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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
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 (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 (aapp) and two Violet (aammpp) minks along with two wild-type minks. Sequencing was performed using BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems), and extension products

<table>
<thead>
<tr>
<th>Primer pair name</th>
<th>Forward and reverse primers (5’-3’)</th>
<th>Repeat motif</th>
<th>Allele size</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAN77</td>
<td>F: TGCTTGGTATCAATTTCCAGA&lt;br&gt;R: CCCCCTGGTATCTCTTGAA</td>
<td>(CA)₆₆</td>
<td>222–226</td>
</tr>
<tr>
<td>RAN118</td>
<td>F: ACTTCCTCCTCACTAAAAACGAG&lt;br&gt;R: AAGCCAGATCTAACGGCTAGT</td>
<td>(CA)₁₂…(GT)₁₂</td>
<td>241–243</td>
</tr>
</tbody>
</table>

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/narcxyl 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 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; Grapholadtsky 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.

Hematologic 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 (thromboelastograph) 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

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.

Figure 2 AA. Normal neutrophils from a peripheral blood smear of wild-type mink; aa. Neutrophils of Aleutian mink displaying giant granules.
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.Leu3156Phe*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.

Acknowledgements

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References


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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