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Point Mutations and Deletions in the *Znfn1a1*/*Ikaros* Gene in Chemically Induced Murine Lymphomas[1]

Anneli Karlsson, Peter Söderkvist, and Shi-Mei Zhuang[2]

Division of Cell Biology, Department of Biomedicine and Surgery, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden

ABSTRACT

The *Znfn1a1* gene encodes a zinc finger protein called Ikaros, which is critical for T-cell development and differentiation. The execution of normal function of Ikaros requires sequence-specific DNA binding, transactivation, and dimerization domains. In this study, exons 3–5 and exon 7 of the *Znfn1a1* gene that encode the functional domains of Ikaros were analyzed for point mutations and deletions in murine lymphomas induced by 1,3-butadiene, 2′,3′-dideoxycytidine, or phenolphthalein. Missense and frameshift mutations were identified in 11% (11 of 104) of the tumors. Interestingly, 8 of the mutations were identified in the NH_2-terminal zinc finger motifs, which are crucial for the DNA-binding function of Ikaros. The other 3 samples carried frameshift mutations in exon 7 that resulted in truncations and abrogation of both transactivation and dimerization domains. One tumor with a missense mutation in the DNA-binding domain also displayed a 45-bp deletion in the dimerization domain. Southern analysis disclosed interstitial homozygous deletions in the functional domains of Ikaros in 4% (3 of 68) of the lymphomas examined. Allelic losses on markers surrounding the *Znfn1a1* gene were detected in 27% (12 of 45) of the tumors analyzed. However, only 2 tumors with allelic losses also showed mutations in the *Znfn1a1* gene, indicating that other tumor suppressor genes located on this region might be involved as well. Our results suggest inactivation of Ikaros in a subset of chemically induced lymphomas and additionally support the contention of tumor-suppressor activity for Ikaros.

INTRODUCTION

The Ikaros protein is an early lymphoid-specific transcription factor and a putative mediator that plays an important role in the development and differentiation of T and B cells (1, 2). Ikaros is encoded by the *Znfn1a1* gene, which is highly conserved in mice and humans, and is located on mouse chromosome 11 and human chromosome 7p13-p11.1. The *Znfn1a1* gene consists of seven exons, of which exons 3–5 encode four NH_2-terminal zinc fingers that enable binding of Ikaros to the GGGA or GGAAA core motifs in the promoter region of target genes (3). A minimum of three zinc fingers in this region is required for high affinity DNA binding and consequently for transactivation of the target gene. Alternative splicing of exons 3–6 of the *Znfn1a1* gene yields at least eight different isoforms. Only three of these isoforms contain three or more NH_2-terminal zinc fingers and can, therefore, function as active isoforms (4, 5). Dimerization between two active isoforms enhances the activity of Ikaros and allows transcriptional activation. However, heterodimers with inactive isoforms that contain less than three NH_2-terminal zinc fingers cannot bind DNA and are transcriptionally inert (6). In contrast with the differential usage of the NH_2-terminal zinc finger motifs, all of the Ikaros isoforms share one transcription-activating domain and two COOH-terminal zinc fingers that are encoded by exon 7. The transcriptional activation domain consists of one acidic and one hydrophobic subdomain. The acidic subdomain may alone function as a weak activator, but maximal activity is attained in cooperation with the hydrophobic subdomain (6). The COOH-terminal zinc fingers mediate dimerization of the Ikaros isoforms.

Studies in mouse models have shown that a homozygous deletion in *Znfn1a1* results in deficiency in T, B, and natural killer cells, as well as their early progenitors (7). Mice with heterozygous deletions, on the other hand, rapidly develop T-cell lymphoma or leukemia. Genetic analysis of the tumors from these mice revealed loss of the wild-type Ikaros allele (8). These results suggest that Ikaros may function as a potential tumor suppressor.

Phenolphthalein, 1,3-butadiene, and 2′,3′-dideoxycytidine are known to induce high incidence of lymphoma in mice (9–11). Phenolphthalein was long used as an ingredient in laxatives, 2′,3′-dideoxycytidine has been approved for treatment of HIV-positive patients, and 1,3-butadiene is a gas extensively used in the plastic industry. To investigate the involvement of Ikaros inactivation in lymphomagenesis, 104 lymphomas derived from the mice exposed to phenolphthalein, 1,3-butadiene, and 2′,3′-dideoxycytidine were analyzed for genetic alterations in the *Znfn1a1* gene. The results disclosed point mutations, insertions, and deletions of *Znfn1a1* in a subset of tumors, indicating that inactivation of Ikaros play a role in the development of these lymphomas.

