A Carbohydrate Sulfotransferase-6 (CHST6) gene mutation is associated with Macular Corneal Dystrophy in Labrador Retrievers

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Abstract
Purpose To locate and identify variants associated with macular corneal dystrophy (MCD) in Labrador Retriever (LR) dogs, in the candidate gene carbohydrate sulfotransferase-6 (CHST6).
Methods The single coding exon of canine CHST6 was sequenced in one affected LR with MCD and one control LR clinically clear of ocular disease. A further 71 control LR with unknown clinical status were sequenced for the putative causal variant in CHST6. A TaqMan SNP genotyping assay was developed and used to screen an additional 84 dogs (five affected LR and 79 clinically clear LR). Finally, the variant was screened in a third cohort of 89 unrelated LR with unknown clinical status to estimate its allele frequency in the population of LR in the United Kingdom.
Results A single nucleotide polymorphism (SNP) was identified within the coding exon of CHST6, resulting in a missense mutation (c.814C>A, p.R272S). All six LR affected with MCD were homozygous for the mutant allele, while 140/151 control LR were homozygous for the wild-type allele and 11/151 were heterozygous for the mutation, indicating an association with MCD (P < 10^-5). The mutant allele was present in the unrelated LR cohort at a frequency of 0.017, suggesting carrier and affection rates of 3.3% and 0.028%, respectively.
Conclusions A missense mutation in the CHST6 gene is strongly associated with autosomal recessive MCD in the LR.

Key Words: canine, CHST6, corneal, dystrophy, macular, MCD

INTRODUCTION

Macular corneal dystrophy (MCD; OMIM 217800) is an autosomal recessive inherited disorder that causes progressive reduced visual acuity in humans.1 Clinically, MCD is characterized by progressive bilateral diffuse clouding of the corneal stroma and the presence of grayish-white, punctate opacities especially in the superficial corneal stroma.1 Histologically, MCD is characterized by the accumulation of glycosaminoglycans (GAGs) within keratocytes and corneal epithelial cells, combined with extracellular deposition of similar material in the corneal stroma and Descemet’s membrane.1,2 An abnormality in the metabolism of keratan sulfate (KS) is implicated in the pathogenesis of MCD.3 In the cornea of patients with MCD, a decrease in corneal glucosamine N-acetyl-6-sulfotransferase (C-GlcNAc6ST) activity results in the formation of poorly sulfated and non-sulfated KS and causes corneal opacity, which in turn results in loss of visual acuity.4,5 Mutations in the carbohydrate sulfotransferase-6 (CHST6) gene encoding C-GlcNAc6ST have been associated with MCD in humans.1

Recently, several related Labrador Retriever (LR) dogs have been diagnosed with similar clinical and histological features to human MCD.6 The purpose of this study was to screen canine CHST6 to identify the underlying mutation in LR dogs affected with MCD, and to estimate the frequency of the mutation in the population of LR in the United Kingdom (UK).

MATERIAL AND METHODS

Animals and sample collection
The affected group included samples from dogs clinically diagnosed with MCD by veterinary ophthalmologists in the UK (n = 3), France (n = 1), Germany (n = 1), and
Finland \((n = 1)\). Clinical signs included multiple bilateral well-demarcated grayish-white punctate corneal opacities with or without corneal neovascularization (Fig. 1). The six affected dogs comprised males and females, with age at diagnosis ranging from 4.5 to 6 years, and histological confirmation of MCD available for three of them. The control group included samples from LR free of eye diseases when examined by veterinary ophthalmologists, and at least 5 years old. The control samples comprised four cohorts: one LR clinically clear of ocular disease; 71 LR of unknown clinical status; 79 LR free of eye diseases and included several dogs related to the affected LR; and 89 LR from the UK of unknown clinical status and unrelated at the parent level to the affected dogs. Control samples were donated by veterinary ophthalmologists, owners, and collaborators to the Animal Health Trust.

Blood samples were collected into EDTA tubes, and genomic DNA was extracted from the whole blood using a Nucleon Genomic DNA Extraction Kit (Tepnel Life Sciences, Manchester, UK) according to the manufacturer’s instructions. For samples collected as buccal swabs, DNA was extracted using a QIAamp® DNA Blood Midi Kit (Qiagen, Manchester, UK) using a protocol modified from the manufacturer’s instructions. All blood and/or buccal swab samples were taken with owner consent.

**CHST6 sequencing**

The exon–intron boundaries of canine CHST6 gene were defined by producing a CLUSTALW7 alignment of the Ensembl-predicted canine CHST6 transcript (ENSCAF00000031950) along with human (ENST00000332272). PRIMER38 was used to design four pair of primers to produce amplicons encompassing the entire coding region of the canine CHST6 gene (CFA5:75,278,935-75,280,136), as well as 900-bp upstream and downstream of the coding region, and two additional internal primers for sequencing, to ensure complete sequence coverage (Table 1).

