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Changing of IKZF1 genotype during Philadelphia-negative precursor-B acute lymphoblastic leukemia progression: a short clinical report

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1. Introduction
Ikaros, a transcription factor encoded by IKZF1 gene, plays essential role in lymphocyte development [1]. Alternative splicing regulates the function of Ikaros, resulting in the production of at least 13 different isoforms. Long isoforms (Ik1–3) are able to bind DNA and considered to be functional. Short isoforms (Ik4–10) are not efficient to bind DNA, and function in a dominant-negative (DN) manner [2,3]. Several studies have shown that focal IKZF1 deletions are frequent genetic alterations in acute lymphoblastic leukemia (ALL) [4–6]. All IKZF1 mutations in ALL are loss-of-function, resulting in null Ikaros, haplosufficient or DN phenotype [7]. Focal IKZF1 deletions in ALL are supposed to be a reason for expression of short, non-DNA-binding Ikaros isoforms which switch their localization in the cell from nucleus to cytoplasm. IKZF1 deletions were first discovered as frequent genetic lesion in BCR-ABL1 positive ALL [8], proved to be a marker of poor prognosis in general ALL group [9,10] and BCR-ABL1 negative ALL [11]. IKZF1 deletions are different from most oncogenes that may occur in leukemic subclones [12,13].

Presented case describes a BCR-ABL1-negative B-cell precursor ALL (BP-ALL) patient with clonal evolution of IKZF1 genotype during disease treatment and progression. Due to the fact that IKZF1 alterations were not taken into account for risk stratification, poor prognosis of the patient has been underestimated.

2. Case report

2.1. Clinical data

The patient was a 17-year-old male who initially presented with weakness, fewer and lymphadenopathy. He was noted to have leukocytosis (21.6 × 10⁹/l) with 75% blasts in the peripheral blood. Clonal karyotype, clinical and blast cell features were established. The blast count in the bone marrow (BM) at the time of diagnosis was 84.0%. Immunophenotyping of BM cells showed 79% blasts expressing HLA-DR, 78% - CD20, 98% blasts were positive with CD19, and 99% – with CD10. It was also noted that 98% cells expressed CD34 and CD22. Based on morphology and immunophenotyping, the patient was diagnosed as BP-ALL, L2, FAB and common B.

Routine BM cytogenetic analysis revealed normal male karyotype (46, XY), however metaphases were not suitable for analysis by G-banding. A multiplex, nested reverse transcription PCR assay for 16 of the most common ALL-associated chromosomal rearrangements (BCR/ABL1, SLL1/TAL1, E2A/PBX, TEL/AML1, and MLL translocations) was negative.

The patient was stratified into standard risk group (SRG), and started the induction therapy according to the multicenter trial ALL-Moscow/Berlin (ALL-MB), 2002 chemotherapy regimen in children with ALL (randomization №1: dexamethasone 6 mg/m² p.o., daunorubicin 45 mg/m² on 8th day). After completing of the induction the patient underwent the 3 stages of consolidation [14].

On day 15 of the induction the BM evaluation was characterized as residual ALL with blasts count more than 1%. The blast count in the BM was 92%. Flow cytometry as well as reverse transcription PCR
showed similar results to those at diagnosis. Repeated routine cytogenetic analysis revealed the 46,?dup(Xp11.2p22.1)Y. del(9)t(9:7)(p13:7)? del(14)(q13q32) [9]/47, idem., +mar[3] complex chromosome abnormality. The relapse initially was classified as S2-risk and treated according to the ALL relapse protocol (ALL REZ 2002) of the Berlin/ Frankfurt/ Münster (BFM) group [15]. At the postinduction day 10 (F2-blok), disease was complicated with prolonged neutropenia, Gram-negative sepsis, right-focal confluent pneumonia and oxygen lack. Despite appropriate treatment and resuscitation procedures within 48 h, patient died from septic shock and multiple organ failure on the 13th day of postinduction (F2-blok).

2.2. PCR analysis and sequencing of intragenic IKZF1 deletions

Genomic DNA was isolated from the BM mononuclear cells using the phenol-chloroform extraction. Most common IKZF1 deletions of exons 1–6 (ΔEx1–6) and 3–6 (ΔEx3–6) were screened by PCR. Used primers were recommended by Liu P. et al [16], except ΔEx1–6, F, that we selected ourselves (Supplementary table 1). PCR was performed using 100 ng of DNA in a 30 μL reaction under the following conditions: 35 cycles of 30 s at 94 °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. Bands were purified from agarose gel and sequenced on ABI PRISM 3130 Genetic analyzer (Applied Biosystems, USA) for the references to color in this figure legend, the reader is referred to the web version of this article.)

At the diagnosis, two different IKZF1 deletions were detected: ΔEx3–6Δ and ΔEx1–6. At the relapse another one Ex3–6Δ deletion was found (Fig. 1).

