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Genetic Mapping and Allelic Loss Analysis in Mouse Thymic Lymphomas of *Helios* and *Aiolos* Belonging to the *Ikaros* Gene Family

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The *Ikaros* gene undergoes bi-allelic changes at a high frequency in γ-ray-induced mouse thymic lymphomas, suggesting the relevance of *Ikaros* to the lymphoma development. Here we test whether *Helios* and *Aiolos*, two other members of the *Ikaros* gene family, are also involved in lymphomagenesis. Genetic mapping showed that *Helios* is located between *D1Mit531* and *D1Mit19* on chromosome 1 and *Aiolos* is between *D11Mit222* and *D11Mit332* on chromosome 11. Analysis using polymorphic markers around the two regions revealed that neither locus exhibited allelic loss in the 78 lymphomas that were induced in *p53* wild-type mice, whereas in 102 *p53*(KO/+) mouse-derived lymphomas *Helios* and *Aiolos* loci showed allelic loss in 8% (8/102) and 33% (34/102), respectively. However, 33 of the 34 lymphomas showing allelic loss at *Aiolos* were *p53*(KO/−) and were accompanied by loss of the *p53* wild-type allele on the same chromosome. Homozygous deletion and mutation analyses failed to detect bi-allelic alterations. These results do not suggest any obvious contribution of *Helios* or *Aiolos* to oncogenesis of the mouse thymic lymphomas.

Key words: Tumor suppressor gene — *Ikaros* — Allelic loss (or LOH) analysis — Somatic mutation — γ-Ray-induced thymic lymphoma

The *Ikaros* gene encodes multiple protein isoforms with zinc finger domains that play an important role in the development of lymphocytes and other hematopoietic cell types.[1–4] Studies of *Ikaros* KO mice suggest that *Ikaros* participates in proliferation of thymocytes and in an oncogenic process.[5] Another gene related to *Ikaros* was identified by the use of degenerate primers complementary to sequences encoding the carboxy-terminal zinc finger of *Ikaros*. This gene, designated as *Aiolos*, exhibits considerable homology to *Ikaros* and its expression is restricted to the lymphoid lineage, like that of *Ikaros*.[6] *Helios* is a third member of the *Ikaros* gene family and is expressed primarily in T-cells.[7, 8] All members of the *Ikaros* family consist of four N-terminal DNA-binding zinc fingers, a conserved bipartite activation domain and two C-terminal zinc fingers, and bind to similar DNA sequences. They interact physically with each other through the carboxy-terminal zinc fingers, as detected by co-immunoprecipitation and also by nuclear staining with antibodies.[5–8]

We have previously demonstrated that *Ikaros* functions as a tumor suppressor gene in the development of γ-ray-induced mouse thymic lymphomas.[9] About 20% of the lymphomas examined underwent bi-allelic DNA alterations of the *Ikaros* gene. As described above, *Helios* and *Aiolos* have sequence homologies to *Ikaros* and show protein-protein interactions, and therefore these two genes might also be involved in lymphoma development. This prompted us to perform genetic mapping and to examine allelic loss in the γ-ray-induced mouse thymic lymphomas. In this paper, we show the chromosomal location of *Helios* and *Aiolos* and demonstrate that neither gene shows frequent allelic loss or bi-allelic changes in the thymic lymphomas.

MATERIALS AND METHODS

Mice and lymphomas MSM is an inbred strain derived from Japanese wild mice, *Mus musculus molossinus*.[10] The details of lymphoma induction were described previously.[11] In brief, the parental *p53*-deficient mouse was originally produced by introduction of a neo-gene fragment into the *p53* gene locus in the ES cells that had been derived from F_1 mouse between C57BL/6(B6) and CBA strains.[12] We have developed a congenic MSM mouse carrying a *p53*-deficient allele by backcrossing this *p53*-deficient mouse to MSM mice for 13 generations. The male mice (N10 to N13 generation) with the genotype of *p53*(KO/+) were mated with BALB/c female mice. The mice were then subjected to γ-ray irradiation, 2.5 Gy four times at weekly intervals, starting at the age of 4 weeks. Development of thymic lymphoma was diagnosed on the basis of observation of labored breathing.

