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# Analysis of Ikaros Family Splicing Variants in Human Hematopoietic Lineages

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**A B S T R A C T**

*Transcription factors from the Ikaros family are involved in lymphocyte differentiation and have a critical role at specific check points of the haemopoietic pathway. However, how developmentally regulated changes are reflected in gene expression programs of lymphocyte differentiation is not well understood. It has been suggested that disregulation of transcription factors from the Ikaros family is associated with the development of different human leukemias. In this work we analyzed the state of Ikaros family members in different leukemic cells with the aim to explore the transcriptional control of human hematopoietic lineages and shed some new light on our understanding of transcription factor significance in human leukemias. By means of RT-PCR and specific primers we investigated the expression of Ikaros, Aiolos and Helios transcription factors and their splicing variants in seven leukemia cell lines derived from different types of leukemia (ALL, CML, AML) and lymphoma (histiocytic lymphoma, Burkitt lymphoma and anaplastic large cell lymphoma). In all of the cell lines examined Ikaros was present in dominant Ik1 to Ik4 isoforms and small Ik6 isoform was absent. Aiolos was expressed in the majority of the cell lines, of both, B and T origin, in the form of the full length Aio1. Helios was also present only in two long isoforms Hel1 and Hel2, and was absent in one third of the lines. Similar distribution of positive and negative expression of Aiolos and Helios found in various types of leukemias could implicate common pathways of their regulation.*

***Key words**: Ikaros, Aiolos, Helios, transcription factors, gene expression, leukemia*

## Introduction

Hematopoietic cells develop from their stem cells and gain their function just as mature peripheral blood cells. Their differentiation is regulated by a number of genes that are either silenced or activated in a sequenced order. This process is extremely complex and constantly subjected to various external influences. Developmental pathway of hematopoietic cells include hierarchical activation/silencing of specific transcription factors, among which Ikaros family are now recognized as important regulators of haemotopoietic and particularly lymphoid differentiation[1,2]. In this process three members of the Ikaros family are involved: Ikaros, Aiolos and Helios. They all have a very similar structure: an amino terminal domain mediating sequence-specific DNA binding and a carboxy terminal domain involved in the formation of homo and heterodimers[3], structure additionally being complicated by isoform formation due to mRNA alternative splicing. Long isoforms usually have at least three of four Zn finger binding domains and can functionally bind DNA. On the other hand some of the splicing occurs in the DNA binding domain resulting in short isoforms (Ik4–Ik8, Hel5–Hel8, Aio4) which lack two or more Zn finger domains. They cannot bind DNA and could act in a dominant negative manner, forming dimers with long isoforms (Figure 1).

As to regulation of lymphoid development, Ikaros transcription factors appears to be involved in the differentiation of both T and B lymphocytes, NK cells, monocytes/macrophages, dendritic cells and neutrophyles. Its abrogation in knock-out mice and in some rare types of leukemia leads to aberrant development of T cell lines. Analysis of Ikaros member downregulation in different

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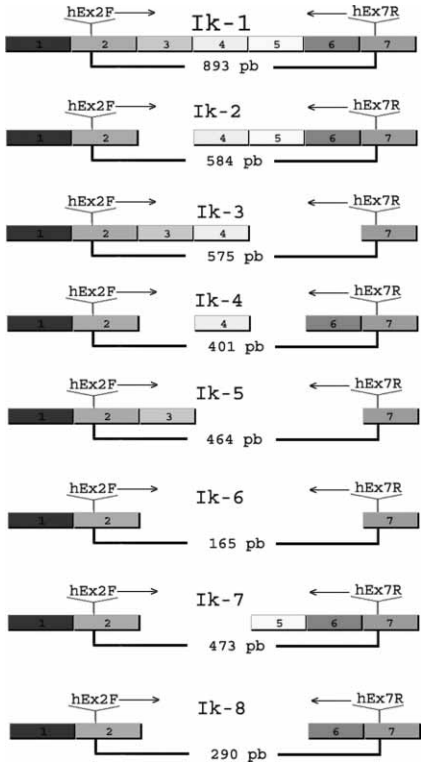


*Fig. 1. Shematic representation of various Ikaros isoforms[17,26].*

types of leukemia suggest that it may also play an important role in nonlymphoid lineages[4].

