Identification of different Ikaros cDNA transcripts in Philadelphia-positive adult acute lymphoblastic leukemia by a high-throughput capillary electrophoresis sizing method

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ABSTRACT

Background
Ikaros is the prototypic member of a Kruppel-like zinc finger transcription factor subfamily that is required for normal hematopoietic cell differentiation and proliferation, particularly in the lymphoid lineages. Alternative splicing can generate multiple Ikaros isoforms that lack different numbers of exons and have different functions. Shorter isoforms, which lack the amino-terminal domain that mediates sequence-specific DNA binding, exert a dominant negative effect and inhibit the ability of longer heterodimer partners to bind DNA.

Design and Methods
In this study, we developed a high-throughput capillary electrophoresis sizing method to detect and quantify different Ikaros cDNA transcripts.

Results
We demonstrated that Philadelphia chromosome-positive acute lymphoblastic leukemia cells expressed high levels of the non-DNA-binding isoform Ik6 that was generated following *IKZF1* genomic deletions (19/46 patients, 41%). Furthermore, a recurring 60 bp insertion immediately upstream of exon 5, at the exon 3/exon 5 junction, was frequently detected in the Ik2 and Ik4 isoforms. This insertion occurred either alone or together with an in-frame ten amino acid deletion that was due to a 30 bp loss at the end of exon 7. Both the alterations are due to the selection of alternative cryptic splice sites and have been suggested to cause impaired DNA-binding activity. Non-DNA-binding isoforms were localized in the cytoplasm whereas the DNA-binding isoforms were localized in the nucleus.

Conclusions
Our findings demonstrate that both aberrant splicing and genomic deletion leading to different non-DNA-binding Ikaros cDNA transcripts are common features of Philadelphia chromosome-positive acute lymphoblastic leukemia.

Key words: *BCR-ABL1*, acute lymphoblastic leukemia, Ikaros.

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The online version of this article contains a supplementary appendix.

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Introduction

Ikaros (IKZF1, Lyf-1) is a member of the Kruppel family of zinc finger DNA-binding proteins. Ikaros is a key regulator of the hemo-lymphoid system and functions by both potentiating and repressing gene expression.[1-3] Mice that are heterozygous for a germline mutation that results in a loss of critical DNA-binding zinc fingers of Ikaros develop a very aggressive form of lymphoblastic leukemia, suggesting that Ikaros has an important leukemia suppressor function.[4] Moreover, Ikaros plays an important role in the recruitment and centromere-associated silencing of potentially leukemogenic growth-regulatory genes.[5] The human *IKZF1* gene, located at 7p12, contains eight exons[6] that can, by alternative splicing, give rise to at least eight isoforms.[7,8] The isoforms differ in the number of N-terminal zinc finger motifs that bind DNA and contain the nuclear localization signals, resulting in isoforms with and without DNA-binding properties. All isoforms share a common C-terminal domain that contains a transcriptional activation domain and two zinc finger motifs that are required for hetero- or homodimerization and for interactions with other proteins. The N-terminal domains contain different combinations of one to four zinc finger motifs. Ikaros proteins with fewer than three N-terminal zinc fingers cannot bind to DNA, and play a dominant negative role in transcription by interfering with the activity of isoforms that can bind DNA.[9-11]

The fact that Ikaros functions as a critical regulator of normal lymphocyte development and the observation of the rapid development of leukemia in mice expressing non-DNA-binding isoforms prompted many studies that investigated whether normal Ikaros expression and function might be altered in human hematologic malignancies. An excess of short Ikaros isoforms has been described in leukemic cells obtained from infants, in childhood B and T acute lymphoblastic leukemias (ALL),[10-13] in *de novo* adult B ALL,[14] in cells from transformed chronic myeloid leukemias (CML)[15] and from *de novo* acute myelomonocytic and monocytic leukemias.[16,17] Recently, data from Mullighan *et al.*[18,19] and preliminary results from our group[20,21] shed light on the mechanisms responsible for the generation of the aberrant Ikaros isoforms. These data demonstrated that expression of non-DNA-binding Ikaros isoforms is due to intragenic *IKZF1* genomic deletions, identified in over 80% of *BCR-ABL1* cases, and not aberrant post-transcriptional splicing.[22] The most frequent genomic alteration involving the *IKZF1* gene was a deletion of an internal subset of exons, most commonly exons 4–7 (Δ4–7). The extent of this deletion correlated with the high expression, as determined by reverse transcriptase polymerase chain reaction (RT-PCR),[19] of the dominant-negative isoform Ik6 lacking the exons from 4 to 7. In this study, we aimed to (i) set up a screening method in order to rapidly identify different Ikaros cDNA transcript variants and (ii) to evaluate their relative expression in samples obtained at diagnosis from 46 adult patients diagnosed with Philadelphia chromosome positive (Ph+) ALL. Ph+ ALL is characterized by clonal proliferation of transformed pre-B cells that express the BCR-ABL oncoprotein;[23] it constitutes 20%-30% of all cases of ALL and has a very poor prognosis.[24,25] Treatment with the tyrosine kinase inhibitor imatinib is currently the standard therapy for CML, which is also caused by BCR-ABL.[26] However, imatinib has shown limited efficacy in the treatment of Ph+ ALL,[27,28] suggesting that BCR-ABL may be only the tip of the iceberg and that other factors may contribute to the pathogenesis of this disease, its resistance to therapy and progression.