MATERIALS AND METHODS

Tumor Induction and DNA Isolation. Tumors were induced in heterozygous *p53*-deficient mice (TSG-p53™) by gavage of phenolphthalein (10) in both B6C3F1 and NIH Swiss mice by gavage of 2′,3′-dideoxycytidine (9), and in B6C3F1 mice by inhalation of 1,3-butadiene (9). In total, 104 lymphomas (31 BLF,[3] 16 DLF, 47 DLS, and 10 PL) were collected, mostly from thymus and spleen, and stored at −70°C until analysis. All of the tumors were of T-cell origin, and DNA was purified as described previously (12). Normal DNA from five different inbred mouse strains including 129/J, C3H/HeJ, AKR/J, Balb/cJ, and C57Bl/6J were purchased from Jackson Laboratory (Bar Harbor, ME).

Mutation Analysis. Exons 3–5 and exon 7 of mouse *Znfn1a1* were amplified by PCR using the following primers: EX3F-AGT AAT GTT AAA GTA GAG ACT CAG and EX3R-GTA TGA CTT CTT TTG TGA ACC ATG for exon 3 (7); FI3-GCT CTC TCT CAG TGC TTA CC and RI4-CTG GGA ACA TGG AAC ACA TG for exon 4 (13); FE5-GTT GGT AAG CCT CAC AAA TGT G and RE5-GAA GGC CCA TGC TTT CCA for exon 5; FE7B-AGG GAG ACA AGT GCC TGT CA and RE7B-CAG CAG CAA GTT ATC CAC GG for the activation domain of exon 7 (13); and IK7DIMF-CGA GCA GCT GAA GGT GTA CA and IK7DIMR1-ATC TTT GTG CTT CAG TGG GG for the dimerization domain of exon 7. Each PCR reaction was carried out in a 20-μl reaction volume with final concentrations of 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 9.0), 0.01% Tween 20, 0.2 mM of each deoxynucleotide triphosphate, 1.5–2.0 mM $MgCl_2$, 0.5 units Taq polymerase (Promega, Madison, WI), and 1 μM of each primer. The amplified DNA was labeled with [α-^{32}P]dATP (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) by a secondary PCR reaction, denatured, and applied to a nondenaturing 6% polyacrylamide gel containing 10% glycerol. Single-strand DNA with an altered electrophoretic mobility was eluted from the gel and reamplified. The

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[2] To whom requests for reprints should be addressed, at Division of Cell Biology, Department of Biomedicine and Surgery, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden. Phone: 46-13-222679; Fax: 46-13-221718; E-mail: shizh@mcb.liu.se.

[3] The abbreviations used are: BLF, 1,3-butadiene-induced lymphoma in B6C3F1 mice; DLF, 2′,3′-dideoxycytidine-induced lymphoma in B6C3F1 mice; DLS, 2′,3′-dideoxycytidiene-induced lymphoma in NIH Swiss mice; PL, phenolphthalein-induced lymphoma in TSG-p53™ mice; LOH, loss of heterozygosity; SSCA, single-strand conformation analysis.

purified PCR product was sequenced with Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech) following the manufacturer's instruction.

LOH Analysis. Forty-five tumor DNAs (14 DLF and 31 BLF) derived from B6C3F1 mice were allelotyped using eight microsatellite markers on the centromeric region of mouse chromosome 11 (Fig. 1). The markers were PCR-labeled with [α-^{32}P]dATP (Amersham Pharmacia Biotech) using primers purchased from Research Genetics Inc. (Huntsville, AL). The labeled PCR products were separated on a denaturing 6% polyacrylamide gel containing 7 M urea. LOH was scored visually as a reduction of >50% intensity of the band from one allele relative to the other in tumor *versus* the corresponding pattern in normal B6C3F1 DNA.

Detection of Large Deletions. Enough DNA for Southern blot analysis was available from 68 lymphomas (27 BLF, 11 DLF, and 30 DLS). Probes for exons 3, 4, 5, and 7 of the *Znfn1a1* gene were generated by PCR using the primers described above and then labeled with [α-^{32}P]dATP (Amersham Pharmacia Biotech) with Prime-It II Random Primer Labeling kit (Stratagene, La Jolla, CA). Each tumor DNA (10 μg) was digested with *Bam*HI and *Hind*III (Promega), then separated on a 1% agarose gel, transferred to a Gene Screen Plus membrane (Pall Gelman Laboratory, Ann Arbor, MI), and fixed by UV cross-linking. The membranes were hybridized to the ^{32}P-labeled probe, washed at 45°C, and then exposed to a BAS III Image Plate (Fuji Photo Film Co., Minami-Ashigara, Japan), which was subsequently analyzed with a Fuji BAS 1000 Laser Image Analyzer (Fuji Photo Film Co.).

