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A frameshift mutation in the *LYST* gene is responsible for the Aleutian color and the associated Chédiak–Higashi syndrome in American mink

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Summary

One of the colors of mink is Aleutian (*aa*)—a specific gun-metal gray pigmentation of the fur—commonly used in combination with other color loci to generate popular colors such as Violet (*aammpp*) and Sapphire (*aapp*). The Aleutian color allele is a manifestation of mink Chédiak-Higashi syndrome (CHS), which has been described in humans and several other species. As with forms of CHS in other species, we report that the mink CHS is linked to the *lysosomal trafficking regulator* (*LYST*) gene. Furthermore, we have identified a base deletion (c.9468delC) in exon 40 of *LYST*, which causes a frameshift and virtually terminates the LYST product prematurely (p.Leu3156Phefs*37). We investigated the blood parameters of three wild-type mink and three CHS mink. No difference in the platelet number between the two groups was observed, but an accumulation of platelets between the groups appears different when collagen is used as a coagulant. Microscopic analysis of peripheral blood indicates giant inclusions in the neutrophils of the Aleutian mink types. Molecular findings at the *LYST* locus enable the development of genetic tests for analyzing the color selection in American mink.

Keywords Aleutian mink, Chédiak-Higashi, frameshift, *lysosomal trafficking regulator*, mutation

Introduction

Chédiak-Higashi syndrome (CHS) is an autosomal monogenic recessive disorder seen in humans (OMIM #214500) and other species (cattle, Kunieda *et al.* 1999; cats, Kramer *et al.* 1977; killer whales, Ridgway 1979; rats, Nishimura *et al.* 1989; mice, Lutzner *et al.* 1967; foxes, Sjaastad *et al.* 1990; and mink, Padgett *et al.* 1964, 1967). CHS is expressed by impaired lysosome degranulation with phagosomes, and this induces changes in the nuclear structures of the leukocytes with the accumulation of large lysosomal vesicles in the neutrophils. This can reduce the bactericidal function, leading to susceptibility to some infections. Furthermore, in melanocytes, the melanosomes are not processed normally, resulting in decreased skin pigmentation. Although American mink are not clinically affected by CHS to the same extent as some other species are, the mink model of the disease is interesting from a biological point of view, as hematologic defects and pigment formation and distribution in the fur have the same origin.

In mink, the leukocyte disorder was first found in animals that were homozygous for the Aleutian coat color allele, which results in a silver-grayish gunmetal appearance (Fig. 1a). The Aleutian color is used in combination with other coat color genes to generate many other colors through selective breeding. Currently, only Sapphire (Fig. 1b) and Violet (also known as moylesapphire) (Fig. 1c) are produced on a commercial scale. Although in the past Aleutian mink were reported to be weaker than normal mink and produce smaller litters of weaker kits (Helgebostad 1963), nowadays the mink color types carrying the mutated locus for Aleutian and CHS have a similar production capacity as the standard dark brown mink (wild-type mink) (yearly statistics – *Dansk Pelsdyravl*, 2010).

Several features of the mink condition partially resemble those of human CHS patients, particularly in relation to the

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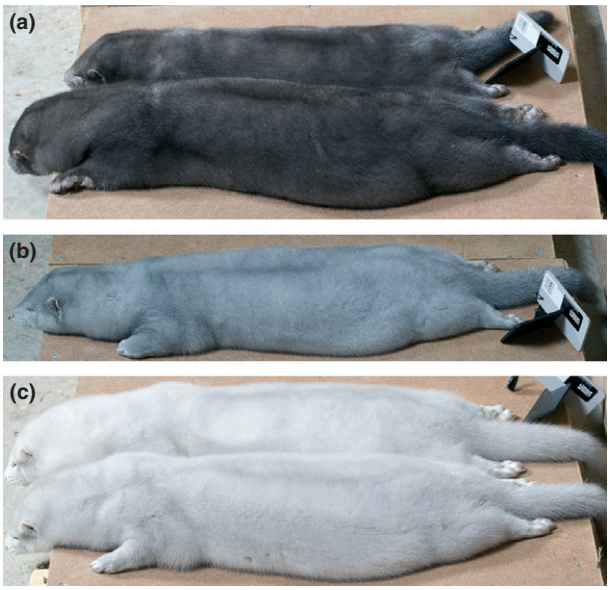

Figure 1 Mink phenotypes homozygous for the *LYST* mutation. (a) Pure Aleutian (aa); (b) Sapphire (aapp); (c) Violet (aammpp). Larger animals are males and smaller ones are females. Photograph: Jesper Clausen.

blood parameters and diluted pigmentation. Nevertheless, Aleutian mink and mink exhibiting derived colors are not known to display any visible clinical abnormalities (farmers & vets – personal communication), thus not representing a welfare issue in mink farming.