Polymerase chain reaction (PCR) analysis Isolation of genomic DNA from lymphomas and brain was carried out by using standard protocols. PCR and separation of PCR

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products by gel electrophoresis were performed as described previously.[9, 11)]

Primer sequences Three primer sequences used for the *Helios* gene were as follows: *Helios* exon 7-1, 5′-ATAAGCTCAGCTTATT-CTCAGG and 5′-ATGTCGC-CATCCGTGGGAAG for detection of a polymorphism and for mutation and homozygous deletion analyses; *Helios* exon 7-2, 5′-GTGTTCAGTTAACTTCTCATACC and 5′-AGAAGTGGGGCCATCCATGT for mutation analysis; *Helios* exon 4, 5′-GAACCTAGGAAATTGTCT-GTGG and 5′-CTCTCTGCCAAAGCTACATGG for homozygous deletion analysis and mutation analysis. Two primer sets for *Aiolos* were as follows: *Aiolos* exon 7-1, 5′-CGGATGATGGACCAAGCCAT and 5′-AGCACAG-AGCCATGTTGAAG for detection of a polymorphism; *Aiolos* exon 7-2, 5′-CGGATGATGGACCAAGCCAT and CCTCCTTCAGAAGA-GGCATCGC for allelic loss analysis. Primers for the *catenin* gene were as described previously.[13)] Microsatellite markers were synthesized according to reported sequences.[14)]

Isolation of BACs BAC clones were isolated by PCR screening of a library that was purchased from Research Genetics, Inc. (Huntsville, AL).

Typing of *p53* *p53* genotyping was carried out as described previously.[9, 15)] One primer (F1-53) located in exon 1 of the *p53* gene, a second primer (R1-53) in a region 5′ to exon 3, and the remaining one (F2-neo) in the neo gene insert. F1-53 and R1-53 amplified a region of the *p53* gene to produce a fragment of 500 bp. F2-neo and R1-53 gave an 800 bp fragment comprising a part of the neo and *p53* gene. Therefore, the 500 bp band and the 800 bp band represent the normal allele and the mutant allele, respectively. F_1 mice of the *p53*(+/+) genotype exhibited two distinct *p53* alleles originating from different mouse subspecies, *Mus m. molossinus* and *Mus m. domesticus*. A primer set amplifying a polymorphic region upstream from the transcription start site was synthesized and used for allelic loss analysis of those lymphomas.

RESULTS

Genetic polymorphisms were searched to map *Helios* and *Aiolos* genes on the mouse chromosome. Since only the cDNA sequence was available for either gene, we sequenced genomic DNA and BAC clones corresponding to *Ikaros* exon 7 by aligning their cDNA sequences with cDNA and genomic sequences of *Ikaros*. As for *Helios*, a base-substitution between BALB/c and MSM genomes, which creates an *Aat*II restriction enzyme cleavage site, was found and accordingly a set of primers was designed to detect the polymorphism. Gel electrophoresis of *Aat*II digests of PCR products with the primers gave an undigested DNA fragment for BALB/c and two digested fragments for MSM (see Fig. 2). The difference was used for genotyping of 131 intersubspecific backcross mice that were obtained by mating (C57BL/6×MSM)F_1 females to MSM males. Likewise, an *Acc*II recognition site polymorphism was found in *Aiolos* by sequence analysis. A set of primers was synthesized accordingly and used for genotyping. Fig. 1 shows the mapping results of the two genes. The *Helios* gene is located between markers *D1Mit531* and *D1Mit19* on chromosome 1, and the *Aiolos* gene is between *D11Mit222* and *D11Mit332* on chromosome 11.

