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Progressive retinal atrophy in Shetland sheepdog is associated with a mutation in the *CNGA1* gene

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Summary

Progressive retinal atrophy (PRA) is the collective name of a class of hereditary retinal dystrophies in the dog and is often described as the equivalent of retinitis pigmentosa in humans. PRA is characterized by visual impairment due to degeneration of the photoreceptors in the retina, usually leading to blindness. PRA has been reported in dogs from more than 100 breeds and can be genetically heterogeneous both between and within breeds. The disease can be subdivided by age at onset and rate of progression. Using genome-wide association with 15 Shetland Sheepdog (Sheltie) cases and 14 controls, we identified a novel PRA locus on CFA13 ($P_{raw} = 8.55 \times 10^{-7}$, $P_{genome} = 1.7 \times 10^{-4}$). *CNGA1*, which is known to be involved in human cases of retinitis pigmentosa, was located within the associated region and was considered a likely candidate gene. Sequencing of this gene identified a 4-bp deletion in exon 9 (c.1752_1755delAACT), leading to a frameshift and a premature stop codon. The study indicated genetic heterogeneity as the mutation was present in all PRA-affected individuals in one large family of Shelties, whereas some other cases in the studied Sheltie population were not associated with this *CNGA1* mutation. To our knowledge, this is the first report of a mutation in *CNGA1* causing PRA in dogs.

Keywords canine, PRA, rod–cone degeneration, Sheltie

Introduction

Retinal dystrophies are a common cause of blindness in purebred dogs. Progressive retinal atrophy (PRA) is a large group of progressive, hereditary, retinal degenerations causing visual impairment and eventually blindness in affected dogs. PRA is often considered the canine equivalent of retinitis pigmentosa (RP) in human beings (Petersen-Jones 1998; Aguirre & Acland 2006). The initial changes in classical PRA are degeneration and loss of rod photoreceptor responses leading to night blindness, followed by a slower loss of cone photoreceptor responses and progressive deterioration of daytime vision.

PRA has been documented in more than 100 dog breeds (Whitley 1988). The age at onset and the rate of progression vary both between and within the different forms of PRA. At least 22 mutations in 19 genes have been identified to be associated with different forms of PRA in more than 50 breeds (Miyadera *et al.* 2012; Winkler *et al.* 2013; Downs *et al.* 2014). The mutations causing PRA in several breeds of dogs remain uncharacterized.

In humans, hereditary retinal degenerations are found in approximately one in 3500 individuals (Hartong *et al.* 2006; Hamel 2007). To date, 192 genes have been identified to be involved in a wide spectrum of retinal degenerations in humans (Daiger *et al.* 2014).

In the Shetland Sheepdog (Sheltie), the only form of retinal disease for which an associated mutation has been identified is Collie eye anomaly (CEA) (Parker *et al.* 2007). Traditionally, CEA has been identified by veterinary ophthalmologists in Sheltie pups 5–10 weeks of age. Screening of adult Shelties has also been recommended in some countries as a result of observations of adult-onset PRA in the breed. PRA in the Sheltie is clinically indistinguishable from PRA in other breeds and shows a wide range in age of onset. The PRA-affected dogs in this study were diagnosed between the ages of 2 and 11 years, with an average of

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5 years. Most of the investigated dogs had their first eye examination at an older age, which means that the age at diagnosis would not reflect the age of onset. Yet another form of retinal degeneration, slowly progressive retinopathy (SPR), has been described in this breed (Karlstam *et al.* 2011). SPR mimics the early stages of PRA ophthalmoscopically, but the progression is considerably slower and reasonable vision is routinely preserved, even in Shelties over 10 years of age. Pedigree information suggests that the modes of inheritance of both PRA and the SPR in the Sheltie are compatible with autosomal recessive inheritance.

Here, we report the identification of a 4-bp exonic deletion in *CNGA1*, *cyclic nucleotide gated channel alpha 1*, a gene whose protein is expressed in the outer segment of rod photoreceptors in the retina (Dhallan *et al.* 1992). The mutation causes a frameshift, resulting in a premature termination codon. We present data supporting that this mutation is responsible for one form of PRA in Shelties. Our results indicate that PRA is genetically heterogeneous in Shelties and that SPR is not genetically linked to the PRA cases.