To date, it has been reported only in humans (OMIM #214500), beige mice (*bg*) (Barbosa *et al.* 1996; Nagle *et al.* 1996; Perou *et al.* 1996) and cattle (Kunieda *et al.* 1999) that CHS is caused by mutations in the *LYST* gene. This gene encodes a membrane-associated protein, which regulates intracellular protein trafficking. The types and positions of the mutations in the *LYST* gene vary widely with humans and between species, and they are of interest from a comparative point of view.

In this study, we have identified the *LYST* gene to be responsible for the mink Aleutian phenotype and the CH-like syndrome. Comparison of *LYST* gene sequences between wild-type and Aleutian mink revealed a phenotype-specific nucleotide deletion that induces a frameshift and a premature stop of the protein product (p.Leu3156Phefs*37). The typical result of such a mutation may be a complete lack of gene expression and of the LYST protein because the non-sense-mediated decay mechanism (NMD) would detect the mRNA containing the premature termination codon and degrade it (Strachan & Read 2010). In addition, examination of blood smears from all the investigated animals indicated giant inclusions in neutrophils correctly segregating with CHS. Supplementary blood analyses of three Aleutian and three wild-type mink also revealed differences in the platelet aggregation *in vitro* induced by collagen.

Materials and methods

Family material, markers and genotyping

A half-sib family originating at the Taastrup Experimental Farm of the Faculty of Life Sciences, University of Copenhagen, Denmark, with 22 offspring that segregated for the Aleutian color (Fig. S1) was genotyped with two microsatellite markers – *RAN77* and *RAN118* (Table 1) – developed from the assembled contig (JF288176) comprising the candidate *LYST* gene (Anistoroaei *et al.* 2011). Genomic DNA was extracted from euthanized mink tongues by using a Promega DNA Extraction Kit (#A1125). Genotyping was performed with fluorescently labeled forward primers (HEX and NED) on an ABI Prism 3130 sequencer (Applied Biosystems), and genotypes were analyzed using GENSCAN analysis (v.3.1.2) software (Applied Biosystems).

Sequences analysis

For analyzing of the *LYST* gene, we initially designed primers on the basis of cDNA and obtained cDNA from CHS mink. Because some of the products could not be amplified and sequenced, genomic DNA was used instead. Thus, primers were designed from the flanking intronic regions of all exons of the *LYST* gene of the mink (JF288176; Anistoroaei *et al.* 2011) with PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table S1). After PCR amplification, the DNA products were sequenced and mutations were first identified by comparing a single Aleutian individual with a wild-type mink genotype and with the reference sequence (JF288176). In the second round, analogous PCR products spanning the relevant mutations were amplified from the DNA of an additional two Aleutian (***aa***), two Sapphire (***aa****pp*) and two Violet (***aa****mmpp*) minks along with two wild-type minks. Sequencing was performed using BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems), and extension products

Primer pair name	Forward and reverse primers (5′-3′)	Repeat motif	Allele size
RAN77	F: TGCTTGGTATCAATTTCCAGA R: CCCTCTGGGTAGTCCTTGAA	$(CA)_{16}$	222–226
RAN118	F: ACTTCCCCACCAAAAGCAG R: AAGCCAGATGCTAAAGGCTAGT	$(CA)_{12}$…$(GT)_{12}$	241–243

Table 1 Microsatellite markers used for testing *LYST* as candidates for Aleutian and Aleutian-derived phenotypes in mink.

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were separated on an ABI 3130 Automated Nucleic Acid Analyzer (Applied Biosystems). DNA sequences were processed using LASERGENE (DNA Star, Inc.), and the sequences were compared using the BLAST bl2seq at NCBI.