In this study we developed a rapid, high-throughput technique based on capillary electrophoresis to detect and quantify different Ikaros transcript variants in the same sample.

Design and Methods

Patients

Forty-six adult patients with *de novo* Ph+ ALL were investigated after we had obtained written informed consent from them. Their median age was 50 years (range, 18-75) and the median blast percentage was 85% (range, 10-100%). Leukemic bone marrow or peripheral blood samples were collected.

Nineteen patients were positive for the p210 BCR-ABL oncoprotein, 24 were positive for the p190 oncoprotein and three for both. Normal mononuclear cells from peripheral blood of healthy normal volunteers and mononuclear cells from bone marrow and peripheral blood from patients with leukemia in remission were also analyzed for Ikaros expression. The human lymphoblastoid SD-1, and the human B-cell precursor leukemia BV-173 cell lines were also included in the analysis. Human cell lines were obtained from DSMZ (Deutshe Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and maintained in culture according to the recommendations from the DSMZ.

Ikaros transcript analysis

Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation and after extraction, 1 μg RNA was used for cDNA synthesis as described previously.[29]

In order to set up a screening system for Ikaros transcript variants, cDNA was amplified with two pairs of oligonucleotides, the forward primer of each couple being conjugated with a fluorescent dye (fluorescein, excitation at 494 nm and emission at 521) at its 5' end yielding amplicons A and B (Table 1). Polymerase chain reaction (PCR) was performed using 1 unit of AmpliTaq Gold DNA polymerase and a final concentration of 1.5 mM $MgCl_2$, on a BIOMETRA Tpersonal thermal cycler set for an initial denaturation at 95°C for 5 min, 25-35 cycles with denaturation at 95°C for 30 s, annealing at 62°C for 30 s, extension at 72°C for 50 s, and a final cycle at 72°C for 10 min and at 60°C for 45 min to stabilize the fluorescence. One microliter of each amplicon was added to 9 μL of formaldehyde (Sigma-Aldrich) containing 0.2 μL of GeneScan 500 (-250) LIZ size standard (Applied Biosystems) and loaded on the ABI Prism 3730 DNA analyzer for automated capillary gel electrophoresis. The results were plotted with AbiPrism GeneMapper v3.5 software (Applied Biosystems). The

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GeneMapper electropheretograms displayed information about transcript length, peak height and peak area. Peak heights are correlated to the quantity of amplified PCR product and were used as an indication of the expression of Ikaros transcript variants in a sample. The relative expression of each Ikaros isoform was expressed as a percent fraction between the height of each peak and the sum of the heights of all peaks in a sample according to the following equation:

Ik isoform A= [A/(A+B)]x100, where A= peak height of isoform A and B= sum of the peak heights of other Ik isoforms expressed in a sample.

Since this method cannot be used for fragments of more than 700 bp, we omitted the Ik1 isoform from the analysis. RNA integrity was confirmed by PCR amplification of the GAPDH mRNA, which is expressed ubiquitously in human hematopoietic cells.

Cloning and sequencing analyses

Nucleotide sequences of all the observed amplicons were validated by repeating the PCR with 5'-unmodified primers and cloning the products into pcR2.1-TOPO vectors using the TOPO TA Cloning Kit and related protocol (Invitrogen, San Diego, CA, USA). TOP10F' strain *E. coli* cells (Invitrogen) were employed as a host for transformation, and colonies containing the recombinant plasmids were screened by PCR with the primer pair for the appropriate amplicon under the same conditions described previously. The PCR products were purifed with QIAquick PCR purification kit (Qiagen, Hilden, Germany) and directly sequenced using an ABI PRISM 3730 automated DNA sequencer (Applied Biosystem, Foster City, CA, USA) and a Big Dye Terminator DNA sequencing kit (Applied Biosystem).

Western blots

Cells were lysed with sample buffer [2% sodium dodecylsulfate (SDS) in 125 mM Tris HCL, pH 6.8]. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 12% gels and then transferred to nitrocellulose membranes (Amersham Biosciences), as described previously.[29]

Subcellular localization studies using confocal laser scanning microscopy

The subcellular localization of Ikaros proteins was examined by immunofluorescence and confocal laser scanning microscopy, as described previously.[29] Representative digital images were processed using the Photoshop Software (Adobe Systems).