2.3. Relative quantity PCR (RQ-PCR) analysis of Ikaros isoforms expression

We estimated expression of all IKZF1 isoforms at the diagnosis by RQ-PCR with settled set of primers and probes relative to process, cells 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, merged and contended.

Control samples showed over 95% of cells with two signals representing both normal IKZF1 alleles, 2.5% with one, and 2.5% with three signals, demonstrating a method error level of 5%. 70% of patient’s BM cells at diagnosis showed deletion of one IKZF1 allele and 26% cells brought both normal alleles. In the BM at relapse 59% of cells were heterozygous for the IKZF1 deletions and 33% of cells with normal alleles were observed (Fig. 2).

2.5. Localization of Ikaros protein

We examined the subcellular localization of Ikaros protein in patient’s BM cells at diagnosis and control ALL BM cells (with wild type IKZF1 gene). Mononuclear BM cells were fixed in fresh solution of 4% paraformaldehyde at 37 °C for 15 min, washed twice, permeabilized in 0.2% Triton-X100 for 10 min at room temperature, washed 2 times. Then PE Mouse Anti-Human Ikaros antibody (BD Pharmingen, USA) was used to stain the cells as recommended by manufacturer. Finally, nuclei were stained by incubating with 0.1 μg/ml propidium iodide (PI) (37 °C, 20 min) and visualized by laser confocal microscope (Leica DML2500, USA). Anti-Ikaros antibody (green, fluorescence excitation maximum of 488 nm and emission maximum of 578 nm) and PI (red, fluorescence excitation maximum of 535 nm and the emission maximum of 617 nm) fluorescence signals were recorded separately and merged.

IKZF1 wild type leukemic cells demonstrated nuclear localization of Ikaros, whereas in patient’s blasts DN isoforms of Ikaros protein localized predominantly in cytoplasm due to the deletion of DNA-binding domain on the N-term of the protein (Fig. 3).

2.4. Fluorescence in situ hybridization (FISH) analysis

Fixed mononuclear BM cells from the patient at diagnosis and at relapse, as well as control ALL BM samples were analyzed for interphase FISH according to manufacturer recommendations. The commercial repeat-free probe Sure FISH 7p12.2 IKZF1 128 kb RD (Agilent Dako, USA) was used. Following the probe hybridization of patient’s BM cells at diagnosis showed deletion of one IKZF1 allele and 26% cells brought both normal alleles. In the BM at relapse 59% of cells were heterozygous for the IKZF1 deletions and 33% of cells with normal alleles were observed (Fig. 2).

Quantitative assessment of minimal residual disease (MRD) was performed by RQ-PCR using patient-specific primer for two identified clonal T-cell receptors and immunoglobulin gene rearrangements (IgH and TCRG) as described previously [17] (Supplementary table 3). MRD level was measured by RQ-PCR in triplicates using serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) of

Fig. 1. Sequences of junctional regions of three different IKZF1 deletions in BM cells genomic DNA of the patient. Positions of primers used for RQ-PCR are underlined by a solid line, probes – the dotted line. Intron sequences before and after the deletion breakpoint colored in green and blue, non-matrix nucleotides – in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
patient’s DNA in normal polyclonal DNA. MRD level was calculated relative to the diagnostic material. MRD level was counted in 4 time points between diagnosis and relapse. The first follow up sample on day 15 of induction was positive (more than 1%), following with two negative points. Two month before the relapse MRD level remained negative, whereas at relapse both targets were positive (Table 1).

2.7. RQ-PCR-based MRD detection by IKZF1 gene deletions

To reveal clonal evolution of IKZF1 deletions from diagnosis to relapse, patient-specific primers were designed to IKZF1 deleted junction. RQ-PCR analysis was performed with consensus second primer and probe (previously described for ΔEx3–6) [18]. Experimental set up was the same as for MRD analysis by Ig/TCR. We found out that both IKZF1 deletions ΔEx3–6d and ΔEx1–6, diagnosed at the first disease presentation, persisted at induction, and then disappeared the same way as Ig/TCR targets. Another IKZF1 deletion ΔEx3–6r detected at relapse was found also at the initial diagnosis and induction on the level of a few percents (Table 1).

3. Discussion

The presence of IKZF1 mutations was confirmed by several methods directed to different levels of manifestation: at the genomic (iFISH, PCR), transcriptomic (RQ-PCR DN isoforms

Fig. 2. iFISH analysis of IKZF1 gene, objectives at 100x magnification. Purple fluorescence signals marked IKZF1 locus, the nuclei stained with DAPI II (blue). One separate purple signal indicates lack of IKZF1 (intragenic deletion). (1–3) Diagnosis; (4–6) Relapse; (7–9) Control BM cells from patient with IKZF1 wild type. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
detection), and protein (cellular localization of Ikaros protein) levels.

