Decreases in Ikaros Activity Correlate with Blast Crisis in Patients with Chronic Myelogenous Leukemia


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Abstract

Gene targeting studies in mice have shown that the lack of Ikaros activity leads to T-cell hyperproliferation and T-cell neoplasia, establishing the Ikaros gene as a tumor suppressor gene in mice. This prompted us to investigate whether mutations in Ikaros play a role in human hematological malignancies. Reverse transcription-PCR was used to determine the relative expression levels of Ikaros isoforms in a panel of human leukemia/lymphoma cell lines and human bone marrow samples from patients with hematological malignancies. Among the cell lines examined, only BV-173, which was derived from a chronic myelogenous leukemia (CML) patient in lymphoid blast crisis, overexpressed the dominant-negative isoform Ik-6. In 9 of 17 samples of patients in blast crisis of CML, Ikaros activity had been reduced either by drastically reducing mRNA expression (4 of 17) or by overexpressing the dominant-negative isoform Ik-6 (5 of 17). Significantly, expression of Ikaros isoforms seemed normal in chronic phase CML patients and patients with other hematological malignancies. In some cases, overexpression of the dominant-negative Ik-6 protein was confirmed by Western blot analysis, and Southern blot analysis indicated that decreases in Ikaros activity correlated with a mutation in the Ikaros locus. In summary, these findings suggest that a reduction of Ikaros activity may be an important step in the development of blast crisis in CML and provide further evidence that mutations that alter Ikaros expression may contribute to human hematological malignancies.

Introduction

Ikaros encodes by alternate splicing a family of zinc-finger DNA-binding proteins necessary for both the development and homeostasis of the lymphoid system (1–4). Ikaros proteins form homo- and heterodimers via the COOH-terminal zinc fingers common to all the isoforms, whereas DNA binding is mediated by the NH2-terminal zinc fingers, the number of which varies among the different isoforms. Isoforms that have less than two NH2-terminal zinc fingers either do not bind DNA or only do so with low affinity; they, thus, have a dominant-negative effect on the DNA-binding capacities of isoforms with more than two NH2-terminal zinc fingers (5). Other potential targets for the dominant-negative Ikaros isoforms are the products of the related genes Aiolos (6) and Helios (7, 8), both of which have expression patterns that overlap with, yet are distinct from, that of Ikaros. Mice homozygous for a deletion in Ikaros of the NH2-terminal zinc fingers produce only dominant-negative Ikaros proteins, resulting in a complete lack of mature T and B lymphocytes and natural killer cells, as well as committed lymphoid progenitors (1). These mice also exhibit exaggerated extramedullary hematopoesis. Mice heterozygous for the dominant-negative mutation generate a normal lymphoid repertoire initially, but as young adults they develop lymphoproliferative disorders and eventually die of T-cell leukemias and lymphomas (3). Mice homozygous for a deletion in Ikaros of the COOH-terminal zinc fingers (i.e., a null mutation in Ikaros), display a phenotype that is less severe than that caused by the dominant-negative mutation. Whereas fetal T lymphocytes, fetal and adult B lymphocytes, natural killer cells and their earliest progenitors are absent, T cells do develop postnatally. However, these T cells also undergo transformation (4). That both Ikaros mutations cause a hyperproliferative phenotype in T cells suggests that a lack of or profound decrease in Ikaros activity leads to T-cell hyperproliferation and T-cell neoplasia. We studied Ikaros isoforms in various leukemia/lymphoma cell lines and leukemic cells from patients with hematological malignancies by RT-PCR2 and immunoblotting. As a result of this analysis, we found a strong correlation between a reduction in Ikaros activity and blast crisis in patients with CML. In 9 of 17 samples examined of patients in blast crisis, Ikaros activity had been compromised, either by severely reducing mRNA expression (4 of 17) or by overexpressing a dominant-negative isoform (5 of 17). In normal volunteers, chronic phase CML patients and patients suffering from other hematological malignancies, Ikaros expression seemed normal. These findings suggest a potential role of Ikaros in the development of blast crisis in CML patients.

Materials and Methods

Cell Lines. Human leukemia/lymphoma cell lines were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS (ICN Pharmaceuticals, Costa Mesa, CA) and 1% penicillin/streptomycin (Life Technologies Inc., Gaithersburg, MD).

