over 18 age volunteers who donate blood in the Center. Fifteen control BM samples were taken during preparation of bone marrow graft for allo-BMT from parents (over 18 age) or siblings (predominantly under 18 age). All samples were obtained according to the local ethical guidelines.

2.2. RNA and genomic DNA extraction and cDNA preparation

Mononuclear cells from BM and PB (control) samples were separated by Ficoll-Paque density gradient centrifugation. Total cellular RNA from MNCs was extracted using the TRI Reagent® RNA Isolation Reagent (Sigma-Aldrich Co LLC, USA) according to the instructions of the manufacturer. RNA quantity and purity was determined using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and gel electrophoresis. A ratio of absorbances at 260 and 280 nm (A260:A280) greater than 1.8 was considered an acceptable indicator of good RNA purity. The integrity of RNA samples was determined based on visual assessment of the 18S/28S ribosomal ratio on agarose gel (two

Table 1
Clinical characteristics of patients.

		Number of cases (%)
All patients		270 (100%)
De-novo		197 (73.0%)
Relapses		73 (27.0%)
Gender[a]		
Male		115 (58.4%)
Female		82 (41.6%)
Age[a]		
0–17 years		179 (90.9%)
≥18 years		18 (9.1%)
Immunophenotype		
T-ALL		39 (14.4%)
B-ALL		231 (85.6%)
WBC ($\times 10^9$/L)		
< 20		132 (48.9%)
20–100		70 (26.0%)
≥100		36 (13.3%)
No data		32 (11.8%)
Fusion genes	Appropriate aberration	
BCR/ABL1	t(9;22)(q34;q11)	9 (3.3%)
MLL translocations	t(11;v)(q23;v)	24 (8.9%)
SIL/TAL1	del(1)(p32p32)	7 (2.6%)
TEL/AML1	t(12;21)(p13;q22)	26 (9.6%)
E2A/PBX	t(1;19)(q23;p13)	7 (2.6%)
Negative	–	188 (69.7%)
No data	–	9 (3.3%)

WBC – white blood cells count.
[a] De-novo cases only.

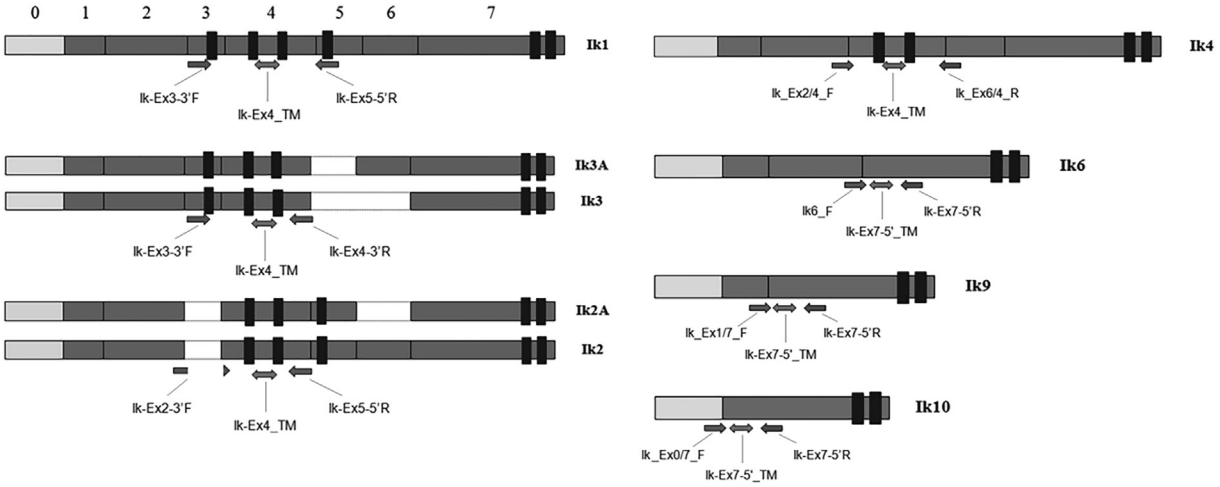

Fig. 2. Positions of primers and probes for RQ-PCR analysis of different Ikaros isoforms.

well-defined peaks on the electropherogram with 18S/28S ratio between 1.8 and 2.0).

1 µg of RNA was annealed with 200 ng of oligo-dT_{18} or random primers by heating to 65 °C for 5 min and cooling to 4 °C. cDNA synthesis was performed in a 20 µl reaction mix with 0.5 mM dNTP Mix, 1 µl RNAsin (Promega) and 1 µl SuperScript™ III Reverse Transcriptase (Life Technologies, USA) at 50 °C for 60 min. To avoid cDNA synthesis bias associated with different primers, each cDNA sample was synthesized in duplicate with random primers and oligo-dT_{18} separately, and then pooled.

2.3. RQ-PCR analysis of Ikaros isoforms expression

Expression levels of different Ikaros isoforms were quantified in 9 separate RQ-PCR reactions plus amplification of control house-keeping gene – Abelson's tyrosine-kinase (ABL) [25]. PCR amplification was performed in 20 µL reaction mix with TaqMan Universal PCR Master Mix (Applied Biosystems, USA), using 500 ng of cDNA, 500 ng of each primer and 150 ng of TM-probe. Amplification was performed on Rotor Gene 6000 Real-Time PCR Machine (Corbett Research, Australia). Cycling conditions: pre-heating 5 min at 50 °C, followed by 10 min 95 °C, then 60 cycles of 15 s at 95 °C, 30 s at 60 °C.

The sequences of primers and probes are described in Results section. We used numbering of coding exons as shown on the scheme in Fig. 1. The combination of primers, probes and dyes used for the RQ-PCR Ikaros isoforms quantification are shown in Appendix A, Table A1, their sequences in Appendix A, Table A2. All reactions were set up in duplicate, including negative controls, RNA-free cDNA synthesis. Expression level was calculated by ΔCt method. ΔCt = Ct (ABL) − Ct (Ik), and expression level = $2^{\Delta Ct}$.

