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James K. Kijas

Barbara Zangerl

Brian Miller

Jacque Nelson

Ewen F. Kirkness

See next page for additional authors

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At the time of publication, author Gustavo Aguirre was affiliated with the James A. Baker Institute for Animal Health, College of Veterinary Medicine. Currently, he is a faculty member at the Vet Med school at the University of Pennsylvania.

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Abstract

PURPOSE: To characterize a novel early onset canine retinal disease, and evaluate the ATP-binding cassette transporter gene *ABCA4* as a potential candidate gene in this and other canine retinal degenerations.

METHODS: Retinal disease was characterized ophthalmoscopically and electroretinographically in two pit bull terrier dogs and their purpose-bred descendants. All 50 exons of the canine *ABCA4* gene were amplified, cloned and sequenced from retinal mRNA of a normal, a carrier and an affected animal, and polymorphisms identified. The latter were used to search for association between *ABCA4* and retinal disease both within the study pedigrees and in additional canine breeds segregating retinal degenerations.

RESULTS: The disease derived from either founder is distinguished by early, severe, and rapidly progressive loss of cone function accompanied by progressive rod loss that is only relatively slower. Cloning and comparative sequencing of *ABCA4* identified six point mutations, none of which were obviously pathogenic. Crossbreeding studies revealed that the diseases in the two founders, although similar, are nonallelic. Pedigree analysis of segregating polymorphisms revealed dissociation between *ABCA4* and both retinal phenotypes.

CONCLUSIONS: The early, severe cone dysfunction in these diseases distinguish them from other forms of canine Progressive Retinal Atrophy. The development of a research population segregating these diseases presents two large animal models for the heterogenous human diseases termed cone-rod dystrophies. Analysis of the canine *ABCA4* homolog gene documented its sequence and identified a set of point mutations that were used to exclude this gene as causal to these canine cone-rod dystrophies.

Disciplines

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Author(s)

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Cloning of the canine *ABCA4* gene and evaluation in canine cone-rod dystrophies and progressive retinal atrophies

James W. Kijas,[1] Barbara Zangerl,[1] Brian Miller,[1] Jacque Nelson,[1] Ewen F. Kirkness,[2] Gustavo D. Aguirre,[1] Gregory M. Acland[1]

[1]James A. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY; [2]The Institute for Genomic Research, Rockville, MD

Purpose: To characterize a novel early onset canine retinal disease, and evaluate the ATP-binding cassette transporter gene *ABCA4* as a potential candidate gene in this and other canine retinal degenerations.
Methods: Retinal disease was characterized ophthalmoscopically and electroretinographically in two pit bull terrier dogs and their purpose-bred descendants. All 50 exons of the canine *ABCA4* gene were amplified, cloned and sequenced from retinal mRNA of a normal, a carrier and an affected animal, and polymorphisms identified. The latter were used to search for association between *ABCA4* and retinal disease both within the study pedigrees and in additional canine breeds segregating retinal degenerations.
Results: The disease derived from either founder is distinguished by early, severe, and rapidly progressive loss of cone function accompanied by progressive rod loss that is only relatively slower. Cloning and comparative sequencing of *ABCA4* identified six point mutations, none of which were obviously pathogenic. Crossbreeding studies revealed that the diseases in the two founders, although similar, are nonallelic. Pedigree analysis of segregating polymorphisms revealed dissociation between *ABCA4* and both retinal phenotypes.
Conclusions: The early, severe cone dysfunction in these diseases distinguish them from other forms of canine Progressive Retinal Atrophy. The development of a research population segregating these diseases presents two large animal models for the heterogenous human diseases termed cone-rod dystrophies. Analysis of the canine *ABCA4* homolog gene documented its sequence and identified a set of point mutations that were used to exclude this gene as causal to these canine cone-rod dystrophies.