Materials and methods

Sample processing

Dogs participating in this study included 200 privately owned Shelties from Norway, Sweden, Denmark and Finland. The dogs had venous blood samples drawn that were collected with the owners' consent during 2000–2013. The samples were obtained from 150 ophthalmoscopically normal dogs, 27 dogs diagnosed with PRA, and 23 diagnosed with SPR. Examinations of and blood sampling from all dogs included in the study were performed in accordance with the legislation in the country concerned and adhered to the guidelines from the Association for Research in Vision and Ophthalmology for use of animals in research. The diagnoses were established by veterinary ophthalmologists who were accredited as national eye panelists. Dogs were diagnosed with PRA upon ophthalmoscopic observation of abnormalities of the retina that included bilateral symmetric pale optic nerve heads, severely attenuated retinal vessels and tapetal hyperreflectivity in addition to clinical observations of visual deficiencies under dark adapted (scotopic) conditions. Dogs were considered to be affected by SPR if they showed bilateral, symmetrical, grayish hyporeflectivity mainly in the periphery of the tapetum but later extending more centrally in the tapetum. Hyperreflection in a substantial part of the tapetal fundus was never observed upon ophthalmoscopic examination in SPR-affected dogs, and neither was obvious pigment migration in the non-tapetal fundus. The size of the retinal vessels varied from normal to only slightly attenuated. Electroretinography (ERG) of the SPR-affected dogs showed either normal or subnormal rod and cone responses but never a complete loss of either rod or cone responses. The extremely slow progression at follow-up examinations, lack of obvious tapetal hyperreflectivity even in old dogs, preserved rod and cone responses of the ERG and relatively normal daylight and dim-light vision in older dogs served as criteria for distinguishing SPR from PRA, although SPR mimics early-stage 'classic PRA' at a single, ophthalmoscopic examination.

Control dogs for the PRA analysis were 6 years of age or older upon ophthalmic examination and showed no signs of any hereditary eye diseases upon ophthalmoscopic and biomicroscopic examination after dilation of the pupils with tropicamide (Mydriacyl; Alcon). The blood samples were collected in EDTA tubes, and genomic DNA was extracted using the E.Z.N.A. Blood DNA mini kit (Omega Bio-Tek), according to the manufacturer's instructions.

SNP genotyping and quality control

The initial dataset consisted of 82 individuals that included 24 PRA cases, 18 retinopathy cases and 40 controls genotyped using the Illumina 170K CanineHD BeadChip. GENABEL package version 1.8.0 (Aulchenko *et al.* 2007), which is a statistical tool implemented in R statistical software (version 3.1.0) (Ihaka & Gentleman 1996), was used for the genome-wide association study (GWAS). Prior to the principal GWAS, we performed iterative quality control (QC) to remove poorly genotyped and noisy data. Non-informative markers (minor allele frequency below 5%), markers having low call rate (below 0.95) and markers out of Hardy–Weinberg equilibrium [first $P < 1 \times 10^{-8}$ and then FDR (false discovery rate) <0.2 (excess autosomal heterozygosity)] were removed from the analysis. Of the initial 173 731 SNP markers, 95 618 (55.04%) markers were included in all analyses. Two individuals (one case and one control) were excluded from the analysis due to exceptionally high identity by state (IBS > 0.95), leaving a total of 80 dogs for further analysis.

Genome-wide association analysis

To examine the population structure, an autosomal genomic kinship matrix was computed. Multidimensional scaling plots were constructed to visualize population structure. Standard *K*-means clustering was computed, and this showed a structure of three subpopulations within the genotyped population (Fig. S1). First, we performed an association analysis in 80 individuals, with 23 PRA- and 18 SPR-affected dogs as cases (total 41) and 39 controls. Due to the clustering, we next performed an association analysis in each of the subpopulations. Finally, we excluded the entire subpopulation 2 containing all SPR cases and performed an association analysis on 15 PRA cases and 14 controls. Genome-wide significance was ascertained by permutation testing ($n = 100\,000$). Manual analysis of SNP genotypes across the region was performed to define a homozygous critical region.

