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Ligneous membranitis in Scottish Terriers is associated with a single nucleotide polymorphism in the plasminogen (PLG) gene

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Summary

Ligneous membranitis (LM) is a rare chronic inflammatory condition of the mucous membranes associated with plasminogen (encoded by PLG) deficiency in affected humans and dogs. In human, the condition is genetic in nature with numerous mutations and polymorphisms in PLG identified in affected individuals and related family members. The condition is uncommonly reported in dogs and, to date, no genetic studies have been performed. We identified related Scottish Terriers (littermates) with severe LM and unaffected relatives ( sire, dam and a sibling from a previous litter). Plasma plasminogen activity was below normal in one affected dog but within normal reference intervals for the other. Sequencing of PLG from the affected dogs revealed a homozygous A>T single nucleotide polymorphism in an intron donor site (c.1256+2T>A). The related, unaffected dogs displayed heterozygous alleles at this position (c.1256+2T/A), whereas no mutation was detected in unaffected, non-related control dogs. This is the first report to identify gene polymorphisms associated with LM in dogs.

Keywords chronic inflammation, fibrinous lesions, hypoplasminogenemia, intron donor site, mucous membranes

Plasminogen has an important role in many physiological processes including embryogenesis, wound healing, fibrinolysis, cell migration and angiogenesis (Drew et al. 1998; Cesarman-Maus & Hajjar 2005; Miles et al. 2014). Ligneous membranitis (LM) is a rare chronic inflammatory disease associated with congenital plasminogen deficiency. The inherited condition is well described in humans (Mingers et al. 1999; Schuster et al. 2001, 2007; Tefs et al. 2006; Rodriguez-Ares et al. 2007); patients commonly present as infants, with ocular, oral and genital lesions (Schott et al. 1998; Tefs et al. 2006; Zare et al. 2010). Patients with LM are frequently found to harbour homozygous or compound heterozygous mutations within the plasminogen gene, PLG, including nonsense, splice-site and frame-shift mutations (Tefs et al. 2003, 2004, 2006; Rodriguez-Ares et al. 2007; Schuster et al. 2007).

Ligneous membranitis has been reported in only six unrelated dogs to date (Ramsey et al. 1995; McLean et al. 2008; Torres et al. 2009). The clinical presentation is similar to that in humans, and low plasma plasminogen activity has been documented in two affected dogs (McLean et al. 2008; Torres et al. 2009).

In this study, two related Scottish Terriers (littermates, one male and one female) presented with biopsy-confirmed LM. Both developed severe proliferative and ulcerative conjunctivitis and gingivitis/stomatitis by 2 months of age. Other clinical signs included increased upper respiratory tract noise, nasal discharge and lymphadenopathy. Clinical pathological findings included neutrophilia, proteinuria and hypoalbuminaemia. No significant clinical improvement was seen despite supportive treatment, and the affected dogs were euthanised due to progressive clinical signs. Post-mortem evaluation of the euthanised dogs revealed multiple abnormalities including severe proliferative fibrinous lesions affecting the trachea, larynx and epicardium and multiple fibrous adhesions throughout the thoracic and abdominal cavities. Clinical signs in the affected Scottish Terriers were similar to those reported previously in a Golden Retriever with LM (McLean et al. 2008) but more severe than those reported in affected...
Dobermanns (Ramsey et al. 1995) and a Yorkshire Terrier (Torres et al. 2009). Hence, this condition may display clinical heterogeneity, which may be breed or family related. A juvenile dog from an earlier litter to the same parents also had been diagnosed with LM and also was euthanised (samples not available for this study).

To further investigate the nature of LM in Scottish Terriers, blood samples were obtained for sequencing of PLG and to determine plasminogen activity. Samples were available for two of three affected dogs reported in this study; three related, unaffected dogs (which consisted of the sire, dam and an adult dog which was the offspring of the same parents from an earlier litter) and four healthy control Scottish Terriers, unrelated to the affected, which were presenting for signs unrelated to LM. Ethical approval was granted by the institute’s ethics committee, and informed consent was obtained from the owners of all dogs included in the study.

Blood was collected via standard venepuncture techniques. Whole blood in EDTA for DNA extraction was stored at −20°C, and citrated plasma for plasminogen assay was immediately separated and frozen at −20°C. Plasminogen assays were performed at the Animal Health Diagnostic Centre, Cornell University, USA. Plasminogen activity was measured by a standard chromatogenic assay validated for dogs (Weltes et al. 1990; Lanevschi et al. 1996). Total genomic DNA was isolated from blood samples using a DNeasy Blood & Tissue Kit (Qiagen) as per the manufacturer’s instructions.