RESULTS

Analysis of Point Mutations in the *Znfn1a1* Gene by SSCA and Direct DNA Sequencing. One hundred and four mouse lymphomas (31 BLF, 16 DLF, 47 DLS, and 10 PL) were analyzed for mutations in *Znfn1a1* exons 3–5 that encode the NH_2-terminal zinc fingers and in exon 7 that encodes the transactivation and the dimerization domains. Eleven of 104 tumors displayed missense or nonsense mutations, small deletions, or insertions in the exons examined (Table 1). Interestingly, 7 tumors (BLF5, BLF9, DLF5, DLS6, DLS10, DLS12, and DLS29) carried mutations in the two zinc finger motifs encoded by exon 4, with 6 missense mutations and 1 small deletion resulting in a frameshift that creates a truncation at codon 105. Furthermore, a nonsense mutation (BLF12) was identified in the zinc finger encoded by exon 5. Taken together, these results suggest prevalent mutations in the NH_2-terminal zinc finger domains. One sample (DLS10) with a missense mutation in exon 4 also showed a 45-bp in-frame deletion in the dimerization domain of exon 7. Surprisingly, all three of the insertions (DLF15, DLS22, and PL2) detected in exon 7 resulted in protein truncation and, in turn, abrogation of the transactivation domain as well as the dimerization domain. Lymphomas derived from the different treatment groups shows similar frequency of *Znfn1a1* mutations, with 10% (3 of 31) in BLF, 13% (2 of 16) in DLF, 11% (6 of 47) in DLS, and 10% (1 of 10) in PL.

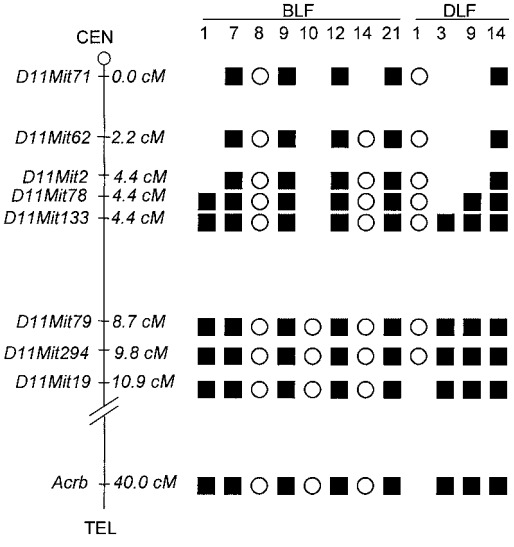

Fig. 1. Schematic figure of allelic losses on chromosome 11 in BLF and DLF. Eight Mit microsatellite markers covering the region where *Znfn1a1* is located were allelotyped in 45 chemically induced lymphomas derived from B6C3F1 mice. One additional marker (*Acrb*) presented in the figure lies within 1 cM of the *p53* gene and has been analyzed previously (12). *CEN*, centromere; *TEL*, telomere. Markers analyzed are shown on the *left* side of the chromosome, and the distance of the marker from the centromere is presented on the *right* side of the chromosome. ■; allelic loss of the C3H allele, ○; allelic loss of the B6 allele. *Absence of circle or square* indicates the retention of heterozygosity.

Silent nucleotide substitutions at the third base of codon 97 were identified in 6 tumors derived from outbred NIH Swiss mice (Table 1). However, this nucleotide variation does not alter the amino acid and has also been identified in Balb/c mice (13), which suggests that it may represent a neutral polymorphism rather than a predisposing mutation.

DNA sequencing in the dimerization domain of exon 7 revealed a 9-bp (nucleotides 1171–1179) deletion in all of the tumors examined, according to the mouse *Znfn1a1* cDNA sequence deposited in GenBank (NM_009578). To examine the possibility of genetic polymorphisms, normal DNA from 129/J, C3H/HeJ, AKR/J, Balb/cJ, C57Bl/6J, and B6C3F1 mice was amplified with primers IK7DIMF and IK7DIMR1, and then sequenced. Interestingly, the 9-bp deletion was also detected in the normal DNA from these six different mouse strains. Nucleotides 1171–1179 are exactly repeated in nucleotides 1180–1188 in the deposited sequence and do not exist in the human sequence (GenBank U40462). Although we could not find the description about mouse strains used for the sequence published (GenBank NM_009578; Ref. 1), our data suggest that these nine base pairs may not exist in mouse *Znfn1a1* cDNA.