The coding region was amplified by polymerase chain reaction (PCR) using HotStarTaq Plus DNA polymerase (Qiagen) in genomic DNA initially from two LR dogs (one affected dog and one control LR clinically clear of ocular disease). PCR products were purified using Multiscreen HTS-PCR filter plates (Millipore, Watford, UK). Amplification products were sequenced on an ABI 3130xl DNA Analyzer using BIGDYE TERMINATOR v3.1 (Applied Biosystems, Foster City, CA), and sequence traces were assembled, analyzed, and compared using the STADEN package.9 To confirm that the putative causal variant was not a common variant within the breed, the affected amplicon was amplified and sequenced, as described above, in an additional cohort of 71 control LR dogs with unknown clinical status.

Polyphen10 and SIFT11 were used for prediction of pathogenicity caused by the putative causal variant. A CLUSTALW7 alignment of CHST6 from multiple species (dog, human, horse, cat, cow, pig, mouse, rat, and rabbit) was generated to assess the level of conservation of the variant position.

**TaqMan assay**

Genotyping of the candidate disease-associated mutation was performed using an allelic discrimination assay

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**Table 1.** Primers used for PCR amplification and sequencing of the canine CHST6 gene

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>Amp</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>External primers used for PCR amplification and sequencing</td>
<td></td>
<td></td>
<td></td>
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<td>CHST6</td>
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<tr>
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<td>GGCCAGTACCTATCATCTTT</td>
<td>823 bp</td>
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<tr>
<td>Internal primers used for sequencing only</td>
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</tr>
<tr>
<td>CHST6</td>
<td>5</td>
<td>2</td>
<td>CAGGTGCTCTACTCGCTGCT</td>
<td>TCCATCAGGTAAGAGAGCTCG</td>
<td></td>
</tr>
</tbody>
</table>

Chr, chromosome; Amp, amplicon.
Mutations in the CHST6 gene are responsible for most cases of MCD in humans.\textsuperscript{1} Marked allelic heterogeneity has been documented in different populations throughout the world; more than 125 CHST6 mutations have been identified in humans with MCD.\textsuperscript{13–26} The most frequent abnormalities are missense or nonsense single nucleotide polymorphisms (SNPs) that alter a conserved part of the protein. Major deletions or insertions have also been reported.\textsuperscript{15} In the present study, all affected dogs with canine MCD were found to be homozygous for a missense SNP mutation in the canine CHST6 gene.

Carbohydrate sulfotransferases (CHST) are a family of enzymes that catalyze the sulfation of specific carbohydrates.\textsuperscript{27} Thirteen CHST enzymes have been identified in humans, including corneal, articular and intestinal, and all

CHST6 mutation causing MCD in Labrador Retrievers

Pedigree analysis
All six affected dogs with MCD were related: two dogs were half-siblings (second degree relatives), while the rest were more distantly related. A common ancestor was identified that was related to all six affected dogs within six to eleven generations. Twenty-eight relatives of the affected dogs were genotyped. They included the father of two affected dogs (\(n=1\), first degree relatives); a full sibling of one affected dog (\(n=1\), first degree relative); and several half-siblings of one or more affected dogs (\(n=5\), second degree relatives). The 21 remaining dogs were distant relatives of the affected dogs (\(n=21\), distant relatives). All first degree relatives (\(n=2\), 100\%), two-second degree relatives (\(n=2\), 40\%), and six distant relatives (\(n=6\), 28.6\%) were carriers for the mutation. Overall, the mutant allele was present in the related LR cohort at a frequency of 0.392, suggesting carrier and affection rates of 15.4\% and 47.7\%, respectively.

Population screening
A total of 89 LR dogs from the UK population and unrelated at the parent level with the affected LR dogs and with one another were screened to determine the mutant allele frequency. Of 89 dogs (178 chromosomes), three were carriers for the mutant allele, indicating that the frequency of the mutation was 0.017. Assuming the mutation to be in Hardy–Weinberg equilibrium within the UK LR population, we can extrapolate that 0.028\% LR will be affected and 3.3\% will carry the mutation.

RESULTS

Candidate gene sequencing
Comparison of the CHST6 sequence of one affected LR with one control LR revealed a total of nine variants. Only 2/9 variants were within the coding region of the CHST6 gene. Only one of these was predicted to be nonsynonymous: a missense mutation (c.814C>A), resulting in an arginine to a serine substitution (p.R272S). Polyphen and SIFT predicted this amino acid change to be pathogenic. Alignment of the CHST6 gene from multiple species revealed complete conservation at both nucleotide (c.814C) and amino acid (p.R272) level.