Hematologic characterization

Peripheral blood smears were stained with a modified Wright's method and analyzed. Approximately 2 ml of whole blood from three wild-type and three Aleutian carriers (two Sapphire and one Violet) was harvested for *in vitro* tests using heparin with ADP, heparin with collagen, heparin with ASPI and serotonin without heparin respectively as antagonists. All the individuals were healthy mature females (ca. 12 months of age). Blood was harvested by heart puncture after ketaminol/narcoxyl deep anesthesia. Animals were subsequently euthanized. The analyses were performed within 1 h from the time of harvesting in a multiplate analysis system (Dynabyte Informationssysteme GmbH). Additionally, a TEG 5000 Analyser (Hemonetics) was used for a thromboelastography test.

Results

Gene description in mink

Based on the analysis of the assembled *LYST* gene genomic contig (JF288176), derived from the CHORI-231 American mink BAC library (Anistoroaei *et al.* 2011), the mink *LYST* gene is predicted to be represented by 53 exons. The coding region consists of 11 403 bp, which translates into 3801 amino acids. A BLAST comparison of mink and human *LYST* sequences revealed nucleotide and deduced amino acid sequence similarities of 88% and 88% respectively, whereas those between mink and dog (the closest relative with a published *LYST* sequence) were 92% and 93% respectively. The contig provided 10 di- and tetranucleotide repeated motifs, which could be subsequently developed into microsatellite markers. *RAN77* and *RAN118* (Table 1) were developed and characterized as markers and were used in this study.

Mapping LYST as the causative gene

The *RAN77* and *RAN118* microsatellite markers were located in mink chromosome 2, close to the centromere, following the latest mink linkage map (Anistoroaei *et al.* subm) upgraded from the Anistoroaei *et al.* (2009) map. Their physical locations also were confirmed on the basis of sequence homology aided by the Zoo-FISH data (Hameister *et al.* 1997; Graphodatsky *et al.* 2000). The genotyping of the two markers for animals within a family segregating for the Aleutian phenotype (Fig. S1) revealed informative polymorphism only for *RAN118*, which yielded an LOD score value of 3.9 with the Aleutian.

Identification of a nucleotide deletion in the LYST gene

A comparison between the Aleutian and wild-type nucleotide sequences of the *LYST* exons and flanking regions revealed seven SNPs inconsistently associated with the Aleutian phenotype distributed on exons 4 (two SNPs), 9 (one SNP), 16 (three SNPs) and 40 (one SNP). The comparison also revealed a base deletion at nucleotide position 9468 in the middle of exon 40. This generates a frameshift mutation starting at amino acid position 3156 and results in a premature stop codon after amino acid 3193 (c.9468delC → p.Leu3156Phefs*37). The mutation showed a perfect co-segregation with the phenotypes of the seven analyzed Aleutian and Aleutian-derived color mink.

Hematology data

As other studies have indicated, both in mink and in other species (Padgett *et al.* 1964; Lutzner *et al.* 1967; Kaplan *et al.* 2008), one of the main abnormalities of CHS is neutrophils with giant inclusion bodies. Our analyses are invariably in agreement with this finding (Fig. 2). Additionally, no difference in the number of platelets was noticed between the two test groups comprising three wild-type mink and three Aleutian. An *in vitro* platelet aggregation test indicated that when collagen was used as antagonist, a great difference was observed between the wild-type group [area under the aggregation curve (AU) between 2836 and 3390] and the Aleutian color types group (AU ranging between 966 and 1790). Aggregation data of whole blood on ASPI, ADP and serotonin did not reveal any significant differences between the two groups of individuals. The TEG (thrombelastograph) test indicated normal and similar blood coagulates, and all the biochemical parameters measured remained within similar ranges. Still, more individuals would be needed for a conclusive statement.