Coding sequence of IKZF1 gene was not screened for point mutations due to the absence of its clinical relevance in ALL. Furthermore, according to the data announced in COSMIC, point mutations in IKZF1 coding sequence are mutually exclusive genetic events with \( \Delta \text{Ex3-Ex6} \) and \( \Delta \text{Ex1-Ex6} \) as well.

Generally, three different intragenic IKZF1 deletions were found in the patient by PCR: \( \Delta \text{Ex3–6}^d \) and \( \Delta \text{Ex1–6} \) at diagnosis and \( \Delta \text{Ex3–Ex6}^r \) at relapse. \( \Delta \text{Ex3–Ex6}^r \) deletion was also revealed in minor leukemic subclone at diagnosis by means of RQ-PCR with patient-specific primer at a lower level (0.087). FISH analysis indicated all deletions as heterozygous. These results can be explained by the presence of three clones of leukemic cells, differ in IKZF1 genotype. Two major clones caring \( \Delta \text{Ex3–6}^d \) and \( \Delta \text{Ex1–6} \) finally were eliminated during treatment, however minor \( \Delta \text{Ex3–Ex6}^r \) clone retained and led to early relapse. This case clearly demonstrated that clonal heterogeneity by IKZF1 gene status and clonal selection during the treatment took place. It is important to note that leukemic cells at diagnosis and relapse were positive for the same IgH and TCRG rearrangements, which proves that all leukemic clones were of common origin, and IKZF1 deletions are not initial, but secondary events. IKZF1 deletion is definitely bound to disease progression, as far as large majority of the cells were IKZF1 deleted at diagnosis, and 26 months later at relapse. So, this or that deletion required for retaining leukemic phenotype and progression. It might appear that mutant \( \Delta \text{Ex3-Ex6}^r \) IKZF1 deletion minor at the diagnosis itself provide to the cells sufficient proliferative or drug resistance advantage. However, to our mind, \( \Delta \text{Ex3-Ex6}^r \) IKZF1 deletion itself does not provide more aggressive phenotype, than \( \Delta \text{Ex3-Ex6}^d \) or \( \Delta \text{Ex1-Ex6} \) deletions, because all of them resulting in the expression of short Ikaros isoforms with identical dominant-negative phenotype. We assume that minor \( \Delta \text{Ex3-Ex6}^r \) clone acquired additional genetic lesion during the treatment.

Our results show that IKZF1 deletions could be used as a target, but reliability of MRD analysis may be impaired by instability of this marker during disease progression.

In primary diagnosis IKZF1 status was not taken into account for risk group stratification. According to protocol ALL-MB-2002 regulations, risk was underestimated and patient was assigned to the SRG. Prognostic value of IKZF1 alterations in ALL, especially BCR-ABL1 negative ALL, is still debated [19]. This case demonstrated the importance of screening of IKZF1 deletions for ALL prognosis.

**Table 1** Measurement of clonal genetic targets by RQ-PCR during follow-up.

<table>
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<th>( \Delta \text{Ex3–6}^d )</th>
<th>( \Delta \text{Ex3–6}^r )</th>
<th>( \Delta \text{Ex1–6} )</th>
<th>IgH</th>
<th>TCRG</th>
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<td>1.22E-02</td>
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<td>3.25E-01</td>
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<td>9.7E-02</td>
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**Fig. 3.** Confocal microscopy was used to examine the localization of wild-type and mutant Ikaros proteins. Nuclei stained with propidium iodine (PI, red), and Ikaros proteins were visualized by PE Mouse Anti-Human Ikaros antibodies (green), objectives at 100x magnification. Micron scale 10 \( \mu \text{m} \). (1–3) Abnormal cytoplasmic compartmentalization patterns of Ikaros protein (green) in patient’s BM cells at diagnosis; (4–6) Nuclear localization of Ikaros protein (green) in control ALL BM cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
4. Conclusion

Our report suggests the screening of IKZF1 gene alterations at diagnosis might be sufficient for prognosis of ALL, especially for BCR-ABL1 negative patients. IKZF1 gene abnormalities can be an independent genetic event which is not initiated by BCR-ABL1 fusion oncogene. In this described case, IKZF1 clonal deletion was the only predictor of relapse and poor prognosis.

From the diagnostic point of view, the analysis of IKZF1 gene deletions is complicated by different localization of chromosome breakpoints. Such methods as SNP-arrays [8,20], CGH-arrays [6,21] and MLPA [22–24] were mostly used for the analysis of this locus. However, the PCR detection is also applicable in most cases and can be supplemented by iFISH, RQ-PCR evaluation of short isoforms expression or protein localization by fluorescent microscopy.

In contrast to the classic chromosomal translocations in ALL, such as BCR-ABL1 or TEL-AML1, which are stable, IKZF1 deletions tend to oligoclonality and demonstrate clonal evolution in the disease progression. This makes IKZF1 deletions less reliable targets for MRD monitoring as compared with clonal Ig/TCR rearrangement.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.leukresrep.2016.06.005.

References