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SHORT COMMUNICATION

STK36 splice site variant in an Australian Shepherd dog with primary ciliary dyskinesia

Abstract

Primary ciliary dyskinesia (PCD) represents a group of diseases characterized by impaired movement of cilia and subsequent health problems in diverse organ systems, notably the respiratory tract. Almost 50 candidate genes for PCD are known in humans. In this study, we investigated an Australian Shepherd dog with a history of recurrent respiratory infections and nasal discharge. A transmission electron microscopy investigation led to the diagnosis of PCD with central pair defect, in which the normal 9:2 arrangement of respiratory cilia was altered and reduced to a 9:0 arrangement. Whole genome sequencing data from the affected dog was obtained and searched for variants in PCD candidate genes that were not present in 918 control genomes from different breeds. This revealed a homozygous single base pair exchange at a splice site of *STK36*, XM_038585732.1:c.2868-1G>A. The mutant allele was absent from 281 additionally genotyped Australian Shepherd dogs. RT-PCR confirmed aberrant splicing in the affected dog with the skipping of exon 20 and the insertion of a cryptic exon, which is predicted to lead to a premature stop codon and truncation of 36% of the *STK36* wild-type open reading frame, XP_038441660.1:(p.Met957Profs*11). *STK36* variants were previously reported to cause PCD in humans and mice. The knowledge from other species together with the absence of the mutant allele in more than 1000 control dogs suggests *STK36*:c.2868-1G>A as the most likely candidate variant for PCD in the investigated case.

Primary ciliary dyskinesia (PCD) belongs to the genetically heterogeneous group of inherited ciliopathies (Horani & Ferkol, 2021). In human patients, variants in almost 50 known PCD candidate genes lead to impaired motile cilia function and consequently to impaired clearance of mucus, bacteria or foreign bodies on respiratory epithelia (OMIM PS #244400) (Afzelius, 1976; Horani & Ferkol, 2021; Magnin et al., 2012). As a result, affected children suffer from recurrent airway infections from an early age (Magnin et al., 2012). As cilia are additionally responsible for body lateralization during embryogenesis, around 50% of patients also show *situs inversus* (Essner et al., 2002).

In domestic animals, molecular studies of PCD cases are rarely performed. The absence of *CCDC39* in Old English Sheepdogs leads to incorrect assembly of inner dynein arm complexes, causing axonemal disorganization and dyskinetic beating (OMIA #001540–9615) (Merveille et al., 2011). Further, a *NME5* frameshift variant was identified in Alaskan Malamutes with PCD and hydrocephalus (OMIA #002206–9615) (Anderegg et al., 2019). Finally, a *CCDC65* nonsense variant was associated with respiratory failure and early death in Lacaune dairy sheep (OMIA #002342–9940) (Ben et al., 2021).

In this study, we examined a male Australian Shepherd dog with a 6-year history of recurrent rhinitis and nasal discharge. According to the breeder, the parents were healthy. The dog was presented for the first time to a veterinarian at 10 weeks of age. At the time, the owner reported a 2-week history of sneezing and yellow-greenish nasal discharge. The clinical signs improved after treatment with broad-spectrum antibiotics and corticosteroids. However, the dog relapsed several times after discontinuation of the treatment and developed multiple episodes of infectious rhinitis with diverse bacterial origins. Testing for lungworms was negative and blood counts were always within normal limits. Further diagnostic testing included routine histopathology of nasal biopsies and a computed tomography that did not help to refine the diagnosis. The dog was treated thereafter with different strategies over time, including oral prednisolone treatment for suspected allergic rhinitis, or a combination of acetylcysteine and oclacitinib to alleviate the clinical signs. At the time of writing, the dog is receiving inhalation therapy with hypertonic saline twice a day; however, clear to milky nasal discharge persists.

Biopsies of nasal and tracheal mucosa were taken for transmission electron microscopy (TEM) analysis. Semi- and ultrathin sections of nasal mucosa revealed

a predominantly regular ciliary arrangement of variable density with partly irregular arrangement of basal bodies. Cilia were of equal length and width, with single compound cilia observed. Frequently cilia with 9:0 tubuli arrangement as well as cilia with indistinct/irregular central tubule pairs were present (Figure 1).