Discussion

The LYST protein and its functions

The *LYST* gene encodes for a lysosomal trafficking regulator protein, which is involved in regulating vesicle's size and trafficking them throughout the cells. Melanosomes are lysosome-like vesicles present in the melanocytes, wherein melanin is synthesized and stored before being transferred to neighboring keratinocytes. Functional studies of different LYST domains and known interacting partners of LYST strongly indicate the role of LYST in determining organelle size by controlling membrane fission events (McVey Ward *et al.* 2003; Durchfort *et al.* 2011). The hallmark of CHS is

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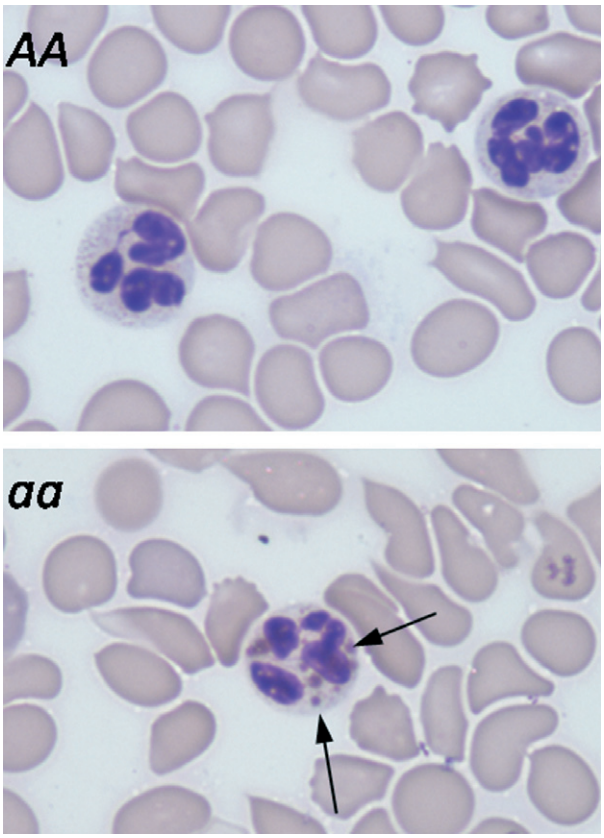

Figure 2 ***AA*.** Normal neutrophils from a peripheral blood smear of wild-type mink; ***aa*.** Neutrophils of Aleutian mink displaying giant granules.

giant inclusion bodies in all granulated cells, giant lysosomes and giant melanosomes. Because lysosomes and lysosome-related organelles are involved in waste processing in the cell as well as in pigment production, blood clotting and immune response (Dell'Angelica *et al.* 2000), their abnormal production would underlie the observed symptoms of CHS. The clinical manifestations of CHS in American mink are almost undetectable in comparison with CHS in humans, who develop a lymphoproliferative syndrome during the so-called accelerated phase. Apart from this difference, the fact that CHS symptoms in mink are less evident than those in humans could also be due to the fact that exposure to infection is limited as the mink farms are normally kept disease free.

Aleutian mink and Aleutian disease infection

In mink, Aleutian disease (AD) or viral plasmacytosis is a lethal infection, caused by a parvovirus, which results in chronic symptoms of progressive weight loss, lethargy, splenomegaly, anemia, rear leg weakness and seizures. AD was first recognized in farm-raised mink in 1956 and was named as such because it was first identified in mink with the Aleutian coat color. Although it was initially assumed that the disease was a result of poor genetics (and linked to the Aleutian color), it was later found that mink of all coat colors were susceptible to the disease. Importantly, however, mink with other coat colors tended to have lower mortality than did Aleutian mink, as the latter exhibit increased bleeding and a putative immunologic deficiency. Padgett *et al.* (1967) suggested that AD is not necessarily linked to the Aleutian genotype and that it may have previously been undiagnosed in other mink color phenotypes. We can presume that the likely explanation for the disease to be initially described and associated with the Aleutian color phenotype is caused by an immune-mediated response to the persistent viral infection that may be dysregulated by the CHS phenotypes owing to the abnormal neutrophils. Therefore, it should be emphasized that there is no exclusive relationship between AD and the CHS characteristics of Aleutian mink phenotypes.

Availability as an animal model

Although several species are known to be affected by CHS (Prieur & Collier 1979), so far only *bg* mice and rats have been considered as animal models of the disease (Nishimura *et al.* 1989; Novak *et al.* 1995). Because mink with CHS (Aleutian and Aleutian-related color phenotypes) are available from many commercial mink ranches, these could also constitute a permanent source of animal models for the syndrome. The approaches may not be directed toward CHS *per se* but focus upon using cells, platelets and tissues of affected humans and mink to elucidate mechanisms and functions of cells and organelles in the absence of the LYST product (Bell *et al.* 1976; Buchanan & Handin 1976; Costa *et al.* 1976).