Bone Marrow Samples. Bone marrow aspirates were obtained from patients with hematological malignancies after obtaining informed consent. Characteristics of patients with CML in blast crisis are shown in Table 1. Additional bone marrow samples from seven normal volunteers, 17 CMLs in chronic

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2 The abbreviations used are: RT-PCR, reverse transcription-PCR; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia.
DECREASED IKAROS ACTIVITY IN CML BLAST CRISIS

**Table 1** Patient characteristics of CML blast crisis

<table>
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<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>Ikaros</th>
<th>Karyotype</th>
<th>MPOa</th>
<th>Phenotype</th>
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<tr>
<td>1</td>
<td>55/M</td>
<td>D</td>
<td>46.XY,9;22(q13;14)</td>
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<td>ND</td>
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<tr>
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<tr>
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<td>D</td>
<td>45.XX,−9;22(q13;14),+20.der22(9;22)(q34;q11)</td>
<td>(−)</td>
<td>CD10+,CD19+,CD33−</td>
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<tr>
<td>5</td>
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<td>ND</td>
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</tbody>
</table>

a MPO, myeloperoxidase; D, down-regulation of Ikaros expression; DN, dominant-negative isoform; ND, not determined; FISH, fluorescence in situ hybridization.

phase, 19 AMLs, 8 ALLs, 6 myelodysplastic syndromes, 3 multiple myelomas, 2 polycythemas vera, and 4 non-Hodgkin’s lymphomas with bone marrow involvement were examined.

**RNA Isolation and RT-PCR Analysis.** RNA was extracted using RNAzol B (Biotex Laboratories, Houston, TX) as recommended by the manufacturer. Total RNA (5 μg) was heat denatured at 70°C for 10 min in the presence of 0.5 μg of oligonucleotide dT primer (Life Technologies Inc.) and chilled on ice. The denatured RNA was reverse transcribed in 20 μl of final volume of 1× reverse transcriptase buffer, 2.5 mM MgCl₂, 125 nM of each dNTP, 10 nM DTT, and 200 units of SuperScript II reverse transcriptase (Life Technologies, Inc.). The reaction mixture was incubated at 42°C for 1 h and then at 70°C for 15 min. PCR was performed using 1 μl of cDNA in 50 μl of 1× PCR buffer, 2.5 mM MgCl₂, 400 nM of each primer, and 5 units of TaKaRa LA Taq (TaKaRa Shuzo, Shiga, Japan) under the following cycling condition: 94°C for 2 min for denaturation, then 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s for 35 cycles (Fig. 1A), followed by 72°C for 7 min. The sequences of the primers used are as follows: Ikaros sense primer, 5′-cacataacctgaggaccatg-3′, and Ikaros antisense primer, 5′-aggctgtagttcagtcgga-3′.

**Sequencing Analysis.** The PCR products were subcloned into the pCR2.1 vector using Original TA Cloning Kit (Invitrogen, San Diego, CA). The sequenc analysis was performed by the ABI 373 DNA Sequencer (Perkin-Elmer Corp., Foster City, CA) using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corp.).

**Immunoblotting.** Nuclear protein extraction and immunoblotting were performed, as described previously (9, 10), with minor modifications. Nuclear extracts (20 μg) from human leukemia/lymphoma cell lines and bone marrow samples were size-fractionated on SDS-polyacrylamide gradient gels and transferred to Hybond nitrocellulose membranes (Amersham International, Buckinghamshire, England). The membranes were incubated in blocking buffer (5% dry milk and 0.1% Tween 20 in PBS) for 1 h and incubated thereafter with anti-Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Ikaros antibody. After extensive washes, the membranes were incubated with peroxidase-conjugated antirabbit IgG (Amersham International) and developed using enhanced chemiluminescence (Amersham International).

**Southern Blotting.** Southern blotting was performed, as described previously (9, 11), with minor modifications. The human Ikaros cDNA used as a hybridization probe was amplified by RT-PCR, confirmed by sequencing analysis, and labeled by High Prime DNA labeling kit (Boehringer Mannheim, Indianapolis, IN) with [α-32P]-dCTP.

**Results.** The expression levels of Ikaros isoforms were analyzed by performing RT-PCR on RNA obtained from a panel of 20 human leukemia/lymphoma cell lines, which included 6 T cell lines and 14 B cell lines, as shown in Fig. 1A. In almost all human leukemia/lymphoma cell lines tested so far the expression of Ikaros isoforms was normal, with the largest isoforms (Ik-1, Ik-2, and Ik-3) being predominant, as described previously (12). However, one exception was the BV-173 cell line (13), which expressed exclusively a small isoform shown by sequencing analysis to be Ik-6 (Fig. 1A and data not shown). This isoform, which is normally expressed at very low levels, lacks the four NH₂-terminal DNA-binding zinc fingers, however, it retains the dimerization domain. Therefore, when overexpressed, Ik-6 can have a dominant-negative effect on the DNA-binding activities of other Ikaros isoforms (5). Because the BV-173 cell line (13) was established from a patient in lymphoid crisis of CML, we used RT-PCR to analyze the expression levels of Ikaros isoforms in bone marrow samples from patients in CML blast crisis. Samples from