DNA-binding assay: electrophoretic mobility shift assay (EMSA)

The Ikaros consensus and mutant sequences (^{32}P-labeled sense probe: 5'-GTTTCTTCAGAGCCTGGGAAACAAGTC-3, containing a known high-affinity Ikaros-binding site (underlined) and ^{32}P-labeled antisense probe: 5'-ATTCTGACTTGTTTCCCAGGCTCGAA-3') were obtained from Sigma Genosys, labeled with T4 polynucleotide kinase (LifeTechnologies, Gaithersburg, MD USA) and [^{32}P]-ATP (NEN, Boston, MA, USA) and purified over a Sephadex G25 (Pharmacia Biotech) column. Forty micrograms of protein nuclear extracts (obtained from full length Ik- and Ik6-positive patients) were incubated with 2 mg poly d(I-C) (Roche Molecular Biochemicals, Indianapolis, IN, USA) and 15 fmol of ^{32}P-labeled probe in 10 mM HEPES, 5 mM Tris, 50 mM KCl, 1.2 mM EDTA, and 10% (vol/vol) glycerol (pH 7.8) for 30 minutes at room temperature. A 200-fold molar excess of unlabeled oligonucleotide (Ikaros consensus or mutant) was added for competition assays. Protein/DNA complexes were resolved on 6% native polyacrylamide gels in 0.25 3 TBE (25 mM Tris, 22.5 mM boric acid, and 0.25 mM EDTA). Gels were visualized by autoradiography using MS-BioMax film and intensifying screens (Kodak, Rochester, NY, USA).

Table 1. PCR primers used for Ikaros transcript analysis.

Amplicon	*Primer name*	*Sequence (5'→ 3')*	*Position*
A	Ikaros F1, Fluorescein-conjugated	ATGGATGCTGATGAGGGTCAAGAC	exon 2
	Ikaros R1	GATGGCTTGGTCCATCACGTGG	exon 8
B	Ikaros F2, Fluorescein-conjugated	GGGGCTGATGACTTTAGGGATTTC	insertion
	Ikaros R1	GATGGCTTGGTCCATCACGTGG	exon 8

Results

Adult Ph+ ALL patients express different wild-type Ikaros transcript variants

The expression of Ikaros was detected in all patients, and the majority of these patients expressed different Ikaros variants at the same time (*Online Supplementary Table S1* and Figure 1A). In 19/46 (41%) Ph+ ALL patients we identified a single peak of 255 bp by fluorescent RT-PCR; by cloning and subsequent sequencing we found that this peak corresponds to an Ik6 isoform in which exon 3 is juxtaposed to exon 8. Since this isoform lacks all zinc fingers required to bind the DNA of target genes, it is considered to be dominant-negative. In the remaining patients (27/46, 59%) we observed the coexistence, in the same PCR sample at the same time, of many fragments ranging from more than 900 bp to less than 200 bp (Figure 1B and 1C). In addition to the already-identified Ik6 isoform in these patients, cloning and subsequent sequencing analysis revealed that the longer PCR product was Ik-1, the full-length isoform containing all exons (945 bp). The 684 bp fragment was identified as an Ik2 isoform that shares three of the four N-terminal zinc fingers (F2, F3 and F4), whereas the 558 bp band corresponded to the Ik4 isoform in which exon 4 and exon 6 are lost. Other spliced Ikaros isoforms were identified: Ik4A containing exons 1, 2, 3, 5 and 8; Ik8 which lacks exons 4, 5 and 6; and a new isoform, termed Ik5A, which contains exons 1, 2, 3, 4, 7 and 8. Furthermore, we identified a new small Ikaros isoform

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that contained just three exons: 1, 2 and 8 (Ik6Δ).

Since patients can express different Ikaros isoforms at the same time, our aim was to determine whether some isoforms could be overexpressed and favored by alternative splicing. We set up a fast, high-throughput method that is derived from microsatellite analysis and based on capillary electrophoresis technology to detect and quantify splice variants. Since the peak heights shown in the electropherograms are correlated to the quantity of amplified PCR product, we used them as an indicator of the relative expression of each Ikaros isoform in a sample. As shown in Figure 1D, in the samples in which more than one Ikaros variant was co-expressed, the isoforms more frequently produced by alternative splicing were Ik2 (median value 19%, SD 5.14), Ik4 (median value 16%, SD 8.46), Ik6Δ (median value 21%, SD 10.45) and Ik8 (median value 11%, SD 3.75). Minor expression of other isoforms was observed: Ik4A (median value 7%, SD 2.75), Ik5A (median value 9%, SD 3.22) and Ik6_ (median value 6%, SD 5.48). In comparison, in normal bone marrow pre-B cells, we observed that the major Ikaros isoforms expressed were Ik2 (median value 23%, SD 3.15), Ik4 (median value 20%, SD 5.20) and Ik8 (median value 9%, SD 4.55). Ik6 expression was observed at very low levels (median value 6%, SD 6.84). Similarly, Ik5A (median value 8%, SD 2.85), Ik4A (median value 7%, SD 2.95) and Ik5 (median value 2%, SD 3.05) were also expressed at very low levels.

