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REVIEW ARTICLE

Pathogenesis and regulation of cellular proliferation in acute lymphoblastic leukemia – The role of Ikaros

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Summary

Acute lymphoblastic leukemia (ALL) is the most common type of leukemia of childhood. Over the last 50 years there have been tremendous scientific advances in understanding the pathogenesis and the mechanisms that control cellular proliferation in ALL. These discoveries led to the development of efficient therapeutic regimens that greatly improved survival of children with ALL. Recently, several genes have been demonstrated to play a key role in tumor suppression and that their deregulation leads to malignant transformation and can affect overall survival. This review summarizes the role of Ikaros (IKZF1) in tumor suppression and regulation of gene expression in leukemia. Deletions and/or mutations of Ikaros have been detected in a large percentage of pediatric and adult ALL and reduced Ikaros function has been associated with poor outcome in ALL. Ikaros function in chromatin remodeling and epigenetic regulation of gene transcription emphasizes the important role of this protein in controlling cellular proliferation. In this review, we particularly focus on the role of signaling pathways in the regulation of Ikaros activity and its transcriptional control in leukemia.

Key words: *casein kinase II, chromatin remodeling, CK2, Ikaros, leukemia*

Introduction

The discovery of the genes that promote or suppress malignant transformation has helped us in gaining insight into the pathogenesis of malignant diseases, in designing targeted therapies, and in identifying novel prognostic markers that aid in determining specific courses of treatment as part of personalized medicine. Despite scientific and ethical challenges [1], we are witnessing tremendous progress in molecular diagnostic approaches [2], the identification of therapeutic pathways [3,4] and in enhanced general understanding of the processes that control malignant transformation and cellular proliferation.

The discovery of the *Ikaros [IKZF1]* gene [5,6] greatly advanced our knowledge of hematopoiesis, immune response and leukemogenesis. Ikaros function is essential for all of these processe [7-10]. The lack of Ikaros in mouse knock-out models results in severely impaired hematopoiesis and immune response [5,11-13], while haplo insufficiency in mice results in the development of T cell leukemia [14]. In humans, the clinical relevance of Ikaros was confirmed when 15% of all B cell pediatric [ALL] was shown to harbor deletions or inactivating mutations in a single Ikaros allele [15]. Ikaros deletions are also strongly associated with BCR-ABL1-positive ALL where 80% of patients with this disease show deletion of an Ikaros allele [16]. Deletion of an Ikaros allele was also shown to be present in 5% of patients with T cell ALL [10,17-19]. In addition to T and B cell ALL, the deregulation of Ikaros activity has also been associated with the development of myelodysplastic syndrome [20], acute myeloid leukemia (AML)

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[21] and adult and juvenile chronic myeloid leukemia (CML) [22], although the role of Ikaros in these diseases has not been studied in detail. The role of Ikaros in the pathogenesis of high-risk leukemia and its value as a prognostic marker was demonstrated by the evidence that patients with Ikaros haplo insufficiency and BCR-ABL1-negative B cell ALL, have a 3-fold increase in chance of relapse after treatment [23-25].

Among the genes that regulate tumor suppression, *Ikaros* deserves a special attention, not only because of its essential role in normal hematopoiesis and leukemogenesis, but also for its complex function in transcriptional regulation and chromatin remodeling. This review will summarize the role of Ikaros in regulating gene expression and will provide insights into upstream pathways that control Ikaros function in tumor suppression.

Ikaros function as a regulator of gene expression

The Ikaros protein has a complex structure with several distinct functional domains (Figure 1). In the N-terminal part of the protein, there are four consecutive zinc-fingers [26]. The first three zinc fingers have the typical C2H2 zinc finger structure that is a feature of the C2H2 domain of the kruppel-like zinc finger proteins. The kruppel-like zinc finger proteins are characterized by the presence of structures that contain two cysteins (C) and two histidines (H) that are covalently bound to zinc. This structure directly binds DNA, and thus, confers DNA-binding activity to proteins that contains C2H2 structures [27]. Crystallography studies have determined that each C2H2 zinc fingers consists of two antiparallel β sheets folded in on an α helix. The amino acids that make direct contact with the major groove of DNA are positioned at the N-side of the helix (positions -1, 2, 3 and 6, at the start of the helix). The binding of these particular amino acids to DNA is sequence-specific, and this has been used to design artificial DNA-binding proteins that can target specific DNA sequences. These kruppel-like C2H2 zinc finger proteins are encoded by the largest group of genes in the human genome. Many of the members of the group are known transcriptional regulators (e.g. SP1), or have a proven role in oncogenesis, (e.g WT1 – Wilms tumor gene) [27].