2.4. PCR analysis of intragenic IKZF1 deletions

Genomic DNA was isolated from the MNCs using phenol-chloroform extraction. Two most common deletions were screened by PCR: deletions of exons 1–6 (ΔEx1–6) and deletions of exons 3–6 (ΔEx3–6). The primers used were adapted from [22], except ΔEx1–6_F, that we designed ourselves. PCR was performed using 100 ng genomic DNA in a 30 µL reaction under the following conditions: 35 cycles of 30 s at 94 °C, 30 s at 62 °C and 1 min at 72 °C, with a final extension step of 5 min at 72 °C. PCR products were primarily examined by 1.5% agarose gel electrophoresis. The presence of prominent band was considered as a deletion, detection of subtle band was considered as 'deletion in subclone'. All defined bands were excised from agarose gel, DNA was isolated and sequenced on ABI PRISM 3130 Genetic analyzer (Applied Biosystems, USA) for junctional identification.

RQ-PCR was used for most cases in addition to traditional PCR to validate the results of the analysis. We used primers and probe combinations recommended previously [26]. For normalization, the same samples were amplified with primers to albumin control gene [27]. Sequences of all primers for *IKZF1* deletions analysis are listed in Appendix A, Table A3.

2.5. Design of RQ-PCR primers and probes for quantification of different Ikaros isoforms

Human *IKZF1* gene is transcribed into at least 13 alternative transcripts. The first aim of this study was evaluation of expression level of different Ikaros isoforms. We designed and optimized the minimal set of primers and probes for reliable, specific RQ-PCR measurement of most Ikaros isoforms.

Sequence data for every Ikaros isoform were collected from the databases http://www.ncbi.nlm.nih.gov/nucleotide/ and http://www.ensembl.org, as well as from sequences obtained in our previous study [11]. The set of primers and probes was limited so that primers and probes were able to detect several isoforms in order to minimize the effect of amplification efficiency variation between different primers and probes (Fig. 2).

The same Ik-Ex4-TM probe was used for all long isoforms and Ik4. Combinations of primers flanking exon 3 and exon 5 allowed discrimination of long isoforms Ik1, 3/3A and Ik2/2A. Specific primers covering exon junction were designed only for Ik4. Short isoforms were amplified with one set of Ik-Ex7-5′TM probe and reverse Ik-Ex7-5′R primer, in pair with isoform-specific forward primers. We introduced different fluorophores for detection of the control gene (FAM), long isoforms (JOE) and short isoforms (ROX) (Appendix A, Tables A1 and A2) to make possible multiplex RQ-PCR detection. In this study, we collected data using singleplex reactions. However, we tried TaqMan probes with equal fluorophores (FAM) showed the same results, so fluorophores did not influence precision of analysis (data not shown).

2.6. Cytogenetic analysis

Conventional cytogenetic analysis was performed on cultured mononuclear BM cells. The cells were cultured in complete RPMI-1640 medium containing L-glutamine (15% fetal bovine serum, 1% antibiotics and 1% L-glutamine) at 37 °C with 5% CO_2 for 24 h. Growing cell cultures were treated with 0.1 µg/ml colcemid (Thermo Fisher Scientific Inc., USA) for 30 min (37 °C, 5% CO_2) and incubated in 0.075 M KCl. After incubation in hypotonic solution, the cells were fixed with 3:1 methanol-acetic acid mix. We used G-banding with trypsin and Gimsa (GTG) to determine the karyotypes. The karyotype

was described according to the guidelines of the International System for Human Cytogenetic Nomenclature (ISCN 2013) [28].

The commercial repeat-free probe Sure FISH 7p12.2 IKZF1 128 kb RD (Agilent Dako, USA) was used for cytogenetic analysis of *IKZF1* aberrations. Staining was performed according to the manufacturer's instructions. Following the probe hybridization process, cell nuclei were identified by DAPI II staining (Abbott Laboratories, Illinois, USA). A minimum of 200 interphase cells were analyzed separately for each probe using the OLYMRUS BX52 microscope (Olympus, USA), equipped with appropriate filters, and coupled to a BioView automated loader systems and software (BioView Ltd., Israel). Digital images were obtained by the use of charge-coupled device camera provided with the microscope: Orange Red (purple), and DAPI II (blue) fluorescence signals were recorded separately and then merged.

2.7. Breakpoint-specific multiplex fluorescent PCR and fragment analysis

Primers were used as described by Caye et al. in such a way that they could be combined in a single multiplex PCR and that the amplicon lengths and fluorescent labeling allowed direct identification of each type of ΔEx1–6, ΔEx3–6, ΔEx1–7 and ΔEx3–7 deletions [15]. A reverse primer GL in intron 1 was added to generate an amplicon of the non-rearranged genomic sequence (731 bp length) as an optional PCR control. PCR conditions and cycling protocol were described by Caye et al. Twenty-fold diluted PCR products were denatured with formamide (Applied Biosystems, USA) 95 °C 5 min and run on an ABI 3130 (Applied Biosystems, USA) analyzer using a fragment size analysis program, and analyses were performed using GeneMapper software (Applied Biosystems, USA).

2.8. Statistical analysis

The distribution of continuous parameters among subgroups was compared using the Mann-Whitney *U* test or Kruskal-Wallis ANOVA test and displayed as box-plots. Two-tailed Fisher's exact test was used to compare categorical parameters among subgroups. p-Values < 0.05 were considered as statistically significant. All statistical analysis was performed using Statistica 6.0 software.

3. Results

3.1. Expression patterns of Ikaros isoforms in ALL and control samples

IKZF1 gene is expressed normally in BM MNCs and PB MNCs, but relative expression of different isoforms varies greatly between BM and PB samples of healthy donors and in some cases of ALL patients. Normal MNCs express long Ikaros isoforms (Ik1, Ikx, Ik2, Ik3) at the levels comparable with the control gene ABL, and exhibit one or two orders of magnitude lower expression levels of short isoforms (Ik4–9). The shortest isoform, Ik10, normally is not expressed. Totally 191 ALL samples were analyzed for Ikaros expression. We observed much wider range of expression levels of several isoforms in ALL compared to control samples. Most of ALL samples had normal profile of Ikaros expression with prevalence of long isoforms, but in some cases, evident overexpression of short isoforms Ik6, 9, 10 was observed (Fig. 3). We did not find significant differences in long isoforms (Ik1, 2, 3), as well as dominant-negative (DN) isoforms Ik4, Ik5, Ik8 expression levels between control and patient groups.