Aiolos transcription factor seems to be involved primarily in B cell differentiation[5,6]. Interplay among Aiolos and other specific transcription factors was found to determine subtle differentiation stages in B cell development[7].

Helios, the third member of Ikaros family, was found to be important in T cell development[8,9]. Thus, constant expression of the full length Helios resulted in an inhibition of T cell development, and the non-DNA binding forms were able to induce lymphoma[10]. The role of Helios in B cell development was also demonstrated[11].

Although still widely unknown, the intricate interplay among these three Ikaros transcription factors, as well as with other transcriptional regulators is started to be revealed. As various types of leukemia are suggested to mirror differentiation stages of hematopoietic cells freezed in their development, in this work we analyzed the state of Ikaros family members in different types of human leukemic cell lines. We compared the expression pattern of their isoforms/splicing variants and discussed their expression in relation to the cell phenotype.

## Methods

### *Cell lines*

Human leukemia/lymphoma cell lines Jurkat (T-lineage ALL), MOLT-4 (T-lineage ALL), NALM-1 (CML in blast crisis), HL-60 (AML), U-937 (histiocytic lymphoma), RAJI (Burkitt's lymphoma) and SU-DHL-1 (anaplastic large cell lymphoma) were maintained in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Co.).

### *RNA extraction, preparation of cDNA*

Total RNA was extracted using Trizol (Invitrogen Co.) and the quality of RNA was monitored through agarose gel electrophoresis. 2 μl of isolated RNA was used for cDNA synthesis using the standard protocol for Superscript II Rnase H Reverse Transcriptase (Invitrogen Co.).

### *Polymerase chain reaction*

A volume of 25 μl for PCR was performed using 2.5 μl of cDNA, 2.5 μl of 10×PCR buffer, 1.8 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM dNTP, 1 μl of each 5 μM primer, 0.2 μl of 5U Ampli Taq gold polymerase (Applied Biosystems) and 15.5 μl of $H_2O$.

The sequences of the primers used are as follows: Ikaros sense primer Hex2F, 5' cccctgtaagcgatactccagatg 3', antisense primer Hex7R, 5' gatggcttggtccatcacgtggga 3'; Aiolos sense primer 5' cactcaggagcagtctgtgc 3', antisense primer 5' agaaggcacgctcttctctg 3'; Helios sense primer Hev S, 5' atggaaacagaggctattgatggc 3', antisense primer Hev 2, 5' agcttttcccccacaaactt 3'; HPRT sense primer (HPRT1), 5' ccaaagatggtcaaggtcgc 3', and antisense primer HPRT 2, 5' ctgctgacaaagattcactgg 3'.

PCR cycling conditions were: 94 °C for 5 min (denaturation); 94 °C for 30 s; 59 °C (Ikaros), 58 °C (Aiolos and Helios) and 52 °C (HPRT) for 30 s; 72 °C for 1 min for 40 cycles; 72 °C for 7 min.

## Results

We compared the pattern of expression of Ikaros, Aiolos and Helios between cell lines originating from different types of human hematopoietic malignancies. In the analysis 7 different leukemic cell lines were included: human T cell leukemia lines Jurkat and MOLT-4 (ALL); human B cell leukemia lines NALM-1and SUDHL-1; lines of myeloid origin HL-60 (AML) and U-937; and RAJI cells originating from a human Burkitt's lymphoma. Figure 2 shows that all cell lines analyzed expressed Ikaros mRNA as a full length Ik1. Most of leukeia cells also express spliced variants Ik2, Ik3 and weakly Ik4. Dominant negative Ik6 was not detected in any of the cell lines. Figure 2 also shows that Aiolos, in the largest full length form of Aio1 was strongly expressed in Jurkat, MOLT-4 and Nalm-1 cells i.e. in cells of B and T origin. In contrary, myeloid cells HL-60 (AML) and U-937 had a very weak expression of the longest isoform Aio1. The Burkitt's lymphoma derived RAJI cells and B cell leukemia SUDHL-1 did not express either full length Aiolos or any form of Aiolos variants. Interestingly, Helios transcription factor was expressed in Jurkat, MOLT-4 and NALM-1, but also in cell strains of