Gene sequencing

Primers for amplification and sequencing of exons of the *CNGA1* gene (NM_001003222) were designed with PRIMER3 (Rozen & Skaletsky 2000) in the introns and exons of genomic DNA (Table S1). The transcribed regions of the gene were sequenced in eight Shelties included in the GWAS, including three PRA cases, three retinopathy cases and two controls. All nine exons of *CNGA1* were amplified by polymerase chain reaction (PCR) using Taq polymerase (Ampliqon) in genomic DNA. Amplification products were sequenced with standard Sanger sequencing on a 3500 × L Genetic Analyzer using Big Dye Terminator v3.1 (Applied Biosystems, Life Technologies). Sequence traces were assembled, analyzed and compared using SEQUENCHER (Gene Codes).

Screening of the detected 4-bp deletion

A PCR primer pair using fluorescent primers (forward: 5′-6-FAM-TCGGCGAGATCAGTATCCTT-3′ and reverse: 5′-CAT TCGGGTGACCTTCTCTT-3′; Eurofins MWG Operon) was designed to further investigate the single exonic sequence variant segregating with the PRA cases. Fragment length polymorphism detection was performed using a 3500 × L Genetic Analyzer and GENEMAPPER® Software (Applied Biosystems, Life Technologies). The entire cohort of Shelties collected ($n = 200$), including 27 PRA cases, 23 retinopathy cases and 150 controls, were screened for the mutation.

Population screening

Additionally, 250 randomly picked Shelties (Norwegian) and 100 randomly picked longhaired Collies (Norwegian) with unknown clinical status were selected for allele frequency investigation and genotyped for the *CNGA1* mutation.

Results

A single region on chromosome 13 is associated with PRA

We initially hypothesized that PRA and SPR were caused by one mutation and performed a GWAS on a dataset of 80 dogs that included 23 PRA cases, 18 retinopathy cases (in total 41 cases) and 39 controls. By applying multidimensional scaling analysis, we observed that the Sheltie study population clearly clustered into three subpopulations (Fig. S1). This initial GWAS revealed a region on chromosome 13 as a possible candidate region ($P_{genome} = 0.15082$ and inflation factor $\lambda = 0.9$).

Due to the clustering and two potentially different phenotypes, we chose to look at each subpopulation separately. Subpopulations 1 and 3, the two subpopulations with most of the PRA cases, both had a common peak at CFA13. Subpopulation 3, with 12 PRA cases and nine controls, had the best values of both $P_{genome} = 0.0025$ and $\lambda = 0.93$. Subpopulation 2, which contained all of the SPR cases, had no peak on CFA13 and therefore was removed from the final analysis.

After removing individuals based on quality assurance and membership in subpopulation 2, 15 PRA cases and 14 controls remained for the final association analysis. GWA analysis was performed on the 29 dogs, and 95 618 SNPs remained for the final analysis after removing markers that did not meet the inclusion criteria defined previously. An association test was performed with inflation factor $\lambda = 1.02$, showing no stratification. The strongest observed signal on chromosome 13, $P_{raw} = 8.55 \times 10^{-7}$ (Fig. 1a) was statistically significant with $P_{genome} = 1.7 \times 10^{-4}$ using 100 000 random phenotype permutations (Fig. 1b). The most significantly associated SNP (*BICF2P405937*) was located at 36 666 622 bp on CanFam 3.1. A total of 62 SNPs on chromosome 13 demonstrated $P_{genome} < 0.05$ (following 100 000 permutations) and the region of strongest statistical association between 33.993 Mb and 46.843 Mb on CFA13 (Fig. 1c). Using homozygosity mapping, we identified a critical region of 7.419 Mb, from 36.868 Mb to 44.287 Mb (Fig. 2).