Allelic loss analysis of *Helios* was carried out using the primers used for the *Helios* mapping and also two markers flanking *Helios*. Fig. 2A shows gel electrophoretic patterns of *Aat*II digests of the *Helios* PCR products and the PCR products of *D1Mit531* and *D1Mit19*. Allelic differences were clearly seen. The frequency of allelic loss was 3.9% (7/180) at the *D1Mit531* locus, 4.4% (8/180) at the *Helios* locus and 3.9% (7/180) at the *D1Mit19* locus; thus the loss frequency at *Helios* was higher than those at the other two loci. Fig. 3A shows a compilation of allelic losses at the three loci in individual lymphomas which allows us to classify the lymphomas into four types: type

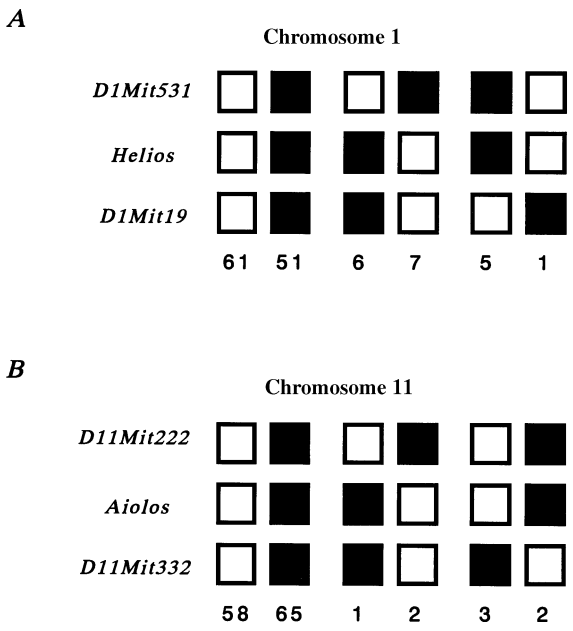

Fig. 1. Distribution of the haplotypes for 131 progeny from intersubspecific backcross mice that were obtained by mating (C57BL/6×MSM)F_1 females to MSM males. (A) *Helios*. (B) *Aiolos*. The loci followed in the cross are indicated on the left. The filled squares represent the C57BL/6 allele, and open squares represent the MSM allele. Each column represents the chromosome identified in the progeny. The numbers of the progeny carrying each type of chromosome are listed at the bottom. The lod scores between *D1Mit531* and *Helios*, between *Helios* and *D1Mit19*, between *D11Mit222* and *Aiolos*, and between *Aiolos* and *D11Mit332* are 20.9, 28.7, 33.1 and 30.1, respectively.

A, lymphomas retaining both alleles of all three loci; type B, lymphomas lacking one chromosomal region of MSM that covers all three loci; type C, lymphomas showing allelic losses at the centromeric two loci but retaining both alleles of the telomeric locus; type D, lymphomas retaining both alleles of the centromeric locus but showing allelic losses at the telomeric two loci. There were two lymphomas, one being a type C lymphoma and the other type D, which might suggest the involvement of *Helios* in lymphomagenesis.

Likewise, allelic loss analysis was done for *Aiolos* in a similar manner. Fig. 2B shows examples of *Acc*II digests of PCR products, together with PCR products of *D11Mit222* and *D11Mit332*, and Fig. 3B summarizes the results of compilation. There was a lymphoma of type E which exhibited allelic loss only at the *Aiolos* locus. The frequency of allelic loss was 19% (34/180) at the *Aiolos* locus, although the loss had a strong preference for the BALB/c allele (see below).

Inactivation of the *p53* gene in lymphomas may affect the allelic loss frequency of *Helios* and *Aiolos* loci because *p53* functions to maintain the genome integrity.[16–19] Besides, the *p53* and *Aiolos* genes reside on the same chromosome 11 and hence single allelic loss events at a