To estimate the level of functional Ikaros isoforms expression and compare groups of patients, we calculated the Ikaros ratio between the sum of functional isoforms (Ik1 + Ik2 + Ik3) and the sum of all isoforms expression levels, as it was proposed in previous studies [11,24]. To define threshold for short isoforms overexpression, boxplots of distributions were built for Ik6, Ik9, Ik10, and the general Ik-Ratio (Fig. 4). Thresholds were set empirically to cut off normal expression levels in normal MNCs from aberrant overexpression in some ALL samples: 0.2 RFU (relative fluorescent units) for Ik6, 0.1 RFU for Ik9 and Ik10, and 0.8 RFU for Ik-Ratio. Determined by this cut-off levels, 35 out of 191 (18.3%) samples were Ik-DN positive (4 T-ALL and 31 BP-ALL). 28 samples were only Ik6+ (14.6%), 25 were only Ik9+ (14%), 6 only Ik10+ (3.5%). 23 samples of 177 examined (13%) expressed more than one short isoform.

There was not significant Ik-DN isoforms overexpression in B-lineage ALL (31/163, 19%) and T-ALL (4/28, 14.3%) (χ^2 $p = 0.55$). However, Ik-DN samples were significantly more frequent among de novo ALL (13.5%), than in relapsed ALL (31%) (χ^2 $p = 0.005$) (Table 2).

3.2. IKZF1 deletions identification

All DNA samples (n = 270) were screened for IKZF1 Ex1–6 and Ex3–6 deletions using PCR method. The criteria for deletion were detection of strong 850–900 bp (ΔEx1–6) or 1200–1300 bp (ΔEx3–6) bands on the agarose gel. In a number of ALL cases, agarose imaging showed a subtle band (Fig. 5). We categorized such result as a deletion if it was reproducible in several PCR reactions or proved to be such by another method, and we hypothesized that it represented deletion in minor leukemic subclone. Moreover, 39 positive or questionable samples were analyzed using breakpoint-specific multiplex fluorescent PCR and fragment analysis. All separate PCR bands were cut out from agarose, purified and sequenced for junctional identification. Finally, 54 ALL samples were analyzed by iFISH analysis. No deletions were detected by RQ-PCR, fragment analysis and iFISH in healthy volunteers' BM samples (n = 15).

Totally, 54 deletions were detected by PCR and/or fragment analysis and FISH in 49 patients. 21 deletions were Ex1–6 (39%), 33 were Ex3–6 (61%). Six patients had both deletions. In one patient, the deletion was diagnosed only according to FISH results. The incidence of *IKZF1* deletions was higher in the relapse group, than in de novo ALL, significant for B-lineage ALL ($p = 0.017$) (Table 3).

IKZF1 aberration was found in five cases of T-lineage ALL. Among them was one biphenotype patient (at primary diagnosis and relapse) with atypical T-cell markers expression. Other three samples had typical cortical T-ALL immunophenotype. *IKZF1* aberrations in these T-ALL patients were proved by PCR, FISH, and Ik-DN overexpression, indicating that *IKZF1* aberrations are associated not only with B-linear ALL.

3.3. Association of IKZF1 deletions with aberrant expression of Ikaros isoforms and discrepancy between different methods

IKZF1 deletions were apparently associated with the aberrant expression of short Ikaros isoforms (χ^2 $p < 0.001$) (Table 4). Quantitative levels of Ik6, 9, 10 were significantly higher in the group of patients with *IKZF1* deletions (Fig. 6). ΔEx3–6 deletions were usually accompanied by overexpression Ik6, or Ik6 and Ik9 isoforms. Expression of Ik10 always occurred in samples with ΔEx1–6 deletions.

Interestingly, expression of DN isoforms Ik4 and Ik8, as well as long isoforms (Ik1, Ik2, Ik3) was not changed significantly in patients with *IKZF1* deletions. There was a marked decrease in expression of Ik1, Ik2, Ik3 in one patient with ΔEx3–6 deletion in both de novo ALL and relapse with presumable loss of heterogeneity.

However, discrepancy between the *IKZF1* deletions in genomic DNA and Ikaros-DN overexpression was observed in some patients. Samples of patients with conflicting results fell into two distinct groups. The first group included patients, who were negative for *IKZF1* deletions detected by PCR-based methods, but positive for Ik-DN expression. The second group comprised patients, who had *IKZF1* deletion proved by PCR and FISH, but negative for Ik-DN. Actually, the third patient group can be also defined by weak detection of deletion (subtle PCR band in agarose gel, small peak in fragment analysis and FISH negative), but Ik-DN overexpression. This latter group we defined as the subclone

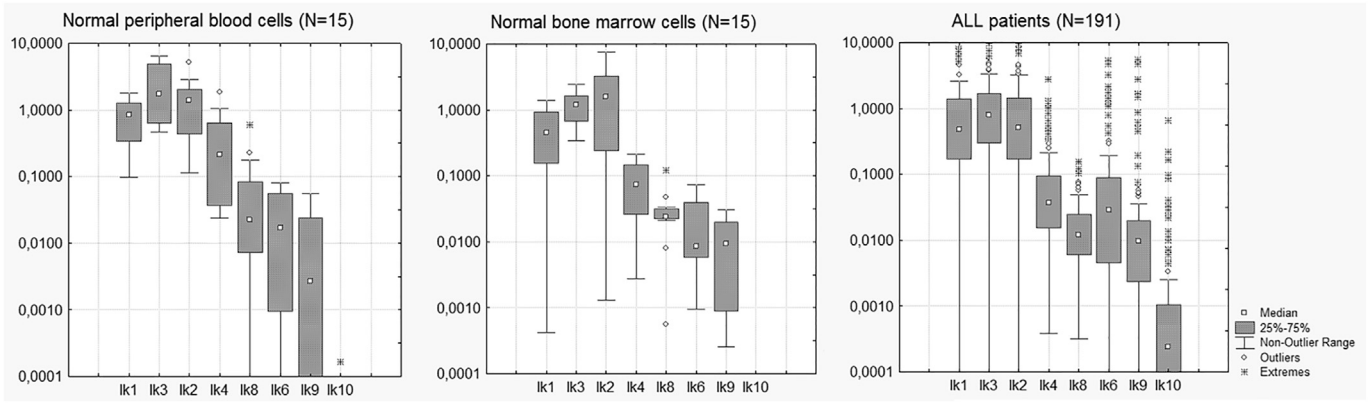

Fig. 3. Profile of different Ikaros isoforms expression in control group and ALL samples.