Ph+ ALL patients express different aberrant Ikaros transcript variants

In addition to wild-type isoforms generated by the alternative splicing of Ikaros pre-mRNA, we frequently identified transcript variants with an atypical length that did not correspond to well-known and characterized wild-type isoforms. By cloning and subsequent sequencing, we found that Ph+ ALL patients may express aberrant spliced Ikaros isoforms already described in normal and T-leukemia cells.[30,31] In the 27/46 (59%) patients who did not express Ik6 alone, we found clones that expressed wild-type Ikaros isoforms (Ik1, Ik2, Ik4, Ik4a, Ik5a, Ik6, Ik6Δ and Ik8) that have been previously described, as well as clones that expressed aberrant Ik2 isoforms [which we called Ik2(ins)] or Ik4/4A/5A isoforms [Ik4(ins), Ik4A(ins), Ik5A(ins), respectively] with a 20 amino acid insertion (TYGADDFRDFHAIIPKSFSR) due to a 60-bp insertion immediately downstream of exon 3 (*Online Supplementary Table S1 and Figure 2A-B*). This alteration was identified alone and together with an in-frame ten amino acid deletion, DKSSMPQKFLG, due to a 30-bp deletion at the end of exon 7. Furthermore, aberrant transcript variants containing only the deletion between the exon 7 and exon 8 were also detected [Ik2(del), Ik4(del), Ik5A(del) and Ik8(del)], increasing the complex scenario of Ikaros isoforms produced by alternative splicing. The observed N-terminal insertions and C-terminal deletions did not cause a frame shift and, therefore, did not change the downstream amino acid sequences.

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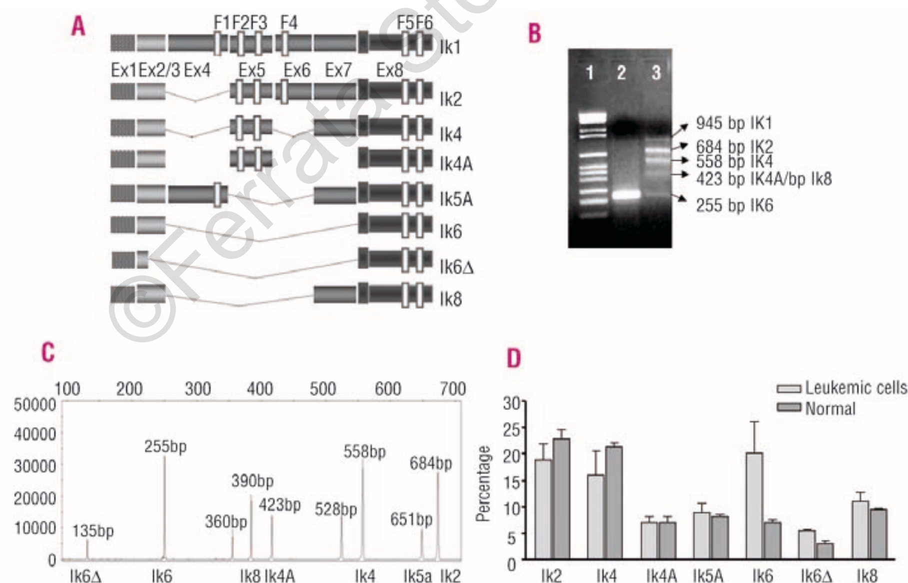

Figure 1. (A) Schematic diagram of the different Ikaros isoforms produced in Ph+ ALL samples by alternative splicing; N-terminal zinc-fingers (F) show DNA-binding activity and C-terminal F mediate dimerization of the protein (Ex= exon). (B) Bands generated by RT-PCR using primers derived on exons 2 and 8 and corresponding to the alternatively spliced products of the Ikaros pre-mRNA transcript. PCR products were detected by ethidium bromide staining of 1.5% agarose gel. The left lane (lane 1) is the molecular size marker, Marker VI Roche; lane 2: Ik6 expression; lane 3: co-expression of Ik1, Ik2, Ik4, Ik4A/Ik8 and Ik6. (C) Electropherogram of Ikaros PCR product performed using a forward primer conjugated with the fluorescein dye at its 5' end. Different Ikaros isoforms are represented in the electropherogram by different peaks. The x-axis displays the computed length of the PCR products in base pairs, as determined automatically by the use of an internal lane standard. The y-axis represents the peak height in fluorescence units. (D) Relative expression of wild-type Ikaros isoforms in Ph+ leukemic cells and normal cells.