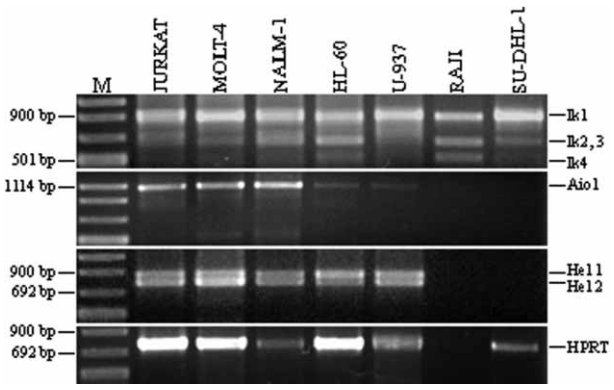


*Fig. 2. RT-PCR analysis of Ikaros, Aiolos and Helios gene expression in freshly isolated human derived cell lines. Amplification products with primers as described in Methods as full length and splicing variants. HPRT is a house keeping control gene. Lane 1 – M (marker, base pairs), Lane 2 – T lymphocytes (Jurkat), Lane3 (T lymphocytes MOLT-4), Lane 4 – mature B lymphocytes (NALM-1), Lane 5 – acute myeloid leukaemia (HL- 60) Lane 6 – immature monocytes U 937, Lane 7 – Burkitt's lymphoma derived cells (RAJI), Lane 7- B lymphocytes (SUDHL-1).*

myeloid origin, HL60 and U-937. Figure 2 also shows the expression of dominant Helios isofroms Hel 1 and Hel 2. Likewise Aiolos, Helios could not be detected in Raji and SUDHL-1 cells.

## Discussion and Conclusion

We analyzed the expression of Ikaros family members, Ikaros Aiolos and Helios in a panel of different types of human leukemic cells, in order to explore if the expression pattern of their splicing variants is related to each other and/or to specific type of leukemic cells.

Ikaros plays an important role in controlling hematopoietic differentiation and has been implicated in lymphoid development and proliferation[12,13], as well as in malignant transformation[14–17]. It has been found that high levels of alternatively spliced Ik6 isoform, lacking DNA binding domain impair the function of Ikaros proteins in a dominant negative manner[18].

In our set of leukemia cell lines, all of the samples were positive for Ikaros expression. Predominant were high molecular weight isoforms, unspliced Ik1, as well as Ik2 and Ik3. Isoform Ik4 was found, but in a low concentration, while dominant negative Ik6 was not detected. The same expression pattern of Ikaros splicing variants could be found in normal blood samples. Previously, analysis of Ikaros transcription factors was performed in several clinical studies on leukemia patients, showing aberrant expression of its isoforms, specifically Ik6, Ik4, Ik7 and Ik8, in different types of leukemia[16,17,19]. Thus, overexpression of short dominant negative Ik6 was found to correlate with the development of the childhood ALL[17] in both, B and T ALL subtypes[20,21]. It has been suggested that aberrant Ikaros splicing could be the consequence of Bcr-Abl translocation, present in infant lymphocytic leukemia[15]. Ik6 variant was also found in adult types of leukemia, CML (chronic myelogenous leukemia), ALL and AML, but in significantly lower number of cases[22,23].

As our samples of leukemic cell lines originated from adult types of leukemia, approximately normal pattern of Ikaros expression is in line with previous findings[24]. We have shown that the expression of Ikaros splicing variants in leukemic cell lines was similar to that obtained from human leukemias, with dominant Ik1 to Ik4 isoforms and absence of Ik6.