deletion. The data for these three groups are summarized in Table 5. Bone marrow blast count indicated to show that it did not compromise the *IKZF1* results.

To test whether deletion in minor subclones may represent an accidental non-pathogenic event, we subjected the control group of 30 healthy donors to the full PCR examination and fragment analysis. Not a single case with positive PCR amplification was detected.

Taken these data together, we concluded to define *IKZF1* status as 'aberrant' in study cohort, when obvious positive results were obtained by at least one method, detection of *IKZF1* deletion on the genetic level or overexpression of Ik-DN.

Table 2
Incidence of Ik-DN expression in ALL cohorts according to immunophenotype.

	Ik-DN overexpression in de novo ALL	Ik-DN overexpression in ALL relapses	p, Chi-square
BP-lineage ALL	16/117 (13.6%)	15/46 (32.6%)	p = 0.006
T-lineage ALL	3/23 (13%)	1/5 (20%)	p = 0.68
Total	19/140 (13.5%)	16/51 (31%)	p = 0.005

3.4. Association of IKZF1 deletions and Ikaros isoforms expression with chimeric oncogenes

Status of *IKZF1* gene was analyzed in association with four chimeric oncogenes: BCR/ABL1, E2A/PBX, TEL/AML1, and MLL rearrangements in the cohort of B-lineage ALL. We found out a strict inverse relationship between *IKZF1* aberrations and TEL/AML1 expression. None of 26 TEL/AML1 positive ALL cases had aberrant *IKZF1*, but 47 *IKZF1* events fell into 205 TEL/AML1 negative group (χ^2 p = 0.006). Interestingly,

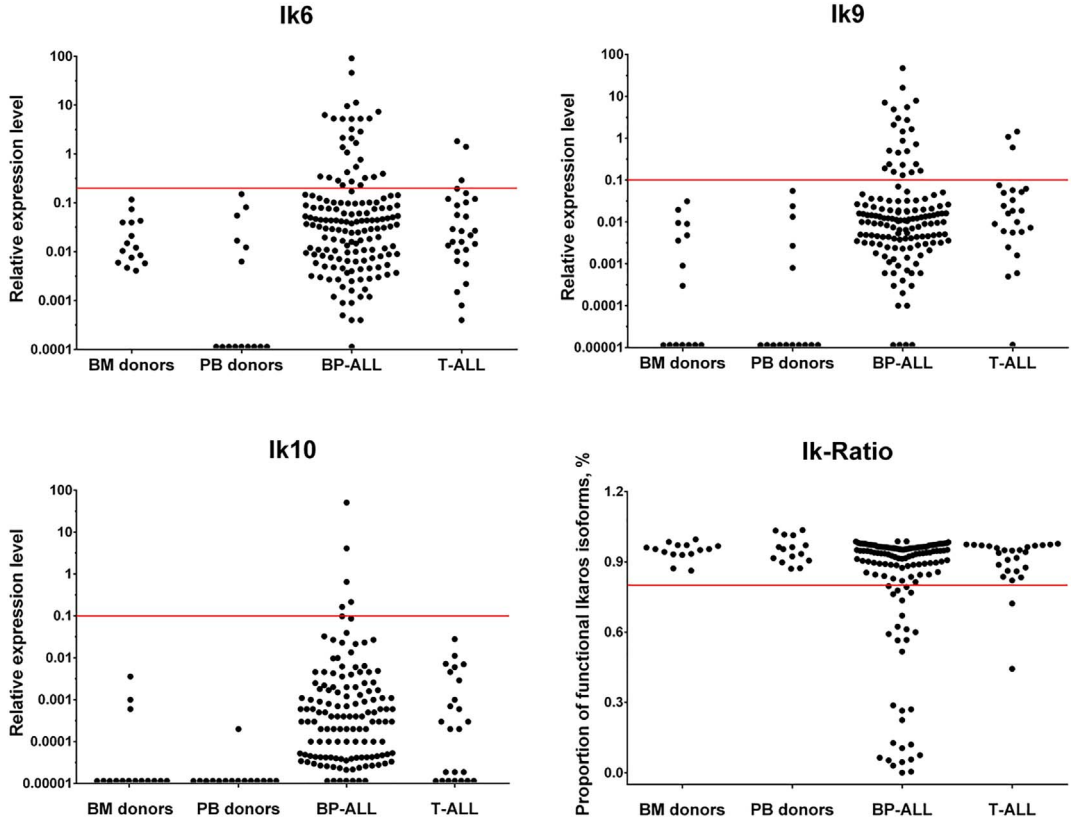

Fig. 4. Threshold levels for aberrant expression of Ik-DN isoforms.

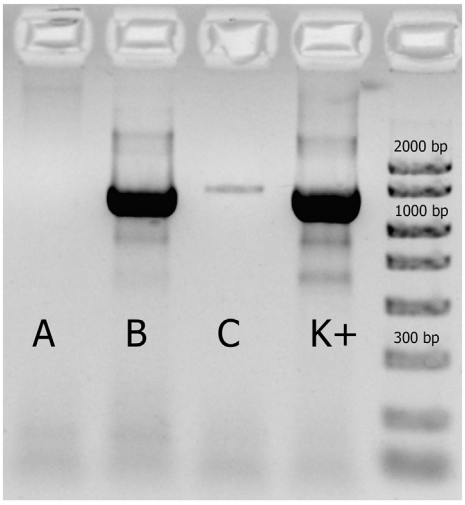

Fig. 5. Agarose gel image shows the absence of deletion in sample A (no band), a single rank band for deletion Ex3–6 (B), single subtle band (C) suspected deletion in minor leukemic subclone and positive control (K+).