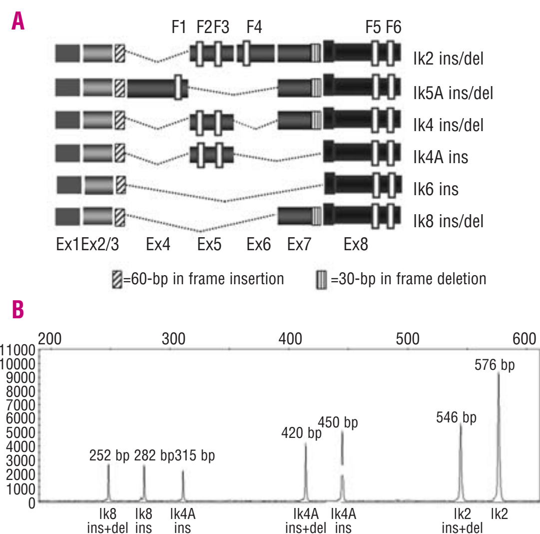

Figure 2. (A) Schematic diagram of Ikaros isoforms with a 60-bp insertion or 30-bp deletion identified in Ph+ ALL samples. (B) Electropherogram of Ikaros PCR product performed using the insertion primer conjugated with the fluorescein dye at its 5' end. Different Ikaros isoforms are represented in the electropherogram by different peaks. The x-axis displays the computed length of the PCR products in base pairs, as determined automatically by the use of an internal lane standard. The y-axis represents the peak height in fluorescence units.

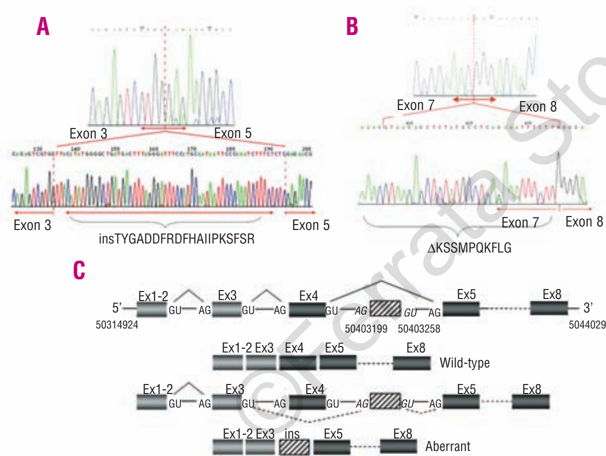

Figure 3. (A) The wild type junction between exon 2 and exon 4 is shown in the upper panel: below there is the aberrant sequence with the insertion of a 60-bp (TTACATATGGGGCTGATGACTTTAGGGATTTCCATGCAATAATTCCCA) between the exon 3/exon 5 junction. (B) The 30-bp deletion at the exon 7/exon 8 junction is shown in the upper panel; below there is the wild-type sequence. (C) Schematic representation of the mechanism determing the insertion of a region inside the intron 3-4 and skipping of exon 4. The nucleotide position of the alternatively spliced insertion is showed according to GenBank accession number NC_000007.12 and starting from 50314924 to 50440293.

The aberrant Ikaros isoforms are due to the selection of an alternative splice donor site and an alternative splice acceptor site

We hypothesized that the 60 bp insertion is due to selection of an alternative splice donor site and an alternative splice acceptor site (Figure 3A). Using the Basic Local Alignment Search Tool (BLAST, *http://www.ncbi.nlm.nih.gov/blast/Blast.cgi*), we found that the 60 bp insertion present in the Ik2(ins) or Ik4(ins) isoforms corresponded to a region in intron 3-4. This sequence is flanked by cryptic splice sites because it starts with AG and ends with GU (i.e., GT in cDNA). In Ik2(ins) or Ik4(ins), the selection of these alternative splice sites may cause skipping of exon 4 and the introduction of an alternative exon (Figure 3C). In the 5A isoform, the 60 bp insertion is in conjunction with exon 3, which adds strength to our hypothesis of its role as an alternative exon. The 60 bp insertion encodes a perfect helix that is followed by a flexible region (KSFSR) located upstream of the DNA-binding zinc fingers (F2 and F3). The presence of this new motif may alter the Ikaros-specific DNA-binding activity significantly. The 30 bp deletion in exon 7 might also have resulted from the selection of an alternative splice site because it starts with a GU sequence (ie, GT in cDNA) at the 5' junction, which could serve as a donor site recognition sequence (Figure 3B).