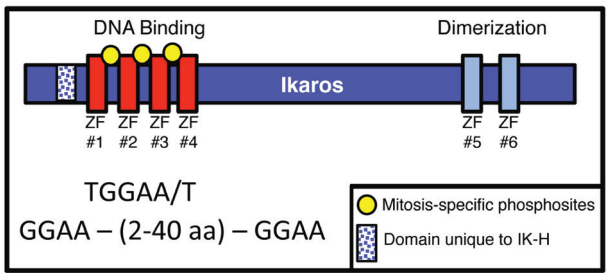

Figure 1. Ikaros is a DNA-binding zinc finger (ZF) protein that regulates gene expression. Four zinc fingers in the N-terminal region of the Ikaros protein control DNA binding. Phosphorylation of the linker regions between these ZFs regulates DNA binding during mitosis. Two ZFs in the C-terminal region of the protein are responsible for dimer formation. Motifs for high affinity Ikaros binding sites are shown. The presence of a 21 amino acid region unique to Ikaros isoform IK-H is hypothesized to positively regulate target genes within pericentromeric heterochromatin.

The fourth zinc finger structure that is adjacent to the above-mentioned C2H2 domains, has a CCHC zinc finger domain. This domain has a zinc finger structure containing three cysteins and 1 histidine that are covalently bound by zinc. These four zinc fingers are responsible for a sequence-specific DNA binding of Ikaros protein. The consensus DNA-binding sequence of Ikaros has been studied extensively both *in vitro* and *in vivo* [28]. These analyses revealed that Ikaros can bind strongly to a single "high affinity" or "optimal" consensus DNA binding sequence TGGGAA/T, although the "core" DNA binding sequence can consist of the "GGGA" or "GGAA" sequence. The presence of two "GGGA" core DNA-binding sequences that are separated by 2-40 random base pairs also constitutes a "high affinity" Ikaros binding site, at least for the human form of Ikaros [29].

The functional role of Ikaros N-terminal zinc finger domains has been studied in detail by mutational analysis, coupled with alanine-scanning mutations of individual amino acids of zinc fingers #2 and #3 [28]. The analysis revealed individual amino acids that were essential for Ikaros binding to its high affinity site (by electromobility shift assay – EMSA), as well as for Ikaros localization to pericentromeric heterochromatin (see below), using confocal microscopy. As expected, the amino acids located at positions, -1, +2, +3 and +6 were essential for Ikaros binding and pericentromeric localization, although additional amino acids within the C2H2 structure were revealed that were necessary for Ikaros binding [28]. These studies showed that zinc fingers #2 and #3 are the most important for Ikaros DNA binding and pericentromeric localization [28].

Recent biological studies have established

the roles of zinc finger #1 and #4 [30]. Creation of mice that lack zinc finger #1 or zinc finger #4, provided an excellent model to determine the role of these structures in hematopoiesis and tumor suppression. The results showed that the mice that were missing zinc finger #1 or zinc finger #4 had specific hematological defects. Each zinc finger was responsible for the regulation of the different stage of lymphoid differentiation. The most striking result was that zinc finger #4, but not zinc finger #1 was essential for tumor suppression to prevent leukemia [30]. These data underscored the importance of intact Ikaros structure for normal hematopoiesis and tumor suppression, as well as the complexity of Ikaros function.