As a genetic etiology was suspected, an EDTA blood sample of the case was taken for DNA isolation and subsequent whole genome sequencing. No samples of parents or littermates were available for analysis. An Illumina TruSeq PCR-free DNA library with ~400 bp insert size was prepared from the affected dog. We collected 172 million 2×150 bp paired-end reads on a NovaSeq 6000 instrument (17.8× coverage). Mapping and alignment to the UU_Cfam_GSD_1.0 (canFam4) reference genome assembly were performed as described (Jagannathan et al., 2019). Filtering for private variants by comparing the sequence data of the affected dog against 918 control genomes from dogs of different breeds yielded 11 homozygous and 51 heterozygous protein-changing variants (Tables S1, S2). We prioritized variants in 47 known PCD candidate genes obtained from the OMIM phenotypic series PS244400. Only one private protein-changing variant was found in a gene previously associated with PCD. This candidate causative variant, XM_038585732.1:c.2868-1G>A, is located at a splice site of the *STK36* gene. It can be designated as Chr37:25167072:G>A (UU_Cfam_GSD_1.0 assembly). We confirmed the presence of the intronic *STK36* variant by Sanger sequencing (Figure 2a, Table S3). Only the case showed a homozygous alternative genotype for this variant, while 281 control Australian Shepherd dogs from the Vetsuisse Biobank were homozygous for the wild-type allele. As the genomic variant affected the canonical AG-dinucleotide at the 3′-splice site of intron 19, we experimentally assessed the consequences on splicing at the transcript level. RNA was extracted from whole blood samples of the case and six unaffected Australian Shepherd dogs using PAXgene RNA tubes. The RNA samples were reverse transcribed into cDNA prior to PCR amplification. RT-PCR bands confirmed the presence of an aberrantly spliced *STK36* transcript in the affected dog, which was subsequently analyzed by Sanger Sequencing (Table S3). While exon 20 comprising 64 bp was skipped, 40 bp of sequence derived from the downstream intron and flanked by cryptic splice sites were included into the aberrant transcript, XM_038585732.1:r.2868_2931delinsCCCTTCTGGAGAACAGACATCACTGTGTCATTAGCAGCAG (Figure 2b). This aberrant splicing event on the mRNA level results in the insertion of a premature stop codon, which leads to truncation of 36% of the wild-type *STK36* open reading frame, XP_038441660.1:(p.Met957Profs*11). *STK36* encodes a serine/threonine protein kinase homologous to the *Drosophila* fused (fu) protein that is essential for the formation of the central pair in motile cilia (Wilson et al., 2009).

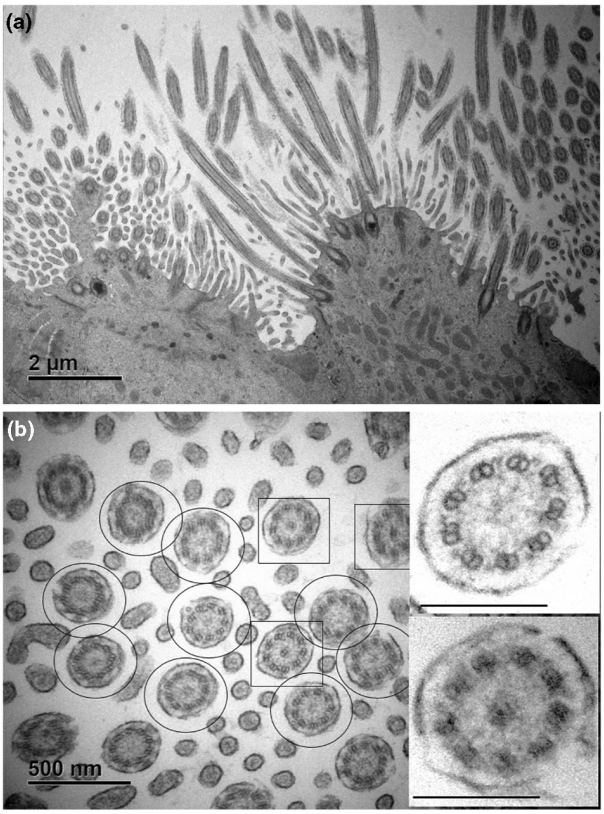

FIGURE 1 Transmission electron microscopy (TEM) findings in the affected Australian Shepherd dog. (a) Regular, but less dense ciliary arrangement in specimens of nasal mucosa with cilia of equal length and width. (b) Cross-sections of cilia with 9:0 tubuli arrangement (circles/top insert) and cilia with indistinct/irregular central tubule pairs (squares/bottom insert). Bar inserts = 200 nm.

In humans, just one PCD patient with an *STK36* variant has been described (Edelbusch et al., 2017). The human patient was homozygous for a single base deletion, leading to a frameshift and premature stop codon, a variant of similar impact to the one discovered herein. Upon TEM of ciliary cross-sections from patient respiratory epithelial cells, abnormal axonemal composition with a 9:0 or 8:0 architecture was found in the human patient (Edelbusch et al., 2017), which strongly resembles the TEM findings of the dog investigated in this study.

Stk36$^{-/-}$ mice also develop a PCD phenotype including respiratory infections and hydrocephalus attributed to loss of function of motile cilia in ependymal cells resulting in altered cerebrospinal flow and altered neurodevelopment (Duy et al., 2022; Merchant et al., 2005). No MRI was done in our case to evaluate for a possible subclinical hydrocephalus. The ciliary dysfunction in *Stk36*$^{-/-}$ mice causes infertility (Merchant et al., 2005). It would have been interesting to investigate sperm motility in our case, but unfortunately, the dog had already been neutered at the time of investigation.