Hereditary retinal degenerations form a diverse spectrum of blinding disorders that affect humans and other mammals. Those diseases recognized in non-human species provide invaluable model systems for studies of disease pathogenesis and the evaluation of treatment strategies. Such models are of particular value when their genetic basis is well understood and they faithfully reproduce the phenotypic characteristics of human disease. Naturally occurring canine hereditary retinal degenerations, such as the T4R opsin mutant dog [1], satisfy these criteria and can facilitate experiments which are currently difficult or impossible in other organisms. The demonstration of gene therapy mediated vision restoration within a canine model of Leber congenital amaurosis (LCA) is a powerful example [2].

Causal mutations for an increasing number of canine hereditary retinal degenerations are being determined. For example, pathogenic mutations have been identified in the canine *PDE6A* and *PDE6B* genes for the α and β subunits of the cGMP specific phosphodiesterase [3,4], the *RPE65* gene[5], the RP GTPase regulator *RPGR* [6], the cone-specific cyclic nucleotide-gated channel β subunit gene *CNGB3* [7] and rhodopsin *RHO* [1]. The resulting disorders model autosomal recessive, autosomal dominant, and X-linked retinitis pigmentosa (*PDE6A* and *PDE6B*, *RHO*, and *RPGR*, respectively), Leber congenital amaurosis (*RPE65*) and achromatopsia (*CNGB3*). With the exception of achromatopsia, these canine diseases are all rod-cone degenerations or dysplasias, in which rod disease, dysfunction, and death precede loss of cones; they are collectively termed Progressive Retinal Atrophy (PRA) and are homologous to human Retinitis Pigmentosa (RP). There is as yet no published animal model corresponding to the human cone-rod dystrophies (CRD), a group of disorders distinguished from rod-cone degenerations, such as RP, by the relatively earlier loss of cone function in CRD. In some CRD patients cone dysfunction markedly precedes rod loss, but in others there is concurrent cone and rod dysfunction. Currently, eight human CRD loci and seven causal genes (RetNet) are recognized.

Mutations in the ATP-binding cassette transporter gene (*ABCA4*) have been associated with a diversity of human retinal disorders. Over 200 unique human *ABCA4* sequence variants have been reported from patients affected with cone-rod dystrophy, Stargardt macular dystrophy, age related macular degeneration (AMD), and autosomal recessive RP [8-11].

The *ABCA4* gene, a member of the ATP-binding cassette (ABC) transporter superfamily, is only expressed in rod photoreceptors of the retina [12] and its precise role in membrane transport remains an open question. The human gene com-

Correspondence to: Gregory M. Acland, Retinal Disease Studies Facility, University of Pennsylvania/New Bolton Center, 382 West Street Road, Kennett square, PA 19348; Phone: (610) 444-1361; FAX: (610) 925-8112; email: gma2@cornell.edu

prises 50 exons covering more than 100 kB of genomic sequence on chromosome 1p22.1 [13]. The canine homolog of this gene has not yet been characterized.

We now report an early onset PRA-like disease derived from pit bull terriers, that shares clinical features with the human cone rod dystrophies. A resource strain of such dogs has now been developed to allow characterization of the disease phenotype and identification of the causal mutation. To evaluate *ABCA4* as a candidate gene in this and other canine diseases, the canine *ABCA4* homolog has been sequenced and mapped on the canine genome.

METHODS

Animals and pedigrees: Two independently acquired dogs were used as founders for the study pedigrees (animals 1 and 3, Figure 1). These founders were both of pit bull terrier ancestry, but were not known to be related to each other. Animal 1 was outbred to two beagle derived crossbred dams (N2, N3) known to be free of retinal degenerative disease alleles. The resulting heterozygous offspring were either interbred, or backcrossed to an affected dog to generate 29 animals segregating the disease phenotype (Figure 1). Animal 2 was bred to a homozygous normal sire (N1) to produce obligate heterozygous offspring which were then interbred to generate 15 F2 animals segregating the disease phenotype (Figure 1). Complementation tests to investigate disease allelism were performed by mating two descendants (animals 14 and 78, Figure 1) of the first founder (animal 1, Figure 1) either directly to the second founder (animal 3, Figure 1) or to one of her affected descendants (animal 78, Figure 1), respectively. The subsequent crossbred strains segregating these retinal degeneration phenotypes are maintained at the Retinal Disease Studies Facility, Kennet Square, PA. All animal procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal tissue for RNA analysis (see below) was collected from three animals; blood samples for segregation analysis was collected from a total of 31 animals. Additional blood samples were obtained from 67 privately owned dogs from the following 11 breeds of dogs with PRA (number of affected individuals given in parenthesis): akita (4), American cocker spaniel (5), American eskimo dog (3), Australian cattle dog