Analysis of Homozygous Deletions in the *Znfn1a1* Gene by Southern Blot. Sixty-eight lymphomas (27 BLF, 11 DLF, and 30 DLS) were examined for homozygous deletion in exons 3–5 and exon 7 of the *Znfn1a1* gene. The tumor DNA was digested with *Bam*HI and *Hind*III, and subjected to Southern analysis with probes specific for exons 3, 4, 5 and 7 of *Znfn1a1*, respectively. Deletions were found in 3 of 68 samples examined (Fig. 2; Table 1). All 3 deletions were identified in DLF tumors. DLF1 and DLF6 showed large deletions covering exons 3–5, whereas DLF14 disclosed absence of exons 5 and 7.

Analysis of Allelic Losses on Mouse Chromosome 11. The *Znfn1a1* gene is located on the centromeric part of mouse chromosome 11, with the genetic distance of ~6 cM from the centromere.[4] Eight microsatellite markers surrounding the *Znfn1a1* gene were used for allelotyping in 31 BLF and 14 DLF. Allelic losses were identified in 8 of 31 (26%) BLF and in 4 of 14 (29%) DLF tumors (Fig. 1 and Table 1). All of the losses except 1 (BLF10) covered the region where *Znfn1a1* is located. The BLF10 showed LOH only in the markers telomeric to the *Znfn1a1* gene but not in the centromeric markers (Fig. 1), which cannot exclude the possibility that one allele of the *Znfn1a1* is lost.

DISCUSSION

The Ikaros protein, encoded by the *Znfn1a1* gene, is a critical transcription factor involved in the development and differentiation of the T-cell lineage. The normal function of Ikaros depends on three domains, including sequence-specific DNA binding, transactivation, and dimerization domains. The DNA-binding domain is located in the NH_2-terminus and consists of four zinc fingers, which are encoded by exons 3, 4, and 5 of the *Znfn1a1* gene. The transactivation and dimerization domains are located in the COOH-terminus and encoded

[4] Internet address: http://www.informatics.jax.org.

Table 1 *Summary of genetic alterations of the Znfn1a1 gene in BLF, DLF, DLS, and PL*

Samples	Mutations in *Znfn1a1* (n = 104)			Deletions in *Znfn1a1* (n = 68)	LOH in chr. 11[a] (n = 45)	Mutations in *p53*[b]
	Exon	Nucleotide	Amino acid			
BLF						
1					+	
5	4	280delG	frameshift (stop at codon 105)			
7					+	N236S
8					+	C173F
9	4	180C>G	C60W		+	frameshift
10					+	H176L
12	5	376C>T	R126X		+	frameshift
14					+	
21					+	
DLF						
1				Exon 3–5	+	
3					+	
5	4	296A>C	D99A			
6				Exon 3–5		
9					+	
14				Exon 5, 7	+	
15	7	785–786insA	frameshift (stop at codon 281)			
DLS						
6	4	223C>G	R75G			
		291G>A	R97R			
10	4	269T>C	L90P			
		291G>A	R97R			
	7	1245–1289del	404–429del			
12	4	296A>C	D99A			
		291G>A	R97R			
22	7	800–801insG	frameshift (stop at codon 281)			
27	4	291G>A	R97R			
29	4	191G>C	G64A			
30	4	291G>A	R97R			
34	4	291G>A	R97R			
PL						
2	7	735–736insC	frameshift (stop at codon 247)			

[a] chr, chromosome; +, presence of LOH.
[b] Mutations of p53 have been analyzed previously (14). Only mutations in the samples with LOH on chromosome 11 are presented.

by exon 7. It has been reported that mice homozygous for a deletion in the Ikaros DNA-binding domain fail to generate lymphocytes, whereas heterozygous mice rapidly develop T-cell lymphoma and leukemia (7).