Overall, the high clinical and ultrastructural similarities between a published human patient, *Stk36*$^{-/-}$ knockout mice and the affected Australian Shepherd

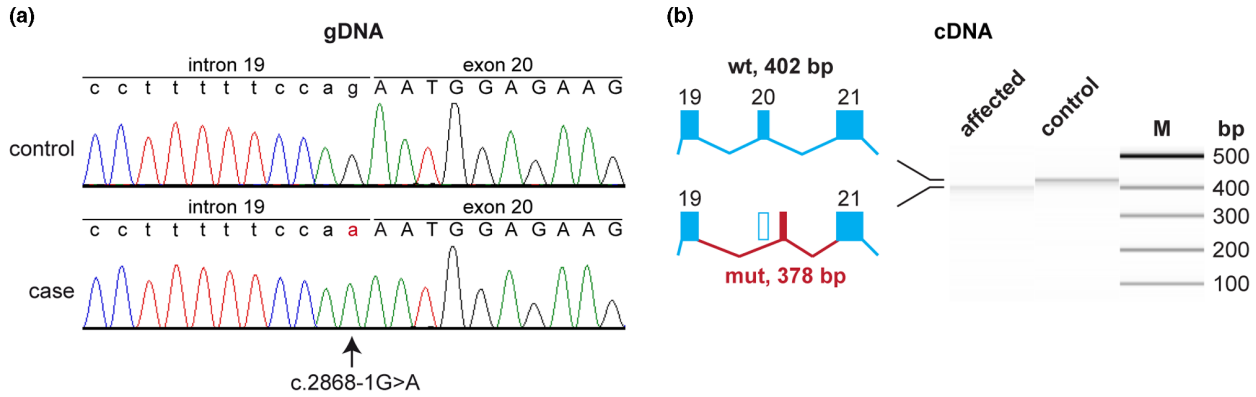

FIGURE 2 Details of the *STK36*:c.2868-1G>A variant. (a) Sanger sequencing electropherograms derived from genomic amplicons of a healthy control and the PCD-affected dog show the base exchange at the last base of intron 19. The variable position is indicated by an arrow, with the altered base marked in red. (b) RT-PCR products obtained from the affected dog and a healthy control. The samples were analysed on a Fragment Analyzer capillary gel electrophoresis instrument. Band sizes correspond to the lengths of the alternatively spliced cryptic exon sequence from intron 20 vs. the normal exon 20.

dog together with the absence of the mutant allele from >900 control dogs of different breeds and an additional 281 Australian Shepherd dog controls imply that *STK36*:c.2868-1G>A is a reasonable candidate causative variant for PCD in the investigated dog. To the best of our knowledge, this is the first report of an *STK36*-associated PCD in domestic animals.

KEYWORDS
animal model, *Canis lupus familiaris*, lung, precision medicine, respiratory disease, splicing

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CONFLICT OF INTEREST
The authors declare no conflicts of interests.

FUNDING INFORMATION
This study received no external funding.

ETHICS STATEMENT
All dogs in this study were privately owned and samples were collected with the consent of their owners. The collection of blood samples from control dogs was approved by the 'Cantonal Committee For Animal Experiments' (Canton of Bern; permit 71/19; approval date 9 September 2019). The collection of samples from the affected dog was performed for diagnostic or therapeutic reasons and did not constitute an animal experiment in the legal sense.

DATA AVAILABILITY STATEMENT
All data are freely available. Accessions for the whole genome sequence data are given in Table S1.

Matthias Christen[1]
Odette Ludwig-Peisker[1]
Vidhya Jagannathan[1]
Udo Hetzel[2]
Ulrike Schönball[3]
Tosso Leeb[1]

[1]*Institute of Genetics, Vetsuisse Faculty, University of Bern, Bern, Switzerland*
[2]*Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland*
[3]*Kleintierpraxis Dr. Ulrike Schönball, Meldorf, Germany*

Correspondence
Tosso Leeb, Institute of Genetics, Vetsuisse Faculty, University of Bern, Bremgartenstrasse 109a, 3001 Bern, Switzerland.
Email: tosso.leeb@unibe.ch

Matthias Christen and Odette Ludwig-Peisker contributed equally (shared first authors).

ORCID
Matthias Christen https://orcid.org/0000-0003-3275-5929
Odette Ludwig-Peisker https://orcid.org/0000-0003-0691-1211
Vidhya Jagannathan https://orcid.org/0000-0002-8155-0041
Udo Hetzel https://orcid.org/0000-0001-9142-560X

Ulrike Schönball https://orcid.org/0000-0003-3738-3589
Tosso Leeb https://orcid.org/0000-0003-0553-4880

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.