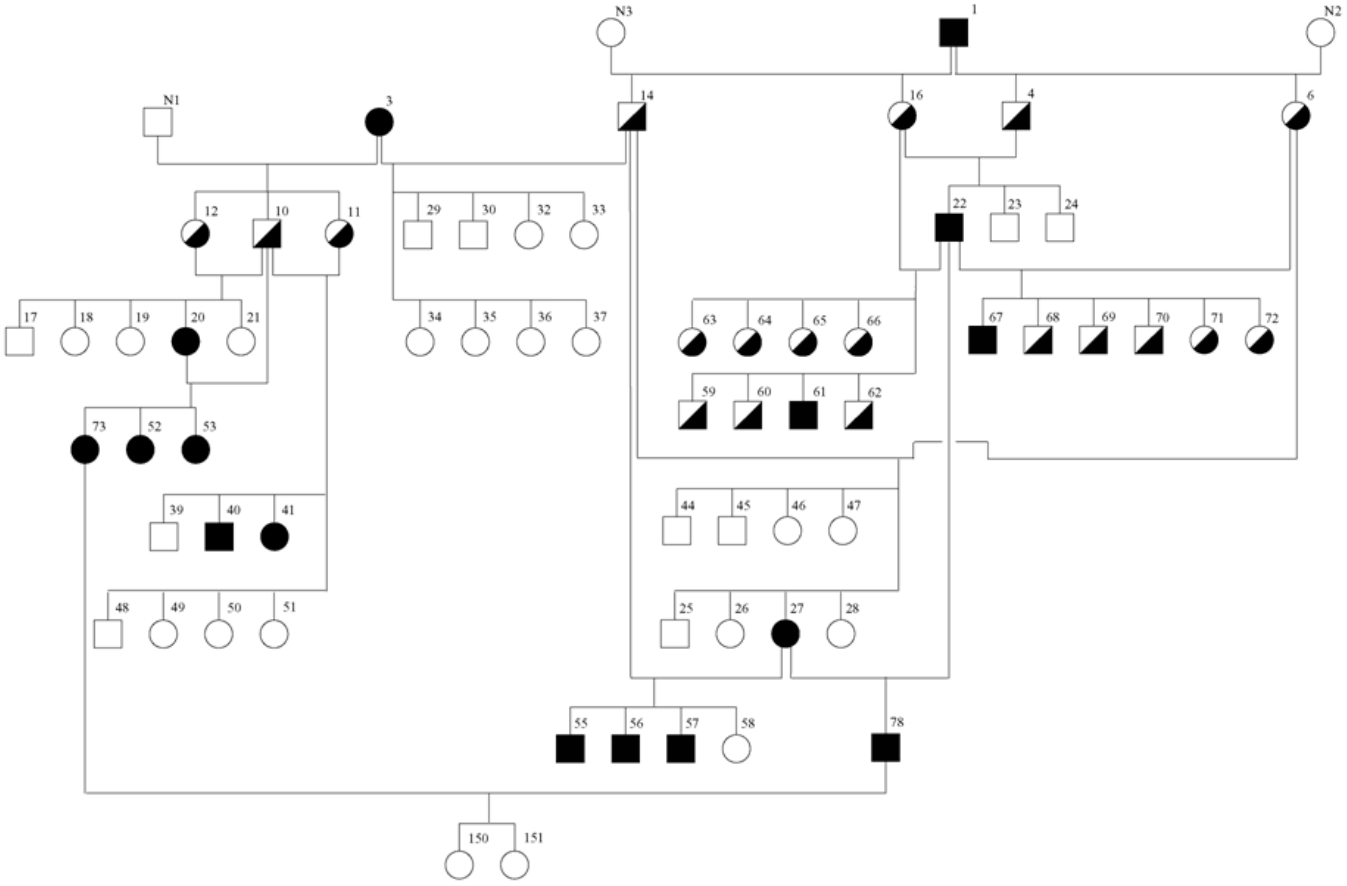

Figure 1. Study pedigree of dogs segregating cone-rod dystrophy. Two affected pit bull terriers (animals 1 and 3) were outcrossed to homozygous normal animals (N1-3). Heterozygous offspring were then either intercrossed or bred to affected animals to generate informative litters. Electroretinography was used to determine the presence of the disease. Affected animals are denoted by solid symbols while asymptomatic animals are illustrated either with half filled (where obligate heterozygotes) or empty symbols (where both parents are non-affected and status at the mutation is unknown). Complementation test-breedings resulted in animals 29-37, and 150-151, all ten of which were non-affected, establishing that the diseases derived from animals 1 and 3, although phenotypically similar, are non-allelic.

(3), basenji (4), English springer spaniel (4), Glen of Imaal terrier (4), Italian greyhound (4), papillon (4), poodle (3) and Tibetan terrier (5).

Phenotypic ascertainment: Diagnosis of retinal phenotype in all colony dogs was established by electroretinography[14,15], and confirmed by clinical examination including indirect ophthalmoscopy. Diagnosis of retinal phenotype in non-colony dogs was by indirect ophthalmoscopy. In selected cases, eyes were enucleated immediately postmortem and fixed either for high resolution retinal morphological examination, or for standard histopathologic examination as described previously [14,16]