Analysis of DNA-binding and non-DNA-binding Ikaros isoforms containing the 60 bp insertion

To obtain a more accurate characterization of the aberrant isoforms, Ikaros cDNA was amplified with a forward oligonucleotide conjugated with a fluorescent dye at its 5' end that was designed to specifically detect forms with the 60 bp insertion following exon 3. RT-PCR products from insertion primer-identified transcripts of different lengths were sequenced and corresponded to hypothetical aberrant isoforms. Among these, the most expressed transcript variants were those of 576 bp (median value 19%, SD 7.41), 546 bp (median value 17%, SD 4.20), 450 bp (median value 14%, SD 4.64) and 420 bp (median value 16%, SD 4.44) which corresponded to Ik2(ins), Ik2(ins+del), Ik4(ins) and Ik4(ins+del) isoforms, respectively (Figure 4). In only three patients (patients 4, 15 and 27 in *Online Supplementary Table S1*), we identified the Ik5A (ins) isoform that was expressed at high levels (median value 29%). The other isoforms, Ik4A(ins), Ik6(ins), Ik8(ins) and Ik8(ins+del), were detected at lower levels. All aberrant isoforms were confirmed by cloning and subsequent sequencing except for Ik6(ins), Ik8(ins) and Ik8(ins+del). It is likely that these isoforms are expressed at very low levels in cases of leukemia (median value 7%, range 3-13%). To determine whether the aberrant human Ikaros splice variants with insertions or deletions were unique to patients with Ph+ ALL at diagnosis, we studied Ikaros expression in acute leukemia cases in remission and in normal human peripheral blood. Our RT-PCR assays with capillary electrophoresis showed that Ikaros transcript variants with inserts or deletions were present in both normal human hematopoietic cells and in acute leukemia cases in remission. However, these isoforms were expressed at low levels (less than 5% considering their expression with respect to the other Ikaros isoforms *vs.* a median value of 15% in leukemic cells) (Figure 4).

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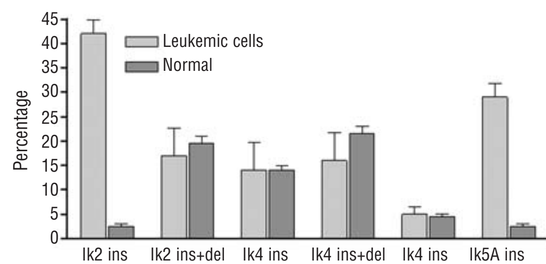

Figure 4. Relative expression of aberrant Ikaros isoforms produced using insertion primers in Ph+ ALL samples and normal cells or cases of acute leukemia in remission. The relative expression of each Ikaros isoform was expressed as a percent fraction between the height of each peak and the sum of the heights of all peaks in a sample according to the following formula: Ik isoform A= [A/(A+B)]x100, where A=peak height of isoform A, and B= sum of the peak heights of other Ik isoforms expressed in a sample. The Ik1 isoform was omitted from the analysis.

Ikaros isoforms in Ph+ ALL showed abnormal subcellular compartmentalization and loss of Ikaros-specific DNA-binding activity

RT-PCR and sequencing results were confirmed by western blot analysis on primary leukemic cells from each adult Ph+ ALL patient expressing small non-DNA-binding and/or aberrant isoforms of Ikaros (Figure 5A). A 57- kDa immunoreactive protein that corresponded in size to Ik-1 and a 47- kDa immunoreactive protein that corresponded to Ik-2 were identified. We confirmed the presence of a smaller immunoreactive protein band of approximately 37 to 40 kDa that corresponded in size to Ik6 in patients who were positive for this non-DNA-binding isoform by RT-PCR. The absence of abundant Ik1 and Ik2 was not caused by generalized proteolytic degradation because a 120-kDa Cbl protein was detected by western blot analysis in the same whole cell lysates. The co-expression of many Ikaros isoforms in the same sample confirmed that the different splice variants identified by RT-PCR are translated. In the patients who were positive for the expression of only Ik6, we confirmed the presence of only one band of approximately 40 kDa.

The subcellular compartimentalization of Ikaros proteins in normal hematopoietic cells and in primary Ph+ leukemic cells was compared by confocal laser scanning microscopy. In normal mononuclear cells, the nuclei were stained brightly by the anti-Ikaros antibody, as shown by a specific punctuate green fluorescent staining pattern. In mononuclear cells from patients who expressed the Ik6 isoform alone, we observed a cytoplasmatic localization of Ikaros, as evidenced by a bright green fluorescent ring surrounding the red nuclei. It is interesting to note that in patients who expressed different Ikaros transcript variants, we identified both nuclear proteins and cytoplasmic proteins. This suggests that full-length Ikaros isoforms localize to the nuclei, whereas the short dominant negative isoforms localize to the cytoplasm (Figure 5B).