Table 3
Incidence of IKZF1 deletions in ALL cohorts according to immunophenotype.

	IKZF1 deletions (PCR/FISH/FA) in de novo ALL	IKZF1 deletions (PCR/FISH/FA) in ALL relapses	p, Chi-square
BP-lineage ALL	25/165 (15.2%)	20/73 (27.4%)	p = 0.017
T-lineage ALL	5/32 (15.6%)	1/7 (14.3%)	p = 0.92
Total	30/197 (15.2%)	19/66 (28.8%)	p = 0.02

Table 4
Coincidence of the IKZF1 deletions and Ik-DN overexpression (general ALL cohort).

	Ikaros isoform expression status (RQ-PCR), N = 191	
	Normal profile	Ik-DN overexpression
IKZF1 deletion	4 (2.1%)	33 (21.8%)
IKZF1 normal	152 (79.5%)	2 (1.1%)

expression of functional isoforms was higher in TEL/AML1 group, significant for Ik1 (p = 0.02) and Ik4 (0.04). On the contrary, a positive relationship between BCR/ABL1 and *IKZF1* aberration was found. 8 out of 9 (89%) Ph + ALL had *IKZF1* aberration, vs. 39 IKZF1 aberrations out of 222 (17.5%) Ph − ALL cases (χ^2 p < 0.001). Expression of long isoforms Ik1, 2, 3, 4, 8 was reduced, but expression of Ik6, 9, 10 was significantly increased in Ph + ALL. MLL rearrangements did not correlate with IKZF1 deletions, however, long isoforms Ik1 and Ik3 (p = 0.01) were expressed at higher level in patients with MLL. No significant association between *IKZF1* status and E2A/PBX was discovered.

3.5. Status of IKZF1 aberrations in paired cases of de novo ALL and relapse

IKZF1 status was analyzed in 45 paired cases (40 with the first relapse and 5 with two relapses). In one patient out of 12 with *IKZF1* deletions at diagnosis, it was not detected at relapse. 11 of 12 patients had *IKZF1* deletions at diagnosis, which reappeared at the relapses. 10 of these 11 patients carried identical deletions at both disease representations, and one patient displayed heterogeneity of *IKZF1* mutations, and clonal evolution after treatment [29]. In this patient, two full-clonal deletions were observed at diagnosis – ΔEx1–Ex6 and ΔEx3–Ex6, most likely biallelic, and overexpression of Ik6, 9, 10. At later relapse in this patient, only one deletion ΔEx3–Ex6 was found, and it was a different deletion from the one detected at diagnosis by sequencing. Both ΔEx3–Ex6 deletions in this patient were measured by RQ-PCR with allele specific primers to the DNA breakpoint. However, de novo ALL and relapse in this patient were identical by clonal IgH and TCRG genes rearrangements. Finally, 33 patients without *IKZF1* deletions at diagnosis remained negative at relapse (Table 6).

Expression of Ikaros isoforms was analyzed in 29 paired cases with RNA available. In 22 cases with normal Ikaros (Ik-DN −) expression and in 6 patients with Ik-DN + at diagnosis, this expression pattern returned back at relapse. One patient overexpressed Ik6 at diagnosis but became Ikaros normal at relapse (Table 7).

4. Discussion

Ikaros is a transcription factor encoded by *IKZF1* gene with well-established role in lymphocyte development and function. Its expression is normally regulated by alternative splicing. Several studies have shown that focal *IKZF1* deletions are frequent somatic copy number alterations in ALL [12,13,30,31]. All *IKZF1* mutations in ALL are loss-of-function, resulting in null Ikaros, haplosufficient or dominant-negative phenotype [32]. Focal *IKZF1* deletions in ALL result in expression of short, non-DNA-binding Ikaros isoforms, responsible for dominant-negative phenotype.

In this study, we performed thorough investigation of the *IKZF1* aberrations in the pediatric ALL cohort by different methods, including monitoring genomic status of *IKZF1* gene by PCR-based methods and FISH, as well as mRNA expression status. The novelty of this study is in quantitative evaluation of different Ikaros isoforms in ALL patients with

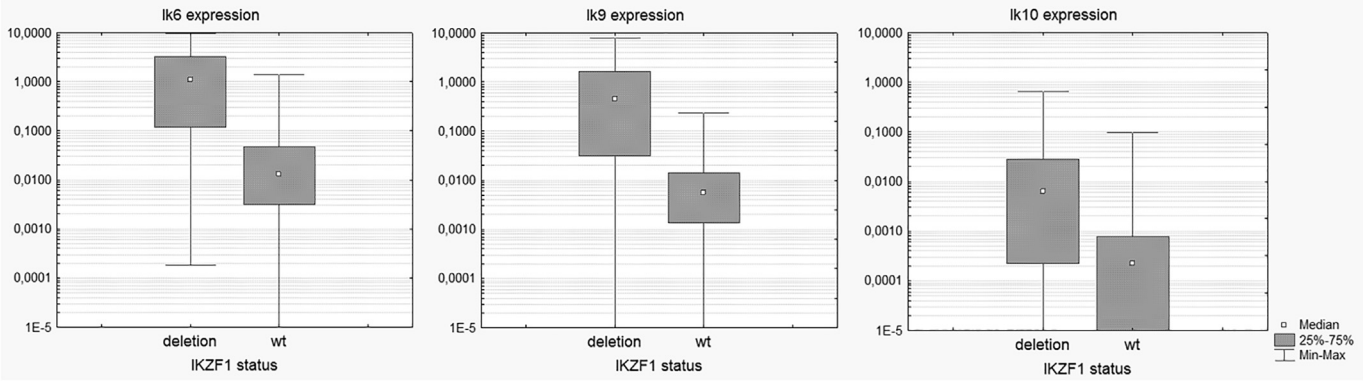

Fig. 6. Expression of short Ikaros isoforms according to *IKZF1* deletions.