![Fig. 1. RT-PCR analysis of the Ikaros isoforms in human leukemia/lymphoma cell lines and bone marrow samples. The PCR products of the Ikaros gene from a panel of human leukemia/lymphoma cell lines (A) and human bone marrow samples (B) were analyzed on a 1% agarose gel with a molecular marker of a 100-bp ladder (Abbott Laboratories). Lanes 1–17 show the PCR products of the patients with CML blast crisis (Table 1, patients 1–17, respectively). PBMC, peripheral blood mononuclear cells from normal volunteer; CML/CML, CML in chronic phase; AMM, leukemic transformation of agnogenic myeloid metaplasia. The PCR products of glyceraldehyde-3-phosphate dehydrogenase are also shown as a control.](https://example.com/fig1.png)
normal volunteers and patients having other hematological malignancies were also analyzed for comparison. In 5 of 17 patients with blast crisis of CML, Ik-6 was the predominant isoform expressed (Fig. 1B and Table 1; patients 4, 5, 7, 10, and 11, respectively), as was confirmed by sequencing analysis (data not shown). However, Ik-6 was not detected in normal volunteers and patients with other hematological malignancies, including 17 samples from patients in chronic phase of CML (Fig. 1B). By the Fisher’s exact test with one-tailed value, the difference of Ik-6 expression between blast crisis (5 of 17) and chronic phase (0 of 17) of CML was statistically significant (P = 0.02). Although the dominant-negative isoform Ik-6 was found to be overexpressed in a significant percentage of blast crisis bone marrow samples (5 of 17), within this same panel of patient samples Ikaros expression was severely reduced at the RNA level in four cases (Fig. 1B; patients 1, 2, 3, and 16). Down-regulation of Ikaros expression and overexpression of dominant-negative proteins both achieve the same result—a reduction in Ikaros activity. Therefore, these results indicate that in 9 of 17 cases of CML patients in blast crisis, Ikaros activity was reduced either by down-regulation of Ikaros mRNA levels or by aberrant overexpression of dominant-negative proteins. In significant contrast, expression of Ikaros isoforms was found to be normal in 17 samples of patients in the chronic phase of CML (two representative samples are shown in Fig. 1B), suggesting that a reduction in Ikaros activity correlates with the onset of blast crisis in CML. Moreover, the mechanism of aberrant overexpression of Ik-6 seems to be restricted to certain forms of leukemia, such as blast crisis of CML, because this phenomenon was not observed in the many other samples analyzed, which included bone marrow samples from 19 AMLs, 8 ALLs, 6 myelodysplastic syndromes, 3 multiple myelomas, 2 polycythemias vera, and 4 non-Hodgkin’s lymphoma with bone marrow involvement.

To confirm that dominant-negative Ik-6 protein was stably overexpressed in the BV-173 cell line and blast crisis bone marrow cells, immunoblotting of nuclear extracts was performed using an antibody specific for Ikaros. Nuclear extracts from human leukemia/lymphoma cell lines showed the presence of Ik-1 as the major isoform and Ik-2 and Ik-3 as the minor isoforms (Fig. 2). However, in nuclear extracts from the cell line BV-173 and in patients who demonstrated high expression of Ik-6 by RT-PCR (Fig. 1B, patients 4 and 5), significant amounts of Ik-6 were detected (Fig. 2). These data, therefore, confirm the results of the RT-PCR analysis (Fig. 1).

To determine whether a genetic lesion could be correlated with the aberrant expression of Ikaros isoforms in those patients, Southern blot analysis was performed using the human Ikaros cDNA as a probe. Although BamH1 digests revealed no differences in the restriction fragments obtained with DNA from normal volunteers and bone marrow from patient 4 (Fig. 3), genomic DNA from BV-173 and patient 3 generated novel bands (indicated by arrowhead and arrow, respectively). PstI digests of genomic DNA also revealed differences in the restriction fragments obtained. A novel band (indicated by arrow) was observed with DNA from both BV-173 and patient 4, and with DNA from patient 3 one germline band (indicated by arrowhead) was not detected.