Candidate gene sequencing

There are 140 genes located within the PRA critical region (CFA13:36.868–44.287 Mb). One of the genes, *CNGA1* (CFA13:43 831 107–43 845 271, CanFam 3.1), is associated with RP in humans and stands out as an excellent candidate gene. All nine exons and splice sites were sequenced in three PRA cases, three retinopathy cases and two control dogs (>6 years of age, all part of the GWAS). A comparison of the sequence data in cases and controls and with the canine reference genome sequence (CanFam 3.1) revealed a deletion of four bases c.1752_1755delAACT in exon 9 in the *CNGA1* gene of the PRA-affected dogs. This mutation results in a frameshift at codon 584 and an early stop codon (Fig. 3). The mutation results in the deletion of 107 of 691 total amino acids of the C-terminal end of the CNGA1 protein and their replacement by eight novel amino acids.

Mutation screening

We screened all 82 dogs present in the original GWAS dataset for the mutation. The remaining cohort of 118 Shelties diagnosed as PRA-affected, retinopathy-affected or healthy were also screened for the mutation. Analysis of the segregation of the c.1752_1755delAACT mutation with PRA in the sampled population indicates that the *CNGA1*-PRA mutation is recessive and fully penetrant in one large family representing 92 of the 200 dogs collected. The