In the C-terminal region of the Ikaros protein, there are two additional C2H2 zinc finger structures (zinc fingers #5 and #6). These domains do not take part in DNA interaction, but rather are essential for the protein-protein interaction of Ikaros [31]. These zinc finger motifs do not have the typical structure characteristic of kruppel-like zinc finger proteins, but rather unique features that have been seen only in a few other genes – Hunchback (in Drosophilla) and TRPS-1 (in humans) [32]. Among members of the Ikaros family are genes that include Aiolos, Helios, Eos, and Pegasus, all of which share the same zinc finger motifs at C-terminal end with a highly homologous amino acid sequence. Each family member can form homodimers as well as heterodimers with its own isoforms or heterodimers with other family members using zinc fingers #5 and #6 [31]. An alanine scanning mutation analysis identified the amino acids that are essential for the dimerization function of this domain (32). It is important to note that Ikaros binds DNA poorly as a monomer, thus dimerization is an essential component of proper Ikaros function.

Additional analysis of Ikaros function in the regulation of gene expression has identified two functional regions. The bipartite activation domain is located in close proximity to the C-terminal zinc finger motifs. It is loosely defined and no alanine scanning mutation studies have been performed. It was determined that this domain has an important role in the positive regulation of basal level expression of Ikaros target genes [31,33].

A novel functional domain comprised of about 21 amino acids has been identified proximal to the N-terminal zinc finger motifs. The most interesting feature is that the full-length Ikaros isoform that contains this domain (human isoform IK-H) is strongly expressed in human lymphocytes and lymphoid leukemia cells, but not in their murine counterparts, raising the possibility that it might have a species-specific function. Deletion mutational analysis, coupled with alanine scanning mutational analysis determined that this domain regulates Ikaros DNA binding affinity toward the different sequences in pericentromeric heterochromatin [29]. Since human pericentromeric heterochromatin has a different structure and higher sequence variability when compared to murine heterochromatin, this provided an explanation for the differential expression of hIK-H in humans as compared to mice. An additional analysis suggested that complexes of Ikaros proteins that contain hIK-H are associated with positive regulation of Ikaros target genes [29,34]. The mechanism of this action has not been determined, although it was suggested that it might be related to the regulation of Ikaros binding to pericentromeric heterochromatin and regulation of the chromatin remodeling and accessibility of transcriptional activators [34,35]. Further analysis is necessary to determine the exact function of hIK-H in the regulation of gene expression.

The primary function of Ikaros is to regulate gene expression and chromatin remodeling. Ikaros binds to the upstream regulatory regions of its target genes and directly regulates their transcription [5]. This Ikaros function was established very early. However, it soon became apparent that the role of Ikaros in the regulation of gene transcription might extend beyond that of a "typical" transcription factor. The discovery that Ikaros localizes at pericentromeric heterochromatin in both murine and human hematopoietic cells revealed that Ikaros-mediated regulation of gene expression occurs *via* chromatin remodeling [36]. Ikaros binds to pericentromeric heterochromatin in a sequence-specific way. It has been observed that Ikaros binding to the upstream regulatory regions of its target genes is associated with their recruitment to pericentromeric heterochromatin and transcriptional repression [36]. Based on these data, a hypothesis has been developed, where by Ikaros represses its target genes by recruiting them into the transcriptionally suppressive environment at pericentromeric heterochromatin, which results in transcriptional repression *via* chromatin remodeling. Subsequently, it was shown that Ikaros can activate its target genes *via* chromatin remodeling, and that activation requires pericentromeric localization of Ikaros [37]. This controversy has been resolved by the discovery that in humans pericentromeric

heterochromatin can contain both transcriptionally repressive and transcriptionally permissive environments [34]. Thus, recruitment of genes into different parts of pericentromeric heterochromatin can result in their repression or activation. It has been suggested that the human hIK-H isoform has an important role in preference toward localization to the permissive region of heterochromatin and that the recruitment of genes by Ikaros complexes that contain hIK-H leads to their activation at permissive regions of pericentromeric heterochromatin [34]. These data underscore the importance of chromatin remodeling in Ikaros-mediated regulation of gene expression.