The ability of nuclear extract proteins from normal mononuclear cells and leukemic cells to show Ikaros-specific, high affinity DNA-binding activity was tested using EMSA. In the extracts from cells expressing the Ik6 isoform alone, only one very weak protein-DNA complex band was found, in contrast to normal cells that expressed full-length isoforms (Figure 5C).

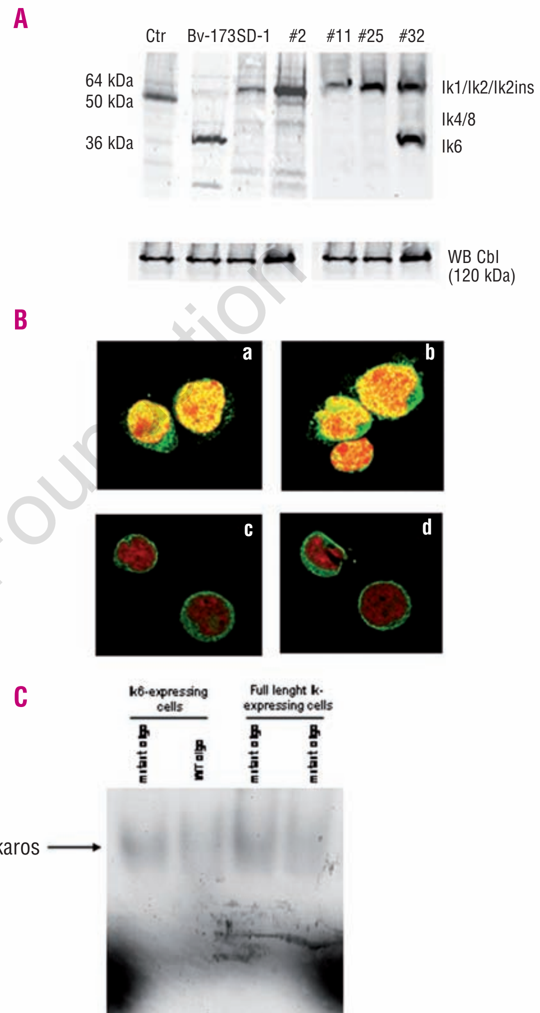

Figure 5. (A) Anti-Ikaros western blots of whole cell lysates from leukemic cell lines (SD-1 and BV-173) and leukemic bone marrow mononuclear cells with Ph+ ALL (#2, #11, #25, and #32). The positions corresponding to the migration patterns of Ik-1 (≈57 kDa), Ik-2/Ik2ins (≈47 kDa) and Ik-6 (≈37 kDa) proteins are indicated. Anti-Cbl western blotting of the whole cell lysates was performed as a control. (B) Expression and subcellular localization of Ikaros proteins in leukemic cells from Ph+ ALL patients. In all images cells were stained with an Ikaros antibody (green) and with propidium iodide (red) to visualize DNA. In (a) and (b) confocal images of leukemic cells from patients expressing full-length Ikaros isoforms showed the characteristic multifocal nuclear localization pattern of Ikaros. (c) and (d) are confocal images of leukemic cells expressing Ik6 isoform and showing cytoplasmic expression of Ikaros (i.e., a bright green fluorescent rim surrounding the completely labeled red nuclei). (C) Competition assays for cells expressing Ik6 and full length Ikaros isoforms. A 200 fold molar excess of unlabeled oligonucleotide (wild type or mutant) was used. Ikaros binding activity was detected in cells expressing full-length isoforms but at very low levels in cells expressing the Ik6 isoform. Nuclear extracts equivalent to 40/μg proteins were used for each reaction; the arrow indicates Ikaros-specific bands.