Cloning of canine ABCA4: Total RNA was isolated from retinal tissue using TRIzol (Invitrogen, Carlsbad, CA) and a single chloroform extraction. First strand cDNA was synthesized in 20 µl reactions using an oligo d(T)16 primer and the GeneAmp RNA PCR kit by following the manufacturers recommendations (Perkin Elmer, Foster City, CA). Nine pairs of canine specific primers were designed to amplify overlapping fragments spanning the 6810 bp *ABCA4* translated region (Table 1). First strand cDNA was amplified in 20 µl reactions containing 0.4 µM both forward and reverse primer (Table 1), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 µM dNTP, and 0.5 U Taq polymerase. For each primer pair the PCR annealing temperature was set at 55 °C for 35 cycles in a thermal cycler (PTC-200, MJ Research, Waltham, MA).

Table 1. Primers used for the RT-PCR based cloning of the canine *ABCA4* gene

Primer	Sequence (5'-3')	STS bp	Exons
17B	ATGTCCTTTCCTGTCGCTGCACC	597	5′UTR-4
18	CAACTGAGTCAGACAGGCCAATGT		
1	ACCTTGTCTCGACTCATGGACACC	1053	4-11
2	GAAGTTCAGCATGGCTTCTGCAG		
3a	CACAGATGACCATGATCAGAGAC	1197	11-16
4a	GGAGCATCATCTTCATGGACATC		
5	TTGGCACTGAGTACCTGGCTCGCT	1077	16-23
6	TCTTGCGCACCAAGGTTAAG		
7	TGTCCACTCACCACATGGACGA	1402	23-33
8b	ATCTGGCCAAGGTGGCTTAA		
9	GAGCAAATTCTGGGTCAAT	796	32-38
10	GATGAAGGTGATGGCACTGCTG		
11	CAGCACAGCCTATGTGGCCTTG	623	38-43
12	CGTCACCTGAGGTCACTGTGGT		
13	GTCTTCTGGGAGTGAATGGAGCT	572	43-47
14	GACTTGAGATGCTGAATGGTGC		
15	GAGGAGTGTGAGGCTCTGTGTA	522	47-3′UTR
16A	GGTCCAATCAGATGACCATAT		
ABCA4-ex13SNPFor	CTGATCCTGTGGAAGACTTCC	134	exon 13
ABCA4-ex13SNPRev	GTAAGGCATCTGCTGGAGATAG		
ABCA4-ex27SNPFor	CAGGGCTCCAATGGCTGCTCT	146	exon 27
ABCA4-ex27SNPRev	TGGTGGAATCGCTTGACCAGCAA		

Nine pairs of canine specific primers were designed to amplify overlapping fragments spanning the 6810 bp *ABCA4* translated region. Primers were designed based on Genbank entries AY427777, AY427778, and AY427779.