Thirteen sets of primers (Table S1) to amplify the 19 identified PLG exons (Petersen et al. 1990) were designed using the PLG nucleotide sequence (GenBank accession no. NC_006583.3) from Canis lupus familiaris (Lindblad-Toh et al. 2005) as a template. The majority of PCR products were generated using high-fidelity KOD DNA polymerase (Invitrogen) with added 1M betaine as required. Where indicated, persistently difficult PCRs were performed with Phusion High Fidelity DNA polymerase (Thermo Scientific). All amplicons were sequenced at least twice from two independent PCRs (performed by Beckman Coulter Genomics). Reads were assembled and mutations identified using DNA BASER SEQUENCE ASSEMBLER v4.13.0 (Heracle BioSoft SRL; www.DnaBaser.com). Mutations were confirmed by further two sequencing reactions from independent PCRs. Sequences encompassing the identified mutation were submitted to GenBank under the following accession numbers: Affected 1 (KP853099), Affected 2 (KP853100), Sire (KP853101), Dam (KP853102), R-control (KP853103), NR-Control 1 (KP853104), NR-Control 2 (KP853105), NR-Control 3 (KP853106), NR-Control 4 (KP853107).

Sequencing of all 19 PLG exons and flanking sequences from nine Scottish Terriers (two affected dogs, sire, dam, a related non-affected control and four unrelated, non-affected controls) found no mutations within any coding regions of PLG. However, the affected dogs demonstrated a single nucleotide change within an intron donor site downstream of exon 10 (c.1256+2T>A) compared to the reference sequence (GenBank accession no.: NC_006583.3). The sire, dam and related control displayed heterozygous alleles (c.1256+2T/A) at this position. All unrelated, non-affected controls displayed wild-type (wt) PLG sequences. A further 23 healthy Scottish Terriers were investigated, and all had a wt genotype at this position (data not shown). Similar splice-site mutations have previously been noted in affected humans with LM (Schuster et al. 2007). The non-carriage of c.1256+2T/A in 24 normal Scottish Terriers indicates that carriers of the mutation are generally not common in this breed.

The identified c.1256+2T>A genotype in affected Scottish Terriers is predicted to adversely affect translation of PLG mRNA downstream of exon 10 and may result in a potential truncation of PLG from amino acid position 419. This would presumably lead to the loss of two kringle domains and the proteolytic trypsin domain, thereby severely affecting PLG functionality (Novokhatny et al. 1984; Law et al. 2012, 2013).

Serum plasminogen activity was determined for the affected dogs, their relatives and healthy controls. Plasminogen activity (reference interval 70–140%) was low (45%) in one affected dog and within reference intervals (95%) in the other (Table 1). Although normal serum plasminogen activity in people with LM has been reported (Naudí et al. 2006; Fuentes-Pérez et al. 2008), the literature for humans supports hypoplasminogenemia as the most likely cause of LM (Schuster et al. 2007). Therefore, it is difficult to explain the apparently normal plasminogen activity in the affected male. This result may reflect inaccuracies in the assay or the canine reference interval:

| Dog | Sex | Age | Status | Plasminogen % (RI: 60–170) | Genotype |
|-----|-----|-----|--------|--------------------------|----------|
| Affected 1 | M | Puppy | Affected | 95 | c.1256+2T>A |
| Affected 2 | F | Puppy | Affected | 49 | c.1256+2T>A |
| Sire | M | Adult | Healthy | 124 | c.1256+2T/A |
| Dam | F | Adult | Healthy | 111 | c.1256+2T/A |
| R-Control | F | Adult | Healthy | 49 | c.1256+2T/A |
| NR-Control 1 | F | Adult | Healthy | 76 | WT |
| NR-Control 2 | F | Adult | Healthy | 101 | WT |
| NR-Control 3 | F | Adult | Healthy | 108 | WT |
| NR-Control 4 | M | Adult | Healthy | 100 | WT |

M, male; F, female; R, related; NR, non-related; RI, reference interval.
however, we were unable to re-assess this as the patient died. Surprisingly, the non-affected adult relative with a c.1256+2T/A genotype displayed low plasminogen activity. A repeat assay, to ascertain whether this result was reproducible, was performed and provided a similar low activity result (Table 1). This dog has no history or clinical signs of LM. Hypoplasminogenemia without the presence of clinical signs has previously been reported in healthy relatives of humans with LM (Tefs et al. 2006). The dam and sire of the affected dogs and all control dogs all had plasminogen activity within reference intervals.

As in some cases of LM in humans, the link between clinical LM and PLG genotype may not be consistently clear in canines. It is possible that plasminogen may be only one factor involved in the clinical syndrome and that the identified PLG mutation is not enough alone to cause clinical signs of LM. This also suggests the potential for other abnormalities in proteins in the fibrinolytic pathway and/or that an environmental factor is involved in development of the condition (Fuentes-Paz et al. 2008). Given the possibility of a truncated plasminogen protein that lacks proteolytic activity in affected individuals, it is possible that other proteins are able to compensate for functions which may also be abnormal in affected patients.

In conclusion, this is the first report of a genetic alteration in a family of dogs with LM, and our findings of a mutation in PLG are similar to data from families of affected humans. A screening programme via PCR and sequencing of the region harbouring the identified SNP in Scottish Terriers could be used for definitive diagnosis of the condition in affected animals and determination of carriage rates in other dog breeds.

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**Supporting information**

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

**Table S1** Primers used to amplify PLG from DNA isolated from the blood of Scottish Terriers and a Boxer.