In the nucleus, Ikaros forms a high-molecular weight protein complex with the histone deacetylase complex NuRD *via* a direct interaction with histone acetylases HDAC1 and HDAC2, as well as with Mi-2β, Sin3A and Sin3B proteins [38]. This observation revealed another mechanism by which Ikaros can regulate transcription of its target genes – by recruitment of the histone deacetylase NuRD complex to the upstream regulatory region of target genes, resulting in alteration in their transcription *via* chromatin remodeling [38]. Global chromatin immunoprecipitation analysis coupled with next generation sequencing of Ikaros and Mi-2β in murine hematopoietic cells revealed a large set of genes that are regulated by Ikaros and the NuRD complex [39].

Ikaros can also form a complex with the SWI/SNF complex *via* direct interaction with the Brg1 protein, which is a component of the SWI/SNF complex [40,41]. The SWI/SNF complex can positively regulate gene transcription. It has been hypothesized that Ikaros can act as a transcriptional activator by recruiting the SWI/SNF complex to the upstream regulatory region of its target genes. However, the Ikaros-SWI/SNF interaction has been studied to a lesser extent *in vivo* than the the Ikaros-NuRD complex, thus additional studies are necessary to determine how frequently Ikaros regulates gene transcription in this manner *in vivo*.

It has been demonstrated that Ikaros can regulate transcription of its target genes by direct recruitment of two proteins – CtBP [42] and CtIPn[43]. Both of these proteins function as transcriptional repressors, thus Ikaros-mediated recruitment of CtBP or CtIP results in downregulation of its target genes.

In summary, the current data strongly suggest that Ikaros can regulate transcription of its target genes in multiple ways – *via* direct activation or repression; by recruitment of transcriptional repressors (e.g. CtBP or CtIP); by recruitment of histone deacetylase chromatin remodeling complex or the SWI/SNF complex; and by recruitment of its target genes to the repressive or permissive regions of pericentromeric heterochromatin. Thus, Ikaros can act both as a transcriptional activator or repressor, and it can regulate expression of a large number of genes *via* various molecular mechanisms.

Regulation of Ikaros activity in leukemia

The Ikaros protein is strongly expressed in most hematopoietic cells. Due to the high expression level, it has been hypothesized that Ikaros function might be regulated by protein modifications. Phosphorylation of Ikaros has been studied most extensively, and a direct functional link between signaling pathways and Ikaros phosphorylation has been discovered. We will summarize below the signal transduction pathways that regulate Ikaros function *via* direct phosphorylation by Casein kinase II (CK2) and their clinical significance in oncology.

Ikaros is phosphorylated at multiple serine and threonine residues, as well as at several tyrosine amino acids. It has been shown that hyperphosphorylation of Ikaros occurs during mitosis [44]. Mutational analysis using phosphomimetic and phosphoresistant Ikaros mutants identified mitosis-specific phosphorylation sites at the serine/threonine residues located within three linkers that connect the four N-terminal, DNA-binding zinc finger motifs. Phosphorylation of the linker regions results in the loss of Ikaros' DNA binding ability, as well as its pericentromeric localization [44]. Once the linker region that connects C2H2 zinc fingers is highly conserved among all members of kruppel-like zinc finger genes [45], further analysis was performed to determine whether mitosis-specific phosphorylation is unique to Ikaros or a global event shared among all C2H2 zinc finger proteins. The results showed that Sp1 and many other zinc finger proteins are phosphorylated during mitosis at the same linker domain [44]. Thus, mitosis-specific phosphorylation of the linker is a global, cell cycle-regulatory mechanism that controls the DNA binding of most, if not all, C2H2 zinc finger proteins during mitosis [44].

Additional studies determined that Ikaros is directly phosphorylated by CK2 at multiple residues [46]. Analysis using phosphomimetic and phosphoresistant Ikaros mutants of phosphosites at the C-terminal end of the protein suggested

that CK2-mediated phosphorylation of these amino acids impairs Ikaros' ability to regulate cell cycle progression at the G1/S check point [46]. These data suggested that CK2 kinase might be an important regulator of Ikaros function in hematopoietic cells.

A major role for CK2-mediated phosphorylation in controling Ikaros activity was established by functional analysis of CK2 phosphosites at the N-terminal end of Ikaros. Results demonstrated that direct phosphorylation of Ikaros amino acids in the N-terminal part of the protein (amino acids #13 and #294) severely reduces the DNA-binding affinity of Ikaros and its pericentromeric localization [47].