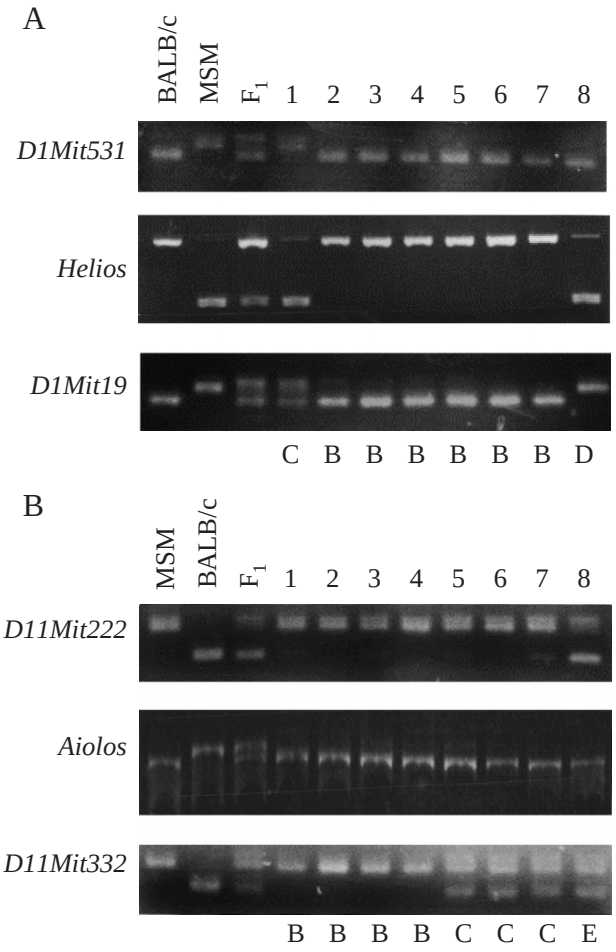

Fig. 2. Allelic loss analysis of *Helios* (A) and *Aiolos* (B) loci. (A) PCR products for *D1Mit531* and *D1Mit19* and *Aat*II digests of the *Helios* PCR products were subjected to gel electrophoresis. (B) PCR products for *D11Mit222* and *D11Mit332* and *Acc*II digests of the *Aiolos* PCR products were subjected to gel electrophoresis. The first three lanes on panels represent control DNA of BALB/c, MSM and F_1 mice, and the other lanes display lymphoma DNA. The number of lymphomas is given arbitrarily. Type of allelic losses in each lymphoma (see legend to Fig. 3) is indicated at the bottom.

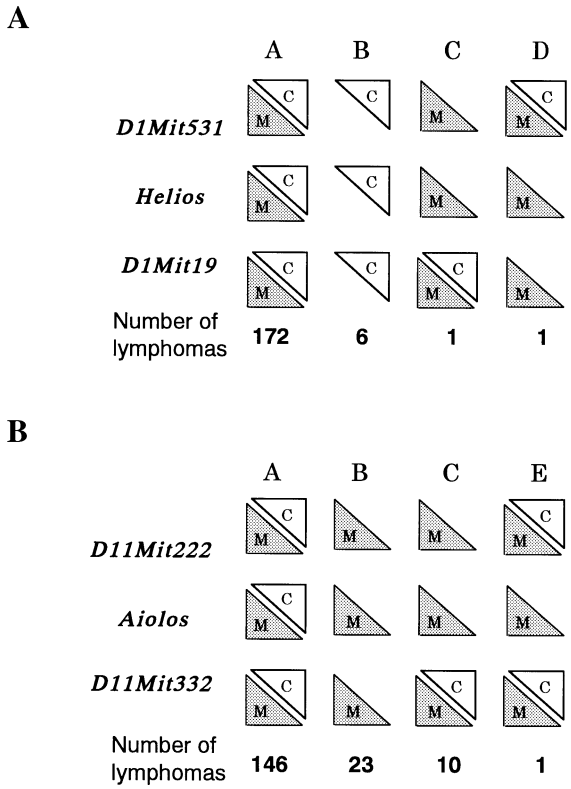

Fig. 3. Chromosomal constitution of lymphomas in the vicinity of the *Helios* (A) and *Aiolos* (B) loci. Three loci used for allelic loss analysis are indicated on the left. Triangles marked by C and M indicate BALB/c allele and MSM allele retained, respectively. Two triangles of C and M represent both BALB/c and MSM alleles retained, and one triangle represents one allele lost. Combination of the status of the alleles allows lymphomas to be divided into four types at both loci: type A, lymphomas retaining both alleles of all three loci; type B, lymphomas lacking one chromosomal region of BALB/c or MSM that covers all three loci; type C, lymphomas showing allelic losses at the centromeric two loci but retaining both alleles of the telomeric locus; type D, lymphomas retaining both alleles of the centromeric locus but showing allelic losses at the telomeric loci; type E, lymphomas exhibiting allelic loss only at the *Aiolos* locus. The number of lymphomas of each type is listed at the bottom.