The LYST mutation

Various CHS clinical phenotypes have been associated with different mutations within the *LYST* gene. The mutations identified in the *LYST* gene of human CHS patients and of *bg* mice (Barbosa *et al.* 1996, 1997; Nagle *et al.* 1996; Karim *et al.* 1997) include various types of frameshift (Table 2) as well as non-sense mutations. Missense mutations in *LYST* have rarely been reported in humans and mice (Karim *et al.* 2002; Runkel *et al.* 2006), but a missense mutation is responsible for CHS in cattle by producing a LYST amino acid substitution (H2015R) (Kunieda *et al.* 1999). In most of the reported cases, the *LYST* gene alterations result in a virtually truncated protein. Karim *et al.* (1997) stated that there is a clear indication that the complete 3801-amino-acid sequence of LYST is required for a CHS-free phenotype in humans. Although RNA expression tests have not been employed in this study, the LYST product is likely to be absent in the CHS animals, as the NMD mechanism is expected to be activated in this case.

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Table 2 Reported frameshift mutations for the *LYST* gene associated with Chédiak–Higashi syndrome. All mutations were reported in human patients. In bold are the two frameshift mutations in the very proximity of the mutation in American mink.

Type and location	Premature termination codon	Reference
Single-bp duplication in codon 40	50	Barbosa *et al.* (1996); Nagle *et al.* (1996)
Single-bp deletion within codon 489	566	Nagle *et al.* (1996)
Single-bp insertion in codon 633	638	Karim *et al.* (1997)
Single-bp insertion in codon 834	898	Dufourcq-Lagelouse *et al.* (1999)
Single-bp deletion in codon 874		Dufourcq-Lagelouse *et al.* (1999)
Two-bp deletion in codon 1024	1030	Barbosa *et al.* (1997)
Single-bp insertion in codon 1028	1029	Barbosa *et al.* (1997)
Single-bp deletion in codon 1772	1773	Certain *et al.* (2000)
Seven-bp deletion in codons 2353–2356	2354	Certain *et al.* (2000)
Single-bp deletion in codon 2518	2519	Certain *et al.* (2000)
56-bp deletion in codons 3035–3053	3036	Certain *et al.* (2000)
10-bp insertion in codon 3076	**3210**	**Certain *et al.* (2000)**
Single-bp deletion in codon 3197	**3258**	**Dufourcq-Lagelouse *et al.* (1999); Certain *et al.* (2000)**

Hematology data

Blood smear analysis on the Aleutian individuals has been carried out in American mink in previous studies (Padgett *et al.* 1964; Blume *et al.* 1969), and our findings are in complete agreement with them. Nevertheless, the TEG and multiplate hematologic investigations performed in this study have never been addressed in this species. Although employed on a small sample size (three wild-type minks and three CHS), there is a clear indication that the platelet aggregation is largely different between the two groups when the whole blood is challenged by collagen.

Conclusion

In this study, a single-bp deletion within exon 40 (c.9468delC) was identified as being responsible for CHS and the Aleutian and Aleutian loci-derived colors in American mink. The mutation causes a frameshift, which results in a premature stop codon 101 nucleotides downstream. The reported mink mutation disrupts the reading frame from amino acid position 3156 onward, introducing a premature stop codon at position 3195 (p.Leu3156Phefs*37). The identification of this mutation in the *LYST* gene provides the basis for establishing a DNA selection tool for American minks that are carriers of the recessive Aleutian color phenotype. The condition in mink can serve as potential animal model for addressing CHS caused by a lack of the LYST protein in humans.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1 Pedigrees segregating for Aleutian phenotypes in American mink. Circles represent females, squares represent males, solid symbols represent *Aleutian color homozygotes (aa)* and clear symbols represent wild-type (homo- or heterozygous; *AA* or *Aa*) individuals. All parents are heterozygous for the *Aleutian with CHS* mutation.

Table S1 Primer sequences for *LYST* exons.

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