The discovery that Ikaros is dephosphorylated *in vivo* by a tumor suppressor – protein phosphatase 1 (PP1), underscored the importance of a precise balance of phosphorylated and dephosphorylated forms of Ikaros for its proper function. The inability of Ikaros to undergo dephosphorylation by PP1 results in its hyperphosphorylation by CK2, loss of DNA binding ability, and pericentromeric localization, a well as its increased degradation *via* the ubiquitin pathway [48]. These data established that two opposite signal transduction pathways – the oncogenic CK2 pathway and the tumor suppressor – PP1 pathway intersect with Ikaros and likely exert their oncogenic or tumor suppressor effects *via* the regulation of Ikaros function [49,50].

Subsequent analysis of Ikaros function in human leukemia cells revealed an additional role for CK2-mediated phosphorylation of Ikaros during the S phase of cell cycle [51].

CK2 is an oncogenic kinase that is overexpressed in AML [52]. Overexpression of CK2 in the T lineage results in the development of T cell leukemia in an animal model [53]. The discovery of the critical role that CK2 has in regulating Ikaros function in leukemia provided insights into the pathogenesis of leukemia and defined a mechanism by which CK2 can contribute to leukemogenesis – *via* inhibition of Ikaros activity. The current model for the role of CK2-mediated phosphorylation of Ikaros in malignant transformation in leukemia is shown in Figure 2. These studies also identified CK2 as a potential therapeutic target and provide a mechanistic rationale for using CK2 inhibitors as a targeted treatment for leukemia.

In summary, the discovery of Ikaros led to an enormous advance in our understanding of the mechanisms that control normal hematopoiesis and malignant transformation. The role of Ikaros as a potential biomarker of high-risk leukemia is currently studied in large sets of patients, and will likely be used in the future as a part of personalized treatment for leukemia. Ikaros function in chromatin remodeling helped us gain insights into the regulation of gene expression in leukemia. Finally, the identification of the mechanism by which CK2 regulates Ikaros function and promotes leukemogenesis is under investigation in studies to develop novel therapeutic agents for leukemia. This story of Ikaros and its role in tumor suppression in leukemia is still developing and we can expect many exciting discoveries in the future.

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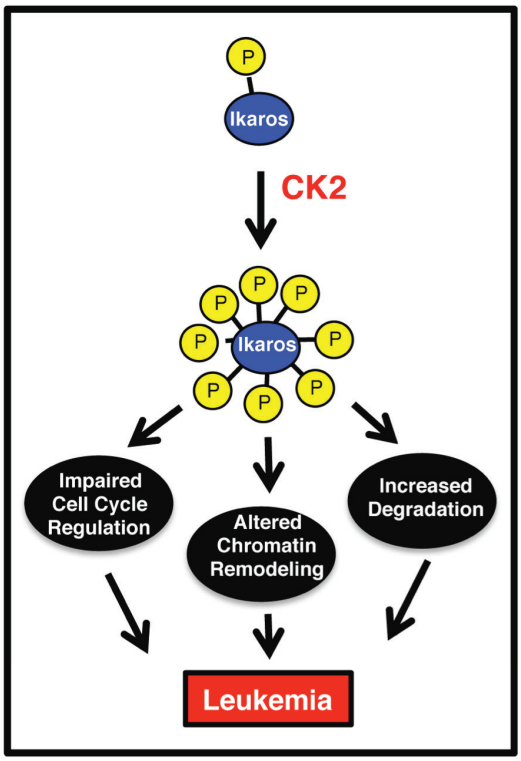

Figure 2. Phosphorylation of Ikaros by CK2 regulates Ikaros tumor suppressor activity. Phosphorylation of Ikaros by CK2 has been shown to 1) inhibit its ability to regulate genes involved in cell cycle control; 2) participate in chromatin remodeling; and 3) increase ubiquitin-mediated degradation of the Ikaros protein. This provides a mechanistic rationale for using CK2 inhibitors as a targeted treatment for leukemia.

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