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

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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; Rodríguez-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; Rodríguez-Ares *et al.* 2007; Schuster *et al.* 2007).

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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 (Welles *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 1 M 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 (KP853 103), NR-Control 1 (KP853104), NR-Control 2 (KP853 105), NR-Control 3 (KP853106), NR-Control 4 (KP85 3107).

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_0065 83.3). 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 (Naudi *et al.* 2006; Fuentes-Páez *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;

Table 1 Patient details, plasma plasminogen activity and detected PLG mutations in Scottish Terriers.

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 and 56	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.

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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-Páez *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 provide compensating 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.