A search for a mutation of the Aiolos phosphorylation domain in lymphocytes from patients with leukemia

We examined whether phosphorylation of Aiolos in primary human lymphocytes is part of the malignant transformation in leukemia. By analyzing mutations at a restriction site we show here that impairment of Aiolos activity in human leukemia is not based on deficient phosphorylation as had been demonstrated in experiments *in vitro*.

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The Aiolos transcription factor belongs to the Ikaros family of zinc finger proteins that specifically bind DNA and function as key regulators of lymphocyte development from the hematopoietic stem cell.[1,2] Aiolos mutant mice develop lymphoproliferative disorders, particularly B-cell lymphoma.[3] In humans the Aiolos gene is mapped to chromosome 17q11.2; the protein product consists of 509 amino acid residues and is highly conserved sharing 86% identity with its mouse counterpart.[4] Given the importance of the Aiolos gene in the lymphopoietic system[3,5,6] and the possible role of Ikaros transcription factors in malignant development[7-9] the aim of this work was to study whether a compromised phosphorylation site (from tyrosine to threonine) of Aiolos in human primary lymphocytes plays a role in leukemogenesis, as demonstrated in mutated cell lines *in vitro*. We screened a possible defect in the Aiolos sequence which *in vitro* leads to the impairment of its function and could, therefore, be one of the multiple hits leading to the development of leukemia. It has been shown in *in vitro* experiments that only tyrosine phosphorylation of Aiolos at position 286 and not at the other three phosphorylation sites is the essential event for its translocation to the nucleus where it is involved in nucleosome remodeling. We therefore investigated whether this phosphorylation site of Aiolos was compromised also in primary human lymphocytes from patients with leukemia and whether this phosphorylation was one of the events that contribute to the development of human leukemia.

All patients met morphological and cytochemical criteria for a diagnosis of acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML) or B-cell chronic lymphocytic leukemia (B-CLL) and their leukemic cells were immunophenotyped by multiparameter flow cytometry (Table 1). We studied lymphocytes from 38 adults with B-ALL, T-ALL, B-CLL or AML at diagnosis and 11 healthy individuals. Each patient signed an informed consent form according to the Helsinki declaration. The study had been approved by the local ethics board.

Samples of leukemic cells were recovered from the interface of density gradients (r=1.077 g/mL) and the total number and percentage of viable cells were determined by hemocytometer counts using trypan blue exclusion. DNA from cells of leukemia patients was purified with TRIzol (Gibco BRL, Paisley, UK) as described by the manufacturer and amplified by means of polymerase chain reaction (PCR) primers specific for Aiolos: F (5'-GCCACTGCTTTGATGTCAGC-3') and R (5'-CTGATGGCGTTATTG ATGGC-3') under the following conditions: 35 cycles of 30 s at 96°C, 30 s at 56°C and 30 s at

Table 1. Leukemia phenotypes.

Leukemia	*Lineage*	*Cells*	*Immunophenotype*	*No. of cases*
	AML	Myeloid cells	MPO±, CD13± CD33±, CD15± CD117±	17
Acute leukemia	ALL B-cell leukemia	Immature B cells	TdT, citCD79a CD19, CD10±, CD20±, cIgM±	8
	T-cell leukemia	Immature T cells	TdT, citCD3, CD7, CD1±, CD2± CD3±, CD5± CD4±, CD8±	8
Chronic lymphocytic leukemia	CLL B-CLL	Mature B cells	CD5, CD19 CD20, CD23 κ or λ (low expression)	7

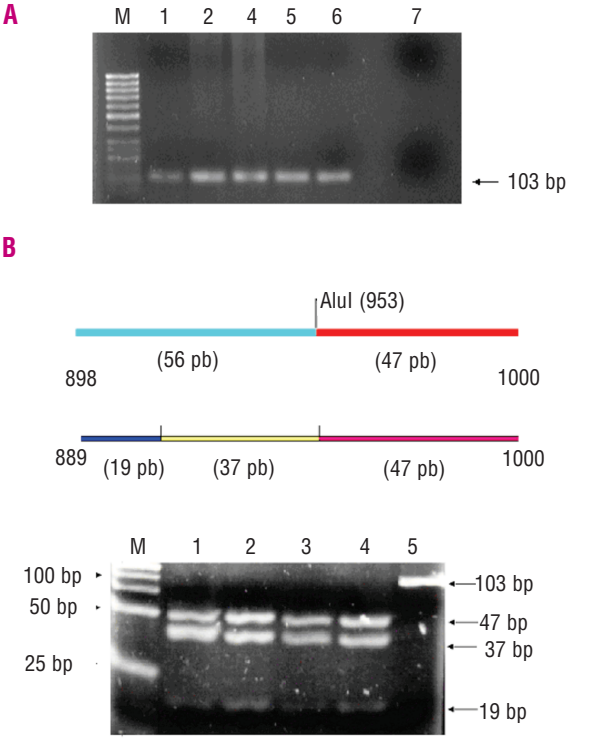

Figure 1. A. Representative results after Aiolos PCR amplification; PCR products (103 bp) of the Aiolos gene of human leukemic cells: M: molecular weight marker VIII (Roche); Lanes 1-5: leukemic cells; Lane 7: negative control (no DNA). **B.** Representative result of *Alu I* restriction; Lanes 1-4: Digested Aiolos PCR products (three fragments: 47bp, 37bp and 19bp); Lane 5: Size of PCR product from human lymphocytes before *Alu I* restriction (103 bp).

72°C, with a final extension step of 5 min at 72°C. The PCR products were first analyzed on 2% agarose gel and stained with ethidium bromide and then digested with *Alu* I enzyme for 1 h at 37°C. Digestion products were size fractionated on 12% polyacrylamide gel and stained with ethidium bromide. *In vitro* results with Aiolos mutants containing threonine instead of tyrosine in position 286, 292, 314 or 342 show that only the tyrosine 286 replacement by threonine inhibits translocation of Aiolos to the nucleus, and therefore abrogates its function, whereas the other mutants are expressed in the nucleus.[10] The aim of our work was to test the *in vivo* condition of the code for tyrosine at position 286 of the Aiolos protein. Since there were no restriction sites at the triplet position for tyrosine 286 we introduced a mismatch and therefore instead of AACT, the PCR amplified DNA molecules had the AGCT sequence, which is a restriction site for the *Alu* I enzyme. This restriction site contains the first base for tyrosine. This approach is an easy, fast and elegant way to test for phosphorylation impairment of Aiolos at the position of interest. Our analysis, based on lymphocyte samples from 38 patients with AML, ALL or B-CLL and from 11 healthy individuals, shows that tyrosine in position 286 of the Aiolos protein in humans is conserved (Figure 1). These results differ from those of the *in vitro* studies and imply that other elements are involved in the development of leukemia *in vivo*.

We conclude that impairment of Aiolos activity in human leukemia is not based on deficient phosphorylation due to a tyrosine replacement. Further research is required to find a possible connection between Ikaros family members and leukemogenesis in human.

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