Table 5
Patients with discrepant or ambiguous analysis results of IKZF1 gene analysis.

Grope	Patient	Phenotype	ALL	BM blasts, %	deletion by PCR	Percent of cells with deletions (FISH)	Ik-DN RQ-PCR	Fusion genes (PCR)	Cytogenetic status of 7 chr	Outcome
1. Hidden deletion	Mo	BP-ALL	Relapse	10	Negative	24%	Ik-6,9	–	n.d.[a]	Relapse2-death
	Vi	BP-ALL	Relapse	77	Negative	n.d.[a]	Ik-9	BCR/ABL	Normal	Death
	Do	BP-ALL	De novo	92.5	Negative	2%	Ik-6	–	n.d.	Remission
2. Silent deletion	Dr	BP-ALL	De novo	98.5	ΔEx1-Ex6	n.d.	Normal	–	n.d.	Remission
	Po	BP-ALL	De novo	93	ΔEx1-Ex6 ΔEx3-Ex6	39%	Normal	–	n.d.	Remission
	Ju	T-ALL	De novo	44.3	ΔEx1-Ex6 ΔEx3-Ex6	42%	Normal	–	tetraploid	Remission
	Az	BP-ALL	De novo	98	ΔEx3-Ex6	5%	Normal	–	n.d.	Remission
3. Subclone deletion or aberrant splicing	Ba	BP-ALL	De novo	94	ΔEx3-Ex6	5%	Ik-6,9	E2A/PBX	Normal	Relapse
	Ba	BP-ALL	Relapse1	51	ΔEx3-Ex6	n.d.	Ik-6,9	E2A/PBX	Normal	Relapse2
	Ba	BP-ALL	Relapse2	95	ΔEx3-Ex6	2%	Ik-6,9	E2A/PBX	Normal	Death
	Ga	T-ALL	De novo	95	ΔEx1-Ex6	5%	Ik-6	–	Normal	Remission
	Ka	BP-ALL	De novo	89	ΔEx1-Ex6	10%	Ik-6,9	–	Normal	Relapse
	Ka	BP-ALL	Relapse	41.2	ΔEx1-Ex6	5%	Ik-6,9	–	Normal	Death
	Ma	BP-ALL	De novo	82	ΔEx3-Ex6	1%	Ik-6,9	–	Normal	Relapse
	Ma	BP-ALL	Relapse	40.3	ΔEx3-Ex6	3%	Ik-6,9	–	Normal	Death
	Ro	BP-ALL	De novo	96	ΔEx3-Ex6	4%	Ik-6	–	Normal	Relapse
	Rm	BP-ALL	De novo	86.3	ΔEx1-Ex6	4%	Ik-6,9	MLL/AF4	Normal	Death
	Su	BP-ALL	De novo	91.5	ΔEx3-Ex6	n.d.	Ik-9	MLL/AF9	Normal	Relapse
	Su	BP-ALL	Relapse	65.7	ΔEx3-Ex6	2%	Ik-9	MLL/AF9	Normal	Death
	Fr	T-ALL	De novo	82.7	ΔEx1-Ex6	1%	Ik-9	–	Normal	Relapse

[a] n.d. – no metaphases or no data obtained.

Table 6
IKZF1 status in paired ALL cases.

	N	IKZF1 status		Conclusion
		De novo ALL	Relapse	
45 paired cases	11 cases	10 deleted	10[a] deleted	91.6% of cases with IKZF1 deletions reappeared at relapse
	1 case	1	0	
	33 cases	33	33	100% of cases without deletions remained negative

[a] One patient displayed heterogeneity of IKZF1 mutations at the diagnosis and the relapse, and clonal evolution after treatment.

Table 7
Ikaros-DN expression in paired ALL cases.

	N	De novo ALL	Relapse	Conclusion
29 paired cases	22 cases	Normal	Normal	In 100% normal Ikaros profile (Ik-DN−) remained at relapse
	6 cases	Ikaros-DN	Ikaros-DN	In 6/7 (86%) of paired cases Ik-DN+ remained at relapse, in
	1 case	Ikaros-DN	Normal	one patient (14%) Ik-DN+ expression changed to normal (Ik-DN−)

or without *IKZF1* deletions by RQ-PCR. In our previous study [11] and a few other publications, PCR- and electrophoresis-based analysis was used for detection of Ik6, or Ik6 and Ik10 [13,22,23]. Our intention was to perform deeper investigation of Ikaros expression profile in ALL samples in comparison with normal blood and bone marrow MNCs.

A minimal set of primers and TaqMan-probes was designed for specific quantification of different Ikaros isoforms. Our results indicate that the most prominent distinctive feature of some ALL cases was overexpression of Ik6, Ik9, or Ik10. Cut-off levels for aberrant Ik6, Ik9, Ik10 isoforms were established. Loss of long transcripts, Ik1, Ik2 or Ik3, found in patients, can be regarded as a negative event. However, it is not very reliable as a diagnostic feature due to possible background amplification from contaminating normal cells in a sample. The reasons of Ik-DN overexpression in ALL are not clearly determined, however, there are two hypotheses on this matter. Firstly, aberrant splicing of Ikaros transcripts may be induced by BCR/ABL1 [33], and secondly, intragenic deletions may be the sole cause of aberrant splicing [13]. Our data demonstrates significantly increased level of short isoforms Ik6, Ik9, Ik10 in ALL cases with *IKZF1* deletions. Apparently, a set of Ik-DN isoforms depends on the type of deletion: ΔEx1–6 leads to appearance of Ik10, and ΔEx3–6 leads to Ik6 and Ik9. However, the cause of expression of Ik6 and Ik9 in patients with ΔEx1–6 remains unclear and, conversely, the same is true for Ik10 expression in one patient with ΔEx3–6 in this study. Theoretically, in the case of ΔEx1–6 leukemia, any other isoform apart from Ik10 can be expressed from the second allele. Whereas in the case of ΔEx3–6, all three short isoforms, Ik6, Ik9 and Ik10, can possibly be expressed with deleted allele by means of alternative splicing.