Following electrophoresis on 2% agarose gels, amplified fragments were excised, purified and directly sequenced using dye terminator chemistry and an ABI 3700 DNA sequencer (Applied Biosystems, Foster City, CA). DNA sequences were aligned using Sequencer software (Gene Codes Corporation, Ann Arbor, MI) and estimates of nucleotide diversity calculated using MEGA version 2.0 [17].

Analysis of ABCA4 polymorphism: A non-synonymous A to C transversion at nucleotide 1880 was detected in the PCR fragment amplified from primers ABCA4-ex13F and ABCA4-ex13R (Table 1) using the conditions described above. Digestion of the 134 bp fragment with *Aci* (GCGG) into two fragments (96 bp and 36 bp) reports the presence of C at position 1880 while presence of the undigested fragment reports presence of A at position 1880. A non-synonymous G to A transition at nucleotide 3958 was detected in the PCR fragment amplified from primers ABCA4-ex27F and ABCA4-ex27R (Table 1) using the same conditions. Digestion of the 146 bp fragment with *Pst*I (CTGCAG) into two fragments (122 bp and 24 bp) reports the presence of G at position 3958 while presence of the undigested fragment reports presence of A at position 3958. An estimate of linkage disequilibrium

Table 2. *ABCA4* genotypes in PRA affected and normal animals

	PRA Affected dogs			Non-affected dogs		
Breed	Number	Exon 13 SNP	Exon 27 SNP	Number	Exon 13 SNP	Exon 27 SNP
akita	2	C/C	A/A	-	-	-
	1	A/C	A/A	-	-	-
	1	C/C	G/G	-	-	-
	-	-	-	1	C/C	A/G
	-	-	-	1	A/C	A/G
American cocker spaniel	4	C/C	A/A	1	C/C	A/A
	1	A/C	A/G	-	-	-
American Eskimo	3	C/C	A/A	2	C/C	A/A
Australian cattle dog	1	A/A	G/G	1	A/A	G/G
	1	A/A	A/G	-	-	-
	1	A/C	A/G	-	-	-
basenji	1	A/A	A/A	-	-	-
	1	A/A	G/G	1	A/A	G/G
	2	A/A	A/G	1	A/A	A/G
English springer spaniel	1	A/C	A/A	1	A/C	A/A
	3	C/C	A/A	2	C/C	A/A
Glen of Imaal terrier	4	A/A	G/G	1	A/A	G/G
	-	-	-	1	A/C	A/G
Italian greyhound	1	A/A	A/A	2	A/A	A/A
	3	A/A	A/G	1	A/A	A/G
papillon	1	A/A	A/G	-	-	-
	1	A/C	A/A	-	-	-
	2	C/C	A/A	2	C/C	A/A
poodle	1	A/A	A/A	-	-	-
	1	A/A	A/G	2	A/A	A/G
	1	A/C	A/A	1	A/C	A/A
	-	-	-	1	A/A	G/G
Tibetan terrier	2	C/C	A/A	2	C/C	A/A
	3	A/C	A/G	-	-	-

Genotypes at two *ABCA4* SNPs determined for a panel of 67 dogs (43 affected by PRA, 24 nonaffected) representing 11 breeds.

(D') between the two positions was calculated from the animal set contained in Table 2. Compound heterozygotes (7/67) were first excluded where unambiguous phase could not be assigned before D' was estimated using DnaSP version 3.53 [18]. Amplification of *ABCA4* associated CFA6 microsatellites C06.636 and 2A11 was performed using previously published fluorescently labeled primers and conditions[19]. Amplified alleles were genotyped using an ABI310 Genetic Analyzer and GeneScan software (Applied Biosystems, Foster City, CA).