Table I. Distribution of *Helios* and *Aiolos* Genotypes in Lymphomas of Four Different *p53* Genotypes

Class	*p53* status[a]		*Helios* allele retained[b]				*Aiolos* allele retained[b]			
	of host	of lymphomas	C/M	C&CC	M&MM	Total	C/M	M&MM	C&CC	Total
[1]	+/+	+/+	75	0	0	0/75	75	0	0	0/75
[2]	+/+	+/−	3	0	0	0/3	3	0	0	0/3
[3]	KO/+	KO/+	19	0	0	0/19	18	1	0	1/19
[4]	KO/+	KO/−	75	6	2	8/83	50	33	0	33/83
			172	6	2	8/180	146	34	0	34/180

a) Four *p53* categories marked on the left include the following lymphomas: [1] +/+, lymphomas showing no inactivation of the two *p53* alleles; [2] +/−, lymphomas exhibiting allelic loss of one *p53* allele. Those tumors were induced in *p53* wild-type mice. [3] KO/+, lymphomas inheriting one *p53*-deficient allele and retaining the wild-type allele; [4] KO/−, lymphomas inheriting one *p53*-deficient allele and lacking the wild-type allele. KO indicates a *p53*-deficient allele; + shows a wild-type allele of *p53*; − represents loss of the *p53* wild-type allele.
b) C/M indicates both *Helios* or *Aiolos* alleles retained; C&CC, BALB/c allele(s) retained; M&MM, MSM allele(s) retained.

site of the chromosome may well involve both loci, which can increase the allelic loss frequency of *Aiolos*. Therefore, the relation between allelic losses of the *Helios* or *Aiolos* locus and the *p53* locus was investigated. *p53* genotyping of lymphomas and the host mice was performed using three sets of primers (see "Materials and Methods"). The typing allowed us to classify 180 lymphomas into four *p53* genotypes: [1] (+/+), both wild-type alleles retained; [2] (+/−), one of the two wild-type alleles lost; [3] (KO/+), the KO allele and the wild-type allele retained; [4] (KO/−), the KO allele retained but the wild-type allele lost. The numbers of lymphomas in these four classes were 75, 3, 19, and 83, respectively.

Table I summarizes the status of the *Helios* and *Aiolos* alleles in lymphomas of the four different *p53* genotypes. It is noteworthy that at either gene locus no allelic loss was found in lymphomas that were induced in *p53* wild-type mice. The eight lymphomas showing allelic loss at the *Helios* locus were all *p53*(KO/−). This may reflect cooperativity between losses of *Helios* and *p53* or the consequence of genomic instability conferred by the *p53* loss. Similarly, 33 of the 34 lymphomas showing allelic loss at the *Aiolos* locus were *p53*(KO/−). The allelic loss had a strong preference for the allele on the BALB/c chromosome that carried a wild-type *p53* allele; all of the losses were of the BALB/c allele. This suggests that almost all of the allelic losses found in *Aiolos* are only the consequence of the wild-type *p53* allele being lost.

Allelic loss frequency was very low at either of the *Helios* and *Aiolos* loci relative to that of *Ikaros*. No notable concordance of losses among the three genes was observed (data not shown). This suggests little involvement of the two genes in the thymic lymphoma development. However, further analyses were done to examine bi-allelic changes in the two genes. In our previous study of *Ikaros*, we found nine lymphomas with homozygous deletion and six mutations in the zinc finger domain regions. Therefore, analysis of homozygous deletion and mutations was done in a similar manner. For the assay of homozygous deletion of *Helios*, two pairs of primers on the putative exons 4 and 7 and a primer set for the *catenin* gene as a positive control were synthesized and used for the eight lymphomas that showed allelic loss. No pattern indicative of homozygous deletion was detected in these samples (data not shown). For mutation analysis of the putative exons 4 and 7, intron-specific primers and primers on the exons were used. We failed to find any mutation in the region. Although preliminary, these results did not provide evidence for involvement of *Helios* in the development of thymic lymphoma. As for the *Aiolos* gene, similar studies were carried out for the 34 lymphomas with allelic loss. No result indicating bi-allelic DNA alteration was obtained (data not shown).