Discussion

In the present analysis, RT-PCR was used to determine the expression levels of Ikaros isoforms in a panel of human leukemia/lymphoma cell lines and in bone marrow samples from CML patients in either chronic phase or blast crisis. Among the panel of cell lines examined, only one cell line, BV-173, which was derived from a CML patient in lymphoid blast crisis, showed aberrant expression of Ikaros isoforms. In this cell line, Ik-6 was found to be the only isoform expressed. Whereas Ikaros expression was normal in 17 chronic phase CML patients, in 9 of 17 cases of CML patients in blast crisis Ik-6 activity had been reduced, either by a severe reduction of Ikaros mRNA (4 of 17) or by aberrant overexpression of the dominant-negative isoform Ik-6 (5 of 17). Due to limiting amounts of bone marrow samples, Western and Southern blot analyses could only be performed on the BV-173 cell line and the samples from patients 3, 4, and 5 (Table 1). However, these experiments demonstrated that the dominant-negative Ik-6 protein was stably expressed at high levels and that reduction of Ikaros expression and overexpression of Ik-6 correlated with the appearance of novel restriction fragments, suggesting that altered expression was due to a mutation in the Ikaros locus. No gross cytogenetic abnormalities could be detected in the 7p11–13 locus in those patients, thus suggesting that the mutation is due to an insertion or deletion rather than to a translocation event. Mapping these mutations and determining how they might affect Ikaros expression must await the generation of a restriction map of the human Ikaros locus.

In the bone marrow samples of patients 4 and 11, larger Ikaros isoforms Ik-1, Ik-2, and Ik-3 could be detected together with high levels of Ik-6. Because these samples contain a mixed population of cells, it is not clear whether the larger isoforms result from normal cells present in the samples or from mutant cells that coexpress Ik-6. We favor the interpretation that the mutant blast cells have acquired a mutation in one Ikaros allele that affects isoform expression. It has been shown in mice that overexpressing a dominant-negative Ikaros isoform as a high-copy-number transgene in cells that express normal levels of larger Ikaros isoforms causes lymphomas.3 Thus, the effect of “loss of heterozygosity” can be achieved by titrating out Ikaros activity through the action of dominant-negative proteins. It is, therefore, consistent that mutation of one Ikaros allele is sufficient to cause blast cell expansion.

Some experiments have suggested that a subpopulation of normal cells expresses high levels of Ik-6 (14). Therefore, the high levels of Ik-6 detected in bone marrow samples of blast crisis patients could be due to the outgrowth of a population of cells that normally expresses high levels of Ik-6. However, our Southern blot analyses, which demonstrate a correlation between Ik-6 overexpression and mutations in the Ikaros locus, argue against this and instead suggest that the expanding blast cell population harbors a mutation in Ikaros that deregulates its expression. Moreover, the relatively high frequency of a severe reduction of Ikaros mRNA seen in blast crisis samples would suggest that progression of CML from chronic phase to blast crisis

3 S. Winandy and K. Georgopoulos, unpublished results.
correlates with mutations that reduce Ikaros activity, rather than mutations that promote the outgrowth of a population that normally expresses high levels of Ik-6. These results, therefore, indicate that, as in the mouse model, Ikaros functions as a tumor suppressor in human hematopoietic cells.

Ikaros has recently been shown to be a component of a higher-order complex that is associated with heterochromatin structures in the nucleus and includes proteins involved in chromatin remodeling, such as Mi2 and histone deacetylases (15, 16). The expression of high levels of dominant-negative Ik-6 could reduce or abolish Ikaros activity by interacting with larger Ikaros isoforms and interfering with their function in these higher-order complexes. Alternatively, reducing the levels of Ikaros proteins by affecting mRNA levels would also be expected to alter the activity of this complex. Ik-6 can also interact with other Ikaros family proteins, such as Aiolos, which is expressed in B and T cells. Thus, in these cell types, Ik-6 would also be expected to have a dominant-negative effect on the activity of Aiolos. Moreover, disrupting Aiolos activity in mice gives rise to lymphoproliferative disorders that are more pronounced in the B lineage (17). Therefore, the development of B lymphoid blast crisis in patients, which overexpress Ik-6, could be due to dominant-negative effects of Ik-6 toward Aiolos activity in lymphoid-committed cells.

In recent studies, both Ikaros and Aiolos have been shown to change nuclear localization patterns on T- and B-cell activation. On entry of the B or T cell into the cell cycle, Ikaros and Aiolos become localized in higher-order chromatin structures where they colocalize with DNA replication foci and components of the DNA replication machinery (15, 17, 18), implying a role in regulating DNA replication. These structures also colocalize with centromeric heterochromatin and nonexpressed genes, thus implying a role in gene silencing (18, 19). Therefore, the function of Ikaros seems to be manifold, affecting gene expression, chromosome propagation, and signaling response thresholds. Destroying one or a combination of these functions may provide a key step in the development of blast crisis in CML patients. A recent study demonstrated that overexpression of dominant-negative Ikaros proteins correlates with infant ALL (20). This suggests that Ikaros may play an important role as a tumor suppressor in other types of human leukemia. Identifying these malignancies and connecting them to the growing body of work elucidating the mechanism of action of Ikaros should provide useful advances in developing new strategies to treat leukemia.

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References

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