Another transcription factor from the Ikaros family, Aiolos, is considered to have an antiproliferative role in B lymphocyte function. However, its role in human lymphoid disorders is still unclear. Different Aiolos splice variants were found in both, normal and neoplastic B lineage cells[25]. Our samples of cell lines, originating from different types of leukemia, were predominantly positive for Aiolos, and expressed the full length form Aio1. Aiolos was expressed in several cell lines of both, B and T origin: in Jurkat, MOLT-4 and NALM-1. In cells of myeloid origin Aio1 was weakly expressed. No Aiolos in RAJI and SUDHL-1 cells was detected, reflecting some differences in its expression pattern depending on the type of leukemia as we have shown before in leukemic patients[25].

Helios, as the third member of Ikaros family, is the least explored. In mice, Helios downregulation was found to interfere with T cell maturation[8,9]. In normal blood Helios is expressed in two isoforms, Hel1 and Hel2 (data not shown). Only in sporadic tumor cell lines and one leukemia patient small spliced and deleted forms of Helios, Hel5, Hel6, Hel7 and Hel8 were found[22]. In the cell lines tested we could detect two splicing variants Hel1 and Hel2, but apparently these cells do not have other deleted or spliced forms of Helios. Considering all of the samples analyzed, originating from different types of leukemic cell lines, it could be concluded that Ikaros was ubiquitously expressed, in normal splice variants. Aiolos, in full-length isoform, was present in all samples except for RAJI and SUDHL-1. Helios, normally expressed in peripheral blood leukocytes, was absent in one third of hematopoietic tumor cell lines. We also observed that several cell lines without Aiolos neither expressed Helios: two B cell strains (RAJI and SUDHL-1). Similar distribution of positive and negative expression of Aiolos and Helios found in various types of leukemias could implicate common pathways of regulation. We conclude that the cell lines used are a suitable model for Ikaros transcription factor analysis and for further studies of mechanisms involved in differentiation and leukemia development.

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## ANALIZA TRANSKRIPCIJSKIH FAKTORA IZ OBITELJI IKAROS U HEMATOPOETSKIM STANICAMA LJUDI

## SAŽETAK

Transkripcijski faktori iz obitelji Ikaros imaju kritičnu ulogu u specifičnim kontrolnim točkama hematopoetskog puta. S obzirom na njihovu važnost u razvoju hematopoetskih stanica upravo se transkripcijski faktori iz obitelji Ikaros dovode u vezu s razvojem leukemija. U ovom radu analizirali smo ekspresiju članova obitelji Ikaros – Ikaros, Aiolos i Helios, u različitim staničnim lozama koje potječu iz leukemija ljudi s ciljem upoznavanja njihove uloge u razvoju leukemija. Metodom reverzne transkripcije (RT-PCR) i specifičnih početnica istražili smo ekspresiju glasničke RNA za Ikaros, Aiolos i Helios kao i oblika nastalih izrezivanjem RNA (»splicing variants«) u sedam staničnih loza nastalih iz različitih tipova leukemija ljudi (ALL, CML, AML) i limfoma (histiocitni limfom, Burkittov limfom i anaplastični limfom velikih stanica). U svim staničnim lozama našli smo da se Ikaros ispoljava u svojem najduljem obliku Ik1 i kraćim izrezanim izoformama Ik2, Ik3 i Ik4 dok izostaje najkraći Ik6 oblik. Aiolos je eksprimiran u većini staničnih loza, kako limfocita B tako i limfocita T, u svojem punom obliku Aio1. Prisutnost transkripcijskog faktora Helios je također predstavljena s dvije najdulje izoforme Hel1 i Hel2 koje, međutim, nedostaju u jednoj trećini staničnih loza. Slična raspodjela ekspresije Aiolos i Helios nađena u različitim tipovima leukemija implicira zajednički put njihove regulacije.