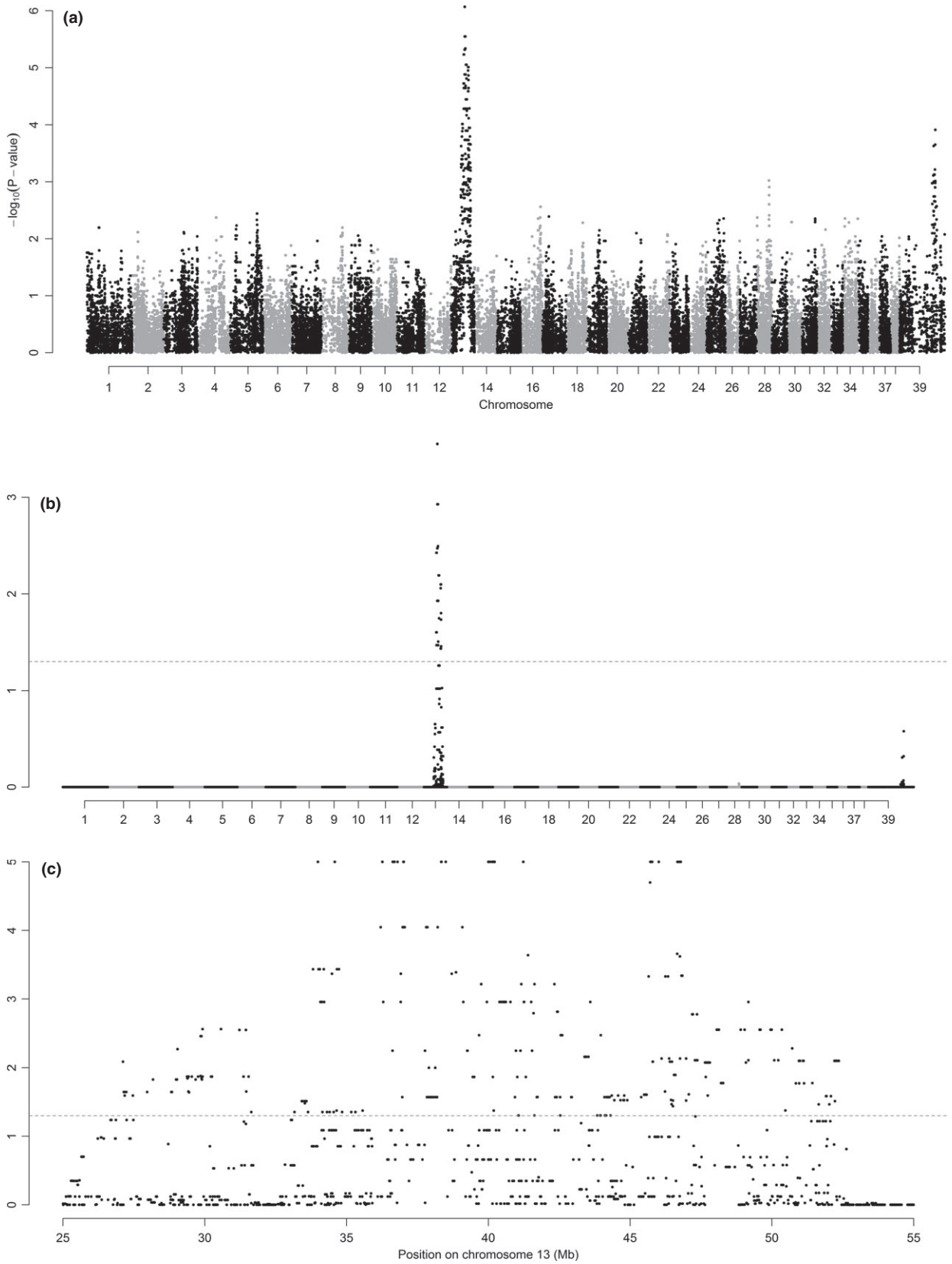

Figure 1 Genome-wide association mapping of PRA in Shelties. (a) Manhattan plot of GWAS on 15 cases and 14 controls using a simple association model. (b) Signal after 100 000 permutations (the gray dashed line represents the 5% significance level). (c) GWAS results in the interval CFA13:25–55 Mb.

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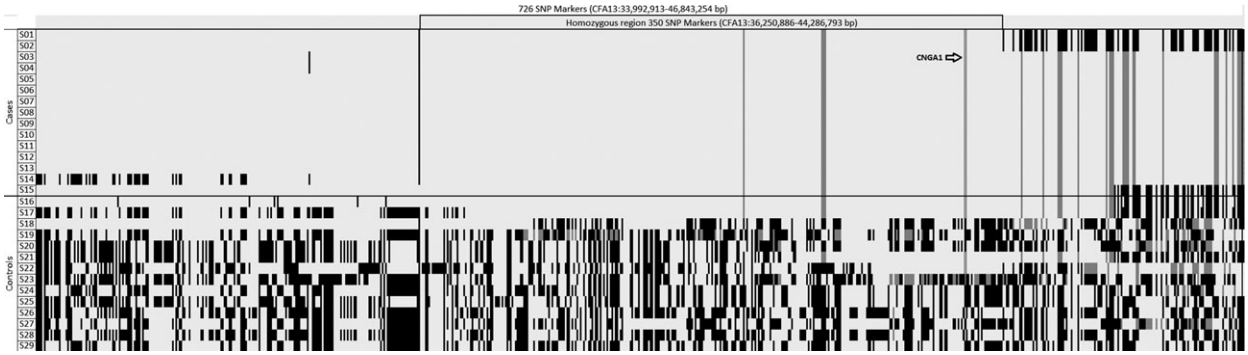

Figure 2 Fine mapping using haplotype analysis. SNP genotypes for 15 PRA cases and 14 PRA controls over the 12.850-Mb region identified during the genome-wide association study (726 SNPs). Light gray blocks represent a SNP marker homozygous for one allele, dark gray blocks represent a marker homozygous for the other allele, and black blocks represent heterozygous markers. All 15 cases share a homozygous haplotype of 416 SNPs (homozygous block), defining the critical region of 7.419 Mb between 36.250 Mb and 44.287 Mb on CFA13, a region containing 140 genes.