DISCUSSION

We previously showed that the *Ikaros* gene undergoes bi-allelic changes at a high frequency in γ-ray-induced mouse thymic lymphomas.[9] In this paper we have tested whether or not *Helios* and *Aiolos*, two other members of the *Ikaros* gene family, are also involved in lymphomagenesis. In contrast to *Ikaros*, no result was obtained which suggests any obvious contribution of *Helios* or *Aiolos* to the development of mouse thymic lymphomas.

The *Ikaros* family of proteins, *Ikaros*, *Helios* and *Aiolos*, are characterized by the presence of four N-terminal DNA-binding zinc fingers, a conserved bipartite activation domain, and two C-terminal zinc fingers.[20] They can activate transcription of reporter genes driven by the *Ikaros* consensus binding site, 5′-GGGAA-3′. Gene disruption

experiments have been carried out for *Ikaros* and *Aiolos* genes. As for *Ikaros*, two distinct strains of *Ikaros*-KO mice have been produced.[5, 21] One carries a deletion of the *Ikaros* DNA-binding domain that displays dominant negative effects on transcription through interaction at the C-terminal domain with proteins of the *Ikaros* family (*Ikaros*-DN type), and the other harbors a deletion of the last translated C-terminal exon (*Ikaros*-C type). Interestingly, heterozygotes of the *Ikaros*-DN mouse strain, but not the *Ikaros*-C strain, exhibit defects in the T lineage, which lead to an abnormal accumulation of CD4/CD8 double-positive thymocytes and ultimately result in T cell leukemia and lymphomas. *Ikaros* controls T cell proliferative responses and probably functions as a growth suppressor, and hence loss of the gene function may confer a growth advantage upon these cells which fosters the development of a tumor.[5] On the other hand, mice homozygous for an *Aiolos*-null mutation exhibit a much less severe lymphopoietic defect than *Ikaros* KO homozygotes do, and the development of B cell lymphomas is frequently seen among aging mutants.[22] The results of gene disruption experiments suggest the involvement of *Ikaros* and possibly *Aiolos* in the development of T cell lymphomas of the mouse. Our previous analysis provided further genetic evidence implicating *Ikaros* in the development of lymphomas in *Ikaros* wild-type mice by showing bi-allelic changes in about 20% of the γ-ray-induced mouse thymic lymphomas.[9]

Immunoprecipitation experiments showed that protein-protein interaction allows *Ikaros* proteins to dimerize with *Helios* or *Aiolos* proteins, as well as themselves. The C-terminal zinc fingers provide a site for these interactions.[6–8] Recent studies of subnuclear localization in B and T cells provide direct evidence of the protein interactions. The Ikaros-Helios complexes localize to the centromeric heterochromatin in T cells,[7, 8] and *Aiolos* dimers with *Ikaros* proteins localize to the same discrete regions within the nucleus of transfected NIH3T3 cells.[6] These complexes in the nucleus are probably important for transcriptional regulation.[7, 8, 23] *Ikaros* isoforms are expressed in most cells of the hematopoietic lineages, including multipotent stem cells.[1–4] *Helios* shows a restricted expression pattern and is transcribed in a subset of cells of the T cell lineage.[7, 8] On the other hand, *Aiolos* is expressed in most of the cell types that express *Ikaros*, but a high expression is seen in mature B cells.[6, 21] These results suggest functional cooperativity of the three proteins and that the cooperativity may be cell type-specific.

As described above, *Ikaros* is a tumor suppressor gene and *Helios* and *Aiolos*, other members of the *Ikaros* gene family, directly interact with *Ikaros* and form functional units in cell nuclei. This raised the possibility that *Helios* and/or *Aiolos* may also function as tumor suppressor genes in the development of thymic lymphomas. However, no evidence for that was obtained in this study of *Helios* and *Aiolos*.

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