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Discussion

The transcription factor Ikaros was identified as a potential regulator of enhancer and promoter elements critical for the expression of lymphoid-specific genes.[8,32] However, its expression is more widely distributed within the developing and adult hematopoietic system since it acts from the earliest stages of hemo-lymphopoiesis and is required for the balanced production and function of a variety of blood and immune cells.[33] Ikaros exerts its effects on development as a set of differentially spliced isoforms that contain two functionally distinct Kruppel-type zinc finger domains. One of these domains is involved in DNA-binding and the other is involved in protein interactions.[34] During the past 10 years, several groups have reported the frequent expression of aberrant Ikaros isoforms in acute leukemias (childhood B and T acute lymphoblastic leukemias,[10-13] *de novo* adult B ALL,[14] CML in blast crisis[15] and *de novo* acute myelomonocytic and monocytic leukemias).[16,17] In 2006, Klein *et al*.[22] suggested that the expression of aberrant Ikaros isoforms occurred at a post-transcriptional level as a result of the action of the BCR-ABL1 fusion protein. Taking advantage of a high-resolution single nucleotide polymorphism array, this hypothesis was completely replaced by the findings from Mullighan *et al*.[19] and our group.[20,21] These studies demonstrated that genomic aberrations of the *IKZF1* locus are responsible for the expression of aberrant Ikaros isoforms. There was a complete concordance between the extent of the deletions and the generation of different Ikaros cDNA transcripts. The most frequent alteration involving the *IKZF1* gene was a deletion of exons 4–7 (Δ 4–7) which lead to the expression of the Ik6 isoform. Given that different Ikaros isoforms can be expressed following genomic deletion and/or alternative splicing and considering the leukemic role of short and aberrant isoforms, it is extremely important to use a sensitive method to detect and quantify the different transcript variants.

In this study, we set up the first fast, high-throughput method to detect and quantify splice variants. The technique is derived from microsatellite analysis[35-37] and is based on capillary electrophoresis technology that is characterized by high detection sensitivity, highly accurate sizing capability and an automated format that requires minimal user intervention. Our results demonstrated that this method can be very useful for screening for different transcript variants at a high resolution and may become a useful tool in different research areas. This technique allows not only screening of different variants, but also the quantification of the same variants in the same experiment. It is, therefore, less labor-intensive than other available techniques, such as real-time PCR.

We were able to characterize all Ikaros isoforms expressed in adult Ph+ ALL at diagnosis and several isoforms that were previously unidentified, such as Ik5A and Ik6Δ. We demonstrated that 41% of Ph+ ALL patients expressed high levels of the DNA-binding dominant negative Ik6 isoform that lacks critical N-terminal zinc-fingers. In addition, this isoform displayed an abnormal subcellular compartmentalization pattern. Nuclear extracts from patients expressing Ik6 failed to bind DNA in a mobility shift assay using a DNA probe containing an Ikaros-specific DNA binding sequence. Furthermore, we recently demonstrated that the expression of Ik6 correlated with BCR-ABL mRNA levels, disease progression, relapse and resistance to imatinib and dasatinib.[29] In 59% of Ph+ ALL patients, we observed the coexistence of multiple splice variants corresponding to the Ik1, Ik2, Ik4, Ik4A, Ik5A, Ik6, Ik6Δ and Ik8 isoforms in the same PCR sample and at the same time. In these patients, we also identified aberrant full-length Ikaros isoforms that were characterized by a 60 bp insertion immediately downstream of exon 3 and a recurring 30 bp in-frame deletion at the end of exon 7 which most frequently involved the Ik2 and Ik4 isoforms. Both the insertions and deletions were due to the selection of alternative splicing donor and acceptor sites. The 60 bp insertion is incorporated into the DNA-binding region and may, therefore, significantly alter the DNA-binding activity of the Ik2 and Ik4 isoforms. The deleted sequence in these aberrant Ikaros isoforms is close to the conserved bipartite transcription activation domain within exon 8 and is adjacent to the C-terminal zinc finger dimerization motifs. The deletion of this peptide determines structural changes that may affect the accessibility of the Ikaros activation domain to members of the basal transcription machinery as well as the stability of such interactions. Furthermore, the ability of these aberrant isoforms to form dimers with other Ikaros isoforms or other proteins may also be impaired, and such impairments may lead to altered DNA-binding or altered subcellular localization of Ikaros. Our studies demonstrated that expression of proteins representing Ikaros insertion forms and DNA non-binding Ikaros isoforms is not limited to malignant cells although there were differences in incidences and percentages between normal and leukemia cells. The most significant and relevant difference in levels of detection was observed for the Ik6 isoform identified as a single isoform only in leukemic cells. Whether and/or how the expression of aberrant Ikaros isoforms contributes to leukemogenesis remains to been determined. However, Kano *et al*.[38] recently showed that Ik6 expression enhances cell survival and reduces apoptotic cell death after inteleukin-3 withdrawal. Understanding the role of altered Ikaros activity in *BCR–ABL1* leukemogenesis could help in the development of more effective therapeutic strategies against this aggressive luekemia.

Authorship and Disclosures

II: collected data and wrote the manuscript; GM and MB designed and supervised the study and gave final approval to the manuscript. AL, RB, SF performed molecular analysis; AB and GB performed western blot analyses; DC, FM EO, FA, FS, SP, CP, PPP, PG, SS, GP, FP, MM, AV, SB and RB contributed to the development of the study and data collection. The authors reported no potential conficts of interest.

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