Radiation hybrid mapping: Nine previously published CFA6 genetic markers were used for radiation hybrid map construction; 2A11, AHTH228, C06.636, FH2164, FH2370, REN149M14, REN152F02, REN37H09 and VCAM1[19,20]. Amplification of each was performed as described on the FHCRC Dog Genome Project / Radiation Hybrid Mapping PCR Protocols webpage. Canine *ABCA4* was mapped using a 146 bp fragment of exon 27 amplified by primers ABCA4-ex27F and ABCA4-ex27R and an annealing temperature of 55 °C. PCR was performed for each marker using DNA from the 92 cell $RH08_{3000}$ canine-hamster panel (Research Genetics, Huntsville, AL). Using Multimap [21], the linkage groups were defined where members returned two point Lod scores higher than 5.0. These marker groups were then ordered in relation to one another based on previously published RH and meiotic maps [19].

RESULTS

Canine model of cone-rod dystrophy: Both founder dogs (Animals 1 and 3, Figure 1) were young adults when first presented for clinical examination. Each dog exhibited marked loss of visually guided behaviour and widely dilated pupils under dim or bright ambient illumination, and was diagnosed ophthalmoscopically as affected with an advanced retinal degeneration. Electroretinographic responses of both dogs were essentially flat lines to stimuli under conditions that normally elicit distinct rod and cone responses (data not shown).

Younger dogs from subsequent generations demonstrated a clinical phenotype markedly different from previously described forms of PRA. Distinct pupillary dilatation, severe visual difficulties under well lit conditions, and intermittent nystagmus were present as soon after weaning as these signs could be evaluated. Electroretinograms (ERGs), recorded from all pups in informative litters as soon after weaning as practicable, demonstrated severe dysfunction of both cones and rods, with cone function consistently more severely impaired than that of rods; in some cases cone function was unrecordable. These findings were, broadly, similarly abnormal in affected dogs derived from either founder. All responses from such young affected dogs (Figure 2, columns 2, 3) were markedly reduced in amplitude compared to age matched non-affected dogs (Figure 2, column 1). Both rod-specific (Figure 2, line 2