Further investigation of this CHST6 variant in the five additional affected LR revealed that all six dogs affected with MCD were homozygous for the mutant allele. Assessment of the 151 control dogs (72 sequenced and 79 assessed with TaqMan assay) revealed that 140/151 were homozygous for the wild-type allele and 11/151 were heterozygous for the mutant allele. A chi-squared test of the data revealed a statistical association of both the mutant allele and the homozygous mutant genotype with MCD (\(P < 10^{-7}\) for both).

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DISCUSSION

Macular corneal dystrophy in dogs has recently been reported\textsuperscript{6} and is clinically and histologically similar to MCD in humans.\textsuperscript{12} Using a candidate gene approach, we have identified a mutation (c.814C>A, p.R272S) in the canine CHST6 gene strongly associated with MCD in LR dogs.

Mutations in the CHST6 gene are responsible for most cases of MCD in humans.\textsuperscript{1} Marked allelic heterogeneity has been documented in different populations throughout the world; more than 125 CHST6 mutations have been identified in humans with MCD.\textsuperscript{13–26} The most frequent abnormalities are missense or nonsense single nucleotide polymorphisms (SNPs) that alter a conserved part of the protein. Major deletions or insertions have also been reported.\textsuperscript{15} In the present study, all affected dogs with canine MCD were found to be homozygous for a missense SNP mutation in the canine CHST6 gene.

Carbohydrate sulfotransferases (CHST) are a family of enzymes that catalyze the sulfation of specific carbohydrates.\textsuperscript{27} Thirteen CHST enzymes have been identified in humans, including corneal, articular and intestinal, and all
share highly conserved regions. The CHST6 gene encodes the corneal, membrane-bound protein (C-GlcNAc6ST), localized to the lumen of the Golgi of keratocytes. It is comprised of 395 amino acids and its function is to transfer N-acetylgalcosamine to KS glycosaminoglycans. C-GlcNAc6ST has a short cytosolic tail at the N-terminal, a single transmembrane span, and a C-terminal domain that contains the sulfate donor binding site, the catalytic domain and an area that determines carbohydrate specificity in vivo. Human patients with MCD have decreased C-GlcNAc6ST activity in their corneas.

The mutation reported in this study (c.814C>A) affects codon 272, which is in a region highly conserved across different CHST proteins and within C-GlcNAc6ST across different species. More specifically, codon 272 of C-GlcNAc6ST is conserved in all seven species examined. The reported SNP mutation causes a change in codon 272 from arginine to serine. Arginine is a basic amino acid and serine is uncharged, and subsequently, this mutation is predicted to cause a conformational change in the C-GlcNAc6ST protein. Interestingly, an identical amino acid change (c.814C>A, p.R272S) has been reported to be associated with MCD in humans; furthermore, other mutations in the same codon have also been reported to be associated with MCD.

The precise organization of the corneal stroma is the most important factor in maintaining corneal clarity. This organization involves the integration of collagen fibrils with selected proteoglycans, such as KS. In the normal corneal tissue, KS exists as a highly sulfated form, and C-GlcNAc6ST transfers sulfate groups to KS. Sulfation of carbohydrates affects the biochemical characteristics of GAGs, such as water solubility and electrical charge. Musselmann et al. reported that variants in the coding region of CHST6 gene decrease the activity of the enzyme leading to a low sulfated form or non-sulfated form of KS. Due to the subsequent reduction in solubility, non-sulfated KS is unable to be completely metabolized, inducing sediment to deposit in the corneal stroma, resulting in the corneal opacity observed clinically.

We genotyped a very modest number (n = 89) of LR from the UK that had been collected for a different parallel study, in an attempt to obtain an approximate estimate of the frequency of the mutation and also to demonstrate the mutation is not a common variant within the LR population. The frequency of the mutation in this limited subset of LR was 0.017. However, the reliability of this estimate is limited due to the very small number of dogs and we were able to analyze from a numerically very large breed. The LR breed consists of different and reproductively isolated lines, including ‘show/confirmation’ lines and ‘working/field trial’ lines, and we suggest that a more comprehensive study would be required to obtain an accurate estimate of the mutation frequency across the different lines of LR. All MCD-affected dogs in this study were reported to be from ‘show/conformation’ lines. It was not possible to determine which lines our 89 unrelated dogs belonged to.

Pedigree analysis and the observation that only dogs homozygous for the CHST6 mutation have MCD are consistent with the disease having an autosomal recessive mode of inheritance. All of the affected LR dogs were related to one another within approximately 10 generations, suggesting the CHST6 mutation may be largely confined to one or a few breeding lines.

CONCLUSIONS

In our study, we have identified a missense mutation in the CHST6 gene (c.814C>A, p.R272S) that is likely the cause of MCD in LR dogs. As a result, a DNA test can be used to eliminate this form of MCD from the breed entirely. In addition, this establishes canine MCD as a large animal model for human MCD, which will enable future investigations into the disease pathogenesis and development of potential therapies.

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REFERENCES


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