We observed minor decrease of long isoforms Ik1, Ik2, Ik3 in patients with *IKZF1* deletions, which can be explained by haploinsufficiency of their expression. Precise mechanism of alternative splicing with intragenic deletions is yet to be explored in the future studies. We did not find overexpression of dominant-negative isoforms Ik4, Ik5 and Ik8 in ALL patients, although Ik4 and Ik8 are supposed to be oncogenic [22,34,35]. Overexpression of Ik4 or Ik8 above the normal level was not found in the studied ALL cohort. However, their expression in leukemia cells varies widely and some variations are similar to that of long Ikaros isoforms. Ik4 and Ik8 are significantly increased in ALL cases without *IKZF1* deletions together with Ik1. Ik4 and Ik8 are significantly lower in Ph-positive leukemia, and higher in cases with TEL/AML1 and MLL fusions. These observations suggest a similarity with Ik1–3 'normal' splicing regulation of Ik4 and Ik8, different from Ik6, Ik9 and Ik10.

Most studies focusing on IKZF1 alterations in ALL, regarded them as a specific feature of B-lineage ALL. However, our results and some other data indicated that Ikaros abnormality also occurred in rare cases of T-ALL [11,31,36,37]. To collect more data, T-ALL patients were included in this study. *IKZF1* deletion together with Ik6 and Ik9 overexpression was found in four patients. However, one of them was biphenotypic, three others showed atypical *IKZF1* status discussed below.

Analyzing our results obtained by different methods, made us

convinced that the aberration of *IKZF1* gene in leukemia from a biological point of view is more complicated than traditional translocations. In contrast to other pathogenic mutations, such as fusion oncogenes, which are usually present in all cells of a tumor, *IKZF1* deletions are a secondary event and may occur in minor subclones of leukemic cells [15]. Moreover, the phenotypic effect of the focal deletions can vary depending on the result of alternative splicing of the deletion allele–from loss of function to a dominant-negative phenotype. Therefore, we combined PCR analysis of mutations with cytogenetic analysis and an estimation of isoform expression. FISH analysis of intragenic deletions showed the lowest sensitivity in about 5% of cells. Another limitation of iFISH is aneuploidy of the 7th chromosome. In disomic state, one signal indicates heterozygous deletion, in monosomy 7, one signal indicates absence of deletion in the remaining chromosome, and in the case of trisomy 7 or polyploidy, even two signals can still be accompanied by a third deleted allele. Application of iFISH requires karyotyping by G-banding to assess the ploidy status of chromosome 7, which is not always possible. In our results, detection of full-clonal deletions by iFISH was 100% confirmed by PCR-based methods. However, FISH-negative cases leave a large margin of underdiagnoses of *IKZF1* aberrations.

RQ-PCR analysis of Ik-6, 9, 10 isoforms overexpression has proved to be the most sensitive and specific method according to our data. Ik-DN overexpression may be the result of various types of intragenic *IKZF1* deletions or other mutations leading to pathogenic phenotype. Being a positive event, Ik-DN overexpression can be detected on the background of normal lymphocytes or in a minor subclone of leukemia cells.

Contradicting results of the *IKZF1* gene status obtained by different methods were combined into three groups, presented in Table 5. Ambiguous results are not the result of low blast cells in the bone marrow sample. The first group included three patients, negative by genomic PCR, but positive for Ik-DN expression. In one patient, deletion was proved by FISH despite low blast cells count. This group can be explained by hidden deletions with breakpoints outside of primers positions.

The second group of four patients had clear deletions, two of them proved by FISH, and at the same time, had normal Ikaros expression profile. Such pattern is the most difficult to explain. We define this group as ‘silent deletions’, as expression of deletion-associated isoforms is silenced by unknown genetic or epigenetic mechanisms. Karyotyping was possible for only one patient, which was shown to be tetraploid. Therefore, the deleted alleles were inactivated and Ikaros expression was compensated by intact allele in additional copies of chromosome 7. While all four patients from this group stay in complete remission, we can speculate that ‘silent deletions’ might be not pathogenic event.

The third group of patients had reduced PCR amplification of deletion and was iFISH negative (except one), but had pronounced Ik-DN overexpression. This contradiction can be explained by deletion in minor leukemic subclones. This conclusion was also reached by Caye et al. [15], who found that MLPA did not detect *IKZF1* rearrangements in 13 of 82 leukemias (15%) with *IKZF1* deletions. The same result was obtained by P. Hoogerbrugge et al. [38], whereby 17 out of 34 deletions were MLPA-negative with an allelic burden of the IKZF1 ΔEx3–6 deletion varying from 28% to < 1%. Alternative explanation for this group is epigenetic changes in splicing, which lead to overexpression of short isoforms. By our data, 9 of 30 (30%) de novo ALL patients and 13 of 49 (26.5%) relapses were assigned into this group. The subclonal origin of *IKZF1* deletions is an obstacle for unambiguous interpretation of diagnostic results on genomic DNA level. It can be assumed that subclonal deletion is a random genetic aberration not associated with leukemia. To test this hypothesis, we performed complete examination of the control group of 30 PB and BM donors. Whereas doubtful peaks below the threshold level appeared in some fragment analysis, Ik-DN expression was never found in healthy donor samples. The clinical implication of these subclonal deletions is still not clear.

Since biology and clinic of leukemia is largely dependent on the expression of pathogenic chimeric oncogenes, *IKZF1* deletions and Ik-DN expression were analyzed in association with BCR/ABL, TEL/AML1, E2A/PBX and MLL fusions. *IKZF1* deletions and Ik6 overexpression were originally described as the most typical for BCR/ABL+ ALL [13,17,22,33,39]. According to our results, correlation between *IKZF1* deletions and BCR/ABL was significant: all patients with BCR/ABL overexpressed Ik-DN isoforms, and all except one had full-clonal deletions. Interestingly, expression of long Ikaros isoforms was reduced in BCR/ABL+ ALL, even in comparison with BCR/ABL- samples with aberrant *IKZF1*, proving the idea of Klein et al. [33] that BCR/ABL influences expression and alternative splicing of *IKZF1*.