Genetic analysis of *Znfn1a1* in chemically induced lymphomas revealed mutations in 11 of 104 (11%) tumors and homozygous deletions in 3 of 68 (4%) samples analyzed (Table 1). Interestingly, 8 of the mutations occurred in the NH_2-terminal zinc finger motifs, which altered the amino acid sequence and may, in turn, change the DNA-binding activity of the Ikaros protein. Among these, 7 mutations were found in exon 4 of the gene, which encodes 2 of the 4 NH_2-terminal zinc fingers. This indicates a mutation cluster in this exon and supports the critical role for these zinc finger domains in the normal function of the Ikaros protein. The three insertions identified in exon 7 were all frameshift mutations that destroyed the protein domains critical for its transactivation and protein-protein dimerization activity. Prevalent missense mutations in the NH_2-terminal zinc fingers, as well as frameshift and nonsense mutations in the activation domain have also been observed in radiation-induced lymphomas (13).

The homozygous deletions of the *Znfn1a1* gene found in 3 samples result in complete loss of the DNA binding domain or transactivation domain, which will obviously abrogate the function of Ikaros. Similar frequency of point mutations and deletions in the *Znfn1a1* gene has been identified in radiation-induced mouse thymic lymphomas (13), indicating the involvement of Ikaros inactivation in the development of chemically and radiation-induced lymphomas.

Allelotyping of the chromosomal region where the *Znfn1a1* gene is located revealed LOH in 26% (8 of 31) of the BLF and 29% (4 of 14)

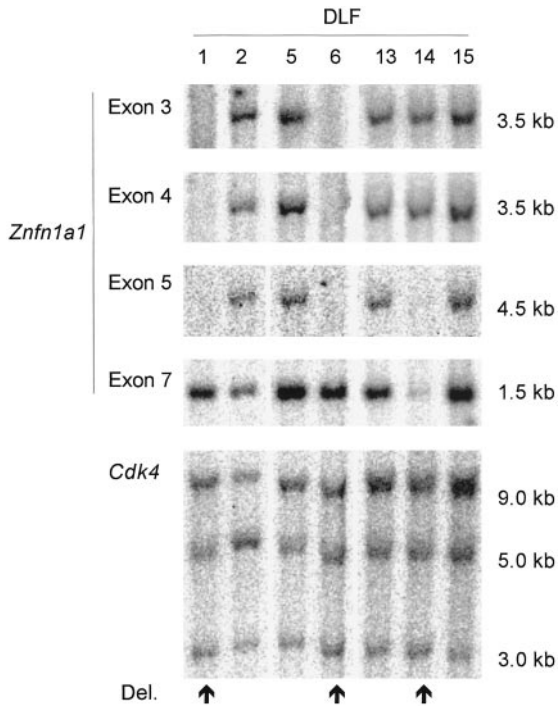

Fig. 2. Southern analysis of homozygous deletion of the *Znfn1a1* gene. The membrane was hybridized sequentially with mouse *Znfn1a1* exons 3, 4, 5, and 7 and *Cdk4* exons 2–8, as indicated on the *left*. The approximate fragment sizes detected by each probe are indicated on the *right*. *Arrows* mark samples with deletions of exons 3–5, or exons 5 and 7, respectively. The very weak band existing in exon 7 in DLF14 indicates contamination of the normal tissue. *Cdk4* is used as control gene.

of the DLF samples (Table 1), implying that allelic loss of *Znfn1a1* may be an important event in lymphomagenesis. However, only 2 of the tumors (BLF9 and BLF12) with LOH showed point mutations in the examined regions of the *Znfn1a1* gene (Table 1). These 2 and additional 3 samples (BLF7, 8, 9, 10, and 12) with LOH displayed point mutations of the *p53* gene in our previous study (14). The *p53* tumor suppressor gene has been mapped to mouse chromosome 11 and is located telomeric to the *Znfn1a1* gene. However, there are still 7 tumors with LOH not displaying *Znfn1a1* or *p53* mutations, indicating that other unknown tumor suppressor genes may be located in this region. It is also possible that other mechanisms could be involved in the inactivation of Ikaros, such as overexpression of the dominant-negative isoforms. In contrast with normal lymphocytes that predominantly express the active isoforms of Ikaros, it has been shown (15–17) that T-type leukemic cells express an increased level of inactive isoforms, which interfere with the active isoforms and exert dominant-negative functions. Unfortunately, we were unable to examine *Znfn1a1* transcripts because of limited tumor material.

Studies in Ikaros knockout mice suggest that inactivation of Ikaros is involved in the development of lymphoma. Here, we have identified point mutations and homozygous deletions in a subset of chemically induced lymphomas, all of which lead to amino acid substitutions or abrogation of the functional domains in the Ikaros protein. Our results additionally support the role of Ikaros as a potential tumor suppressor.

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