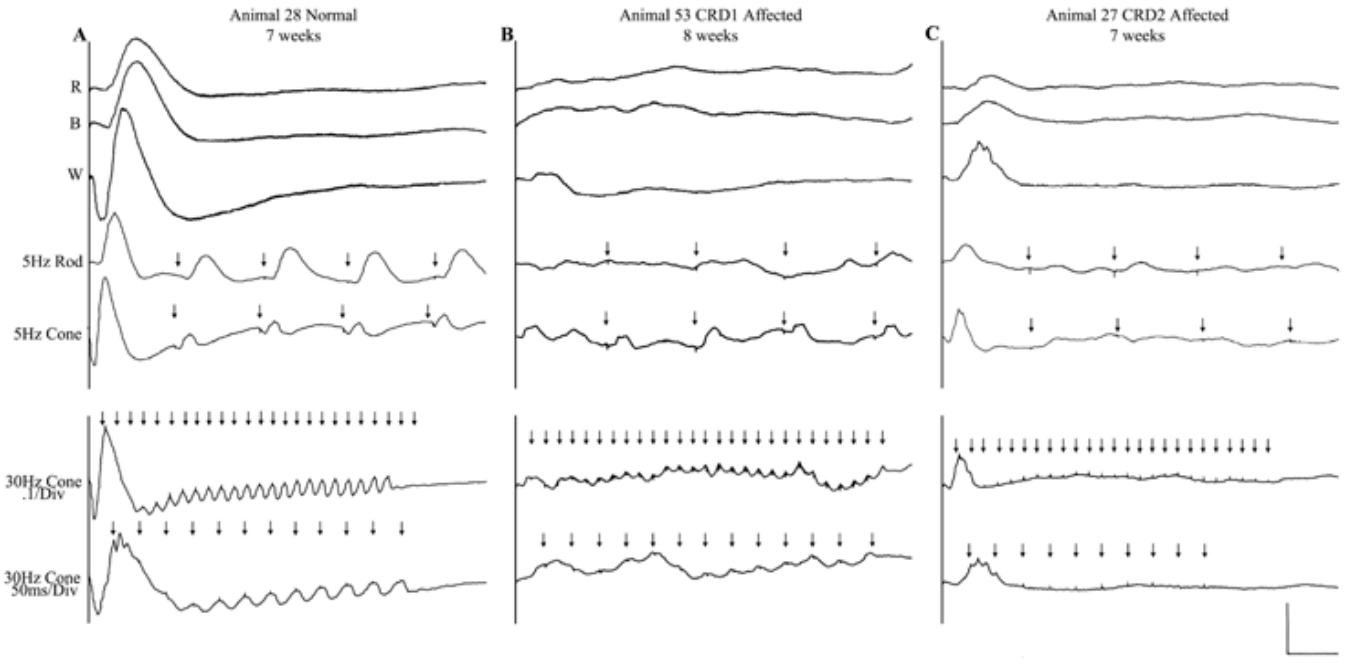

Figure 2. Electroretinograms from normal and cone-rod dystrophic dogs. Electroretinograms recorded from representative normal (column 1) and cone rod dystrophic (columns 2, 3) dogs. Each vertical panel presents the responses to a red flash (line 1); a blue flash (line 2); a white flash (line 3); 5 Hz low intensity white light flashes (line 4); and 30 Hz high intensity white light flicker (lines 5, 6): These elicit rod-specific (lines 2, 4), cone-specific (lines 5, 6, 7) and mixed rod-cone responses respectively. Animal 53 derives its disease from one founder (Animal 3, Figure 1) and Animal 27 from the other (Animal 1, Figure 1). Because these diseases are nonallelic they are termed *crd1* and *crd2*, respectively. Although both cone and rod responses are markedly reduced in both affected dogs, the reduction in cone response amplitudes, relative to the loss of rod function, is far more severe than in previously described canine rod-cone degenerations and dysplasias. Residual cone function, at this age, is better preserved in animal 53 than Animal 27, a consistent albeit slight difference between CRD1 and CRD2 affected animals.

and 4) and cone-specific (Figure 2, lines 5, 6 and 7) stimuli produced tiny or virtually absent responses from affected dogs. Stimuli eliciting mixed rod-cone responses (Figure 2, lines 1 and 3) were also markedly reduced in amplitude in affected dogs compared to non-affected. Ophthalmoscopic evidence of retinal thinning became apparent in affected pups between 3 months and 6 months of age. By 12 months of age all affected dogs demonstrated fixed dilated pupils, advanced generalized retinal degeneration on ophthalmoscopic examination, non-recordable ERG responses, and were clinically blind. Morphologic examination of affected eyes examined to date reveal only diffuse severe retinal degeneration in dogs over 6 months of age (data not shown).

Eight pups (Animals 29-37, Figure 1) were produced by breeding one of the founders (Animal 3, Figure 1) to an obligate heterozygous son (Animal 14, Figure 1) of the other founder. Two further pups (Animals 150, 151, Figure 1) were produced by breeding affected descendants of either founder to each other. Each of the 8 pups from the first litter was examined clinically and electroretinographically at 11 weeks and 5 months of age, and their retinas were examined morphologically at 5 months of age. The 2 pups from the second litter were examined clinically and electroretinographically at 8 weeks of age. All observations were within normal limits and none of the ten pups was affected with the retinal disease segregating in other litters of the study pedigree (data not shown).

Cloning of canine ABCA4: The genomic *ABCA4* sequence present within the December 2001 freeze of the human public draft sequence (found on the UCSC Genome Bioinformatics Site) was used to search the x1 database of the canine genome constructed by The Institute for Genome Research (TIGR). Of the 50 exons contained within the human query, partial or complete sequence hits were returned for 23 canine exons. This enabled design of canine specific primers for RT-PCR amplification of *ABCA4*. cDNA derived from both *crd* and normal retinal mRNA was amplified using nine primers sets (Table 1). Comparison of the amplified fragments from each mRNA preparation revealed products of the same length, indicating normal *ABCA4* splicing within the dystrophic reti-