TEL/AML1 oncogene was associated with normal status of IKZF1. TEL/AML1+ ALL did not have IKZF1 deletions or Ik-DN expression, and level of long isoforms Ik1, Ik2 and Ik3 was enhanced. Probably, this chimeric oncogene does not effect on Ikaros splicing, but their pathogenic role in blast transformation is similar to that of IKZF1 aberrations, making TEL/AML1 an alternative to IKZF1. The same tendency was found for MLL rearrangements. Both TEL/AML1 and MLL translocations are initial events for ALL, occurring in embryogenesis or infancy. Since IKZF1 mutations are a second event, which occurs with the development of leukemia and their appearance in MLL or TEL/AML1 positive leukemia probably is no longer necessary.

Paired cases of de novo diagnosis and relapses were intentionally collected in this study to analyze stability of *IKZF1* deletions and Ik-DN expression during disease progression. We observed a stable inheritance of aberrations between primary ALL and relapse. In 45 paired cases, *IKZF1* genotype was stable between diagnosis and relapse except two cases. In the first, putative subclonal deletion and Ik6 overexpression were lost at relapse. In the second case, changes in both alleles of *IKZF1* have happened. This case is of special interest, because primary ALL and relapse occurred in different clones, as assessed by *IKZF1* status, but originated from the same clone, as deduced from clonal IgH and TCRG genes rearrangements [29]. This case proves the idea that *IKZF1* alterations may be secondary and on-going events, and leukemia is a subject of complex clonal evolution.

5. Conclusion

We applied RQ-PCR analysis to investigate Ikaros isoforms expression and established cut-off levels for Ik6, Ik9 and Ik10 overexpression in ALL. Ik-DN expression was associated with *IKZF1* deletions and BCR/ABL, but not TEL/AML1 and MLL chimeric oncogenes. We described the limitations of PCR-based and iFISH-based analyses of *IKZF1* intragenic deletions, and established that RQ-PCR detection of Ik-DN is the most reliable method. One third of the *IKZF1* deletions were identified in minor suclones of leukemic cells. We observed a stable inheritance of aberrations between primary ALL and relapse, however, the type of deletion may change during disease progression. Investigation of Ikaros aberrations in connection with resistance to chemotherapy and disease outcome will be continued in our next study.

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Appendix A

Table A1
The combination of primers, probes and dyes used for the RQ-PCR Ikaros isoforms quantification.

Primer/probe	Sequence
ABL-1	TGGAGATAACACTCTAAGCATAACTAAAGGT
ABL-TM	FAM-CCATTTTTGGTTTGGGCTTCACACCATT-BHQ1
ABL-2	GATGTAGTTGCTTGGGACCCA
Ik-Ex3-3′F	GGTTCACAAAAGAAGCCACAC
Ik-Ex5-5′R	TAGCTTCGGCCACAATATCC
Ik-Ex4-3′R	GGAGTGCGTCCTCAGGTG
Ik-Ex2-3′F	AGAGTGACAGAGTCGTGGGAGA
Ik-Ex4_TM	JOE-AGCCCTTCAAATGCCACCTCTGCAAC-BHQ1
Ik-Ex2/4_Ik4_F	AGAGTGACAGAGTCGTGGGAGA
Ik-Ex6/4_Ik4_R	TTTCTTCTTTAATGACGGAGTGC
Ex7-5′R	AGCTGGCGCTGCTGTCGT
Ex7-5′TM	ROX-CAAGGGCCTGTCCGACACGCC-BHQ2
Ik5_F	CACAAAAGAAGCCACACTGGG
Ik6_F	AAGAGTGACAGAGTCGTGGGGGA
Ik8_F	AGAGTGACAGAGTCGTGTCATTAAAG
Ik-Ex1/7_F	AAGACATGTCCCAAGTTTCAGGGG
Ik-Ex0/7_F	GCGCGACGCACAAATCCACGG

Table A2
Sequences of primers and TaqMan probes used for the RQ-PCR Ikaros isoforms quantification.

Transcript	Forward primer	Reverse primer	TaqMan probe	Dye
ABL	ABL1	ABL2	ABL_TM	FAM
Ikaros 1	Ik-Ex3-3′F	Ik-Ex5-5′R	Ik-Ex4_TM	JOE
Ikaros 1, 3, 3A[a]	Ik-Ex3-3′F	Ik-Ex4-3′R	Ik-Ex4_TM	JOE
Ikaros 2, 2A	Ik-Ex2-3′F	Ik-Ex5-5′R	Ik-Ex4_TM	JOE
Ikaros 4, 4A	Ex2/4_Ik4_F	Ex6/4_Ik4_R	Ik-Ex4_TM	JOE
Ikaros 6	Ik6_F	Ex7-5′R	Ex7-5′TM	ROX
Ikaros 5	Ik5_F	Ex7-5′R	Ex7-5′TM	ROX
Ikaros 8	Ik8_F	Ex7-5′R	Ex7-5′TM	ROX
Ikaros 9	Ik_Ex1/7_F	Ex7-5′R	Ex7-5′TM	ROX
Ikaros 10	Ik_Ex0/7_F	Ex7-5′R	Ex7-5′TM	ROX

[a] The expression of Ikaros isoforms 3 and 3A was calculated as the difference between the value obtained with these primers pair and the value of Ik1 expression.

Table A3
Sequences of primers for IKZF1 deletions analysis.

ΔEx3–6 deletion	
ΔEx3–6_F	CCACAGGGCAAGTCATCCACATTTTG
ΔEx3–6_R	CAGACCATAGAGTCCCTCCTAGGGGAAAAA
ΔEx1–6 deletion	
ΔEx1–6_F	TTCCTCCTCTAATCTTTGGACTTG
ΔEx1–6_R	CAGACCATAGAGTCCCTCCTAGGGGAAAAA

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