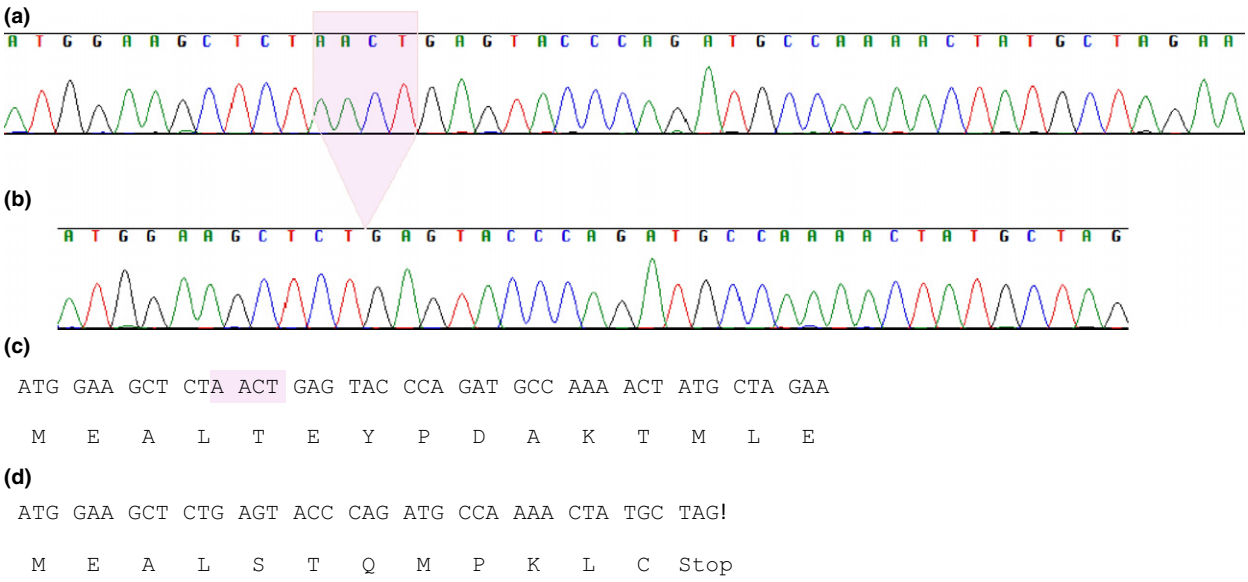

Figure 3 Overview of the *CNGA1* mutation. Sanger dideoxy sequencing traces for part of *CNGA1* exon 9 are shown for an unaffected (a) and affected (b) Sheltie. Panels (c) and (d) show the codon and amino acid alignments for the unaffected and affected Sheltie, respectively. The 4-bp deletion causes a frameshift and premature stop codon within eight residues.

mutation did not segregate with the disease in nine PRA cases and 23 retinopathy cases collected (not included in the final GWAS), and the remaining 76 unaffected dogs were homozygous or heterozygous for the wild-type allele.

Population screening

We screened 250 additional random Shelties and 100 random Collies with unknown eye examination status for the c.1752_1755delAACT mutation. Only one among the 250 Shelties tested was observed to be heterozygous for the mutation.

Discussion

PRA in Sheltie has not previously been associated with any identified genetic variants. Using a GWAS approach, we have identified a novel candidate variant in the *CNGA1* gene that is likely to be responsible for PRA in one group of Shelties. This is supported by the involvement of the gene in human RP and the nature of the mutation, a deletion of 4 bps causing a frameshift, an early stop codon and truncation of the protein.

A large number of the dogs used in the GWAS were known to be related through established pedigrees, and because

samples were collected from different pedigrees, the presence of population stratification was expected. However, after the removal of a pedigree containing the SPR cases, we performed a final association test with 15 cases and 14 controls, with an acceptable inflation factor ($\lambda = 1.02$). The population structure and the cryptic relationship of the sampled animals were therefore not accounted for in the final analysis. The relatedness among the study population and the limited size of the study cohort may account for the relatively wide region detected (7.419 Mb). The GWAS was significant after 100 000 permutations [$-\log 10 (8.55 \times 10^{-7}) = 6.07$] and also by Bonferroni correction accounting for the number of haplotype blocks (2400) in the canine genome [$-\log 10(0.05/2400) = 4.68$] or number of genes (22 000) [$-\log 10(0.05/22\,000) = 5.64$]. A Bonferroni correction for the number of SNPs [$-\log 10(0.05/95\,618) = 6.28$] is excessively stringent in dog breeds, for which extensive linkage disequilibrium means that each SNP is not an independent test (Lindblad-Toh *et al.* 2005; Tang *et al.* 2014). Because collection of samples initially was planned to perform a family-based linkage analysis, many of the cases and controls were related and a number of the controls were carriers of the mutation. In fact, eight of 14 were carriers, not adding to the statistical power.

CNGA1 is known to cause RP in humans (Dryja *et al.* 1995). *CNGA1* is highly expressed in the eye in humans and is localized to the rods in the retina. Canine *CNGA1* has previously been mapped and sequenced as a candidate gene for PRA in dogs, but no disease-causing mutations were found in these studies (Veske *et al.* 1997; Zhang *et al.* 1997).

Several mutations in *CNGA1*, causing RP, have been found in humans (Dryja *et al.* 1995; Paloma *et al.* 2002; Zhang *et al.* 2004). A frameshift mutation causing RP in humans in codon 645 of CNGA1, truncating the last 32 amino acids in the C-terminus, was found to encode a protein that is predominantly retained inside the cell instead of being targeted to the plasma membrane (Dryja *et al.* 1995). The 4-bp deletion found in Shelties is located at the canine codon 584, homologous to the human codon 581 which is in a highly conserved region (Fig. S2). The deletion may lead to a dysfunctional protein similar to the human *CNGA1* mutated protein.

A recent study in humans indicates that approximately one in four to five individuals from the general population may carry a null mutation responsible for human retinal disease (Nishiguchi & Rivolta 2012). This indicates that the risk of a consanguineous couple having a child with a genotype resulting in blindness is relatively high, compared with other inherited conditions. Almost 200 genes have been identified to be involved in human retinal disorders, and the number of genes known to be involved in canine retinal disorders is likely to increase. A high locus heterogeneity for retinal disorders may also exist in dogs, also within some breeds, and may explain the clinical cases of PRA in Shelties without the detected mutation.

I When the collection of blood samples for the project began, the Shelties were thought to be affected by one type of general PRA. The age of diagnosis varied between 2 and 11 years of age, with an average age of diagnosis at 5 years. It is important to be aware that many of the older dogs had their first eye examination at a very high age, because we screened available relatives after finding young affected dogs. This means that the age of diagnosis would not be well associated with the age of onset. When screening for PRA began, it soon became apparent that the breed suffered from an additional form of retinal degeneration, SPR, that progresses more slowly and does not cause such obvious visual impairment as does PRA (Karlstam *et al.* 2011). SPR is slowly progressive and resembles early stages of PRA ophthalmoscopically. ERG is currently the best method to distinguish between the two diseases, but ERGs were not obtained from the dogs sampled in the early phase of this project.

The PRA cases lacking the *CNGA1* mutation have been ophthalmologically screened for CEA and have been subjected to genetic testing of the prcd-PRA and the rcd2-PRA mutations. The dogs have been observed to be clear of these mutations. These nine dogs can be divided into two groups regarding the age of diagnosis, as five dogs were diagnosed between 2 and 4 years of age and four dogs were 7–8 years of age. The genetic basis of the other PRA (*CNGA1*–wild type) cases and the genetic basis of SPR in this breed warrant further investigation. More than 100 breeds are affected by canine retinal disorders, and in most breeds, these diseases have been shown to be genetically heterogeneous (Downs *et al.* 2011, 2013, 2014; Miyadera *et al.* 2012; Winkler *et al.* 2013; Downs & Mellersh 2014). Our results indicate that there is at least one other variant accounting for other forms of PRA in Shelties. Several different analyses have been run (results not shown) to identify regions associated with both the remaining PRA and the SPR cases, together and separately, so far without success. The dogs affected by the SPR do not seem to have any common genetic cause with any of the PRA cases.

The identification of the 4-bp deletion in *CNGA1* in some Shelties affected by PRA, which is likely to be responsible for one PRA variant in this breed, will enable breeders to work toward the elimination of this particular form of PRA. Genetic testing and further studies may reveal whether this mutation is responsible for an early-onset PRA variant. In addition, Shelties with the CNGA-1 mutation may be a valuable large animal model for comparative studies on gene therapy of hereditary retinal degenerations.

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Supporting information

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

Figure S1. Multidimensional scaling (MDS) plot of the complete dataset. The Sheltie population ($n = 80$) is visualized with a MDS plot displaying the formation of three subpopulations.

Figure S2. *CNGA1* human–dog–mouse multiple sequence alignment using CLUSTAL W (1.83).

Table S1. Primers for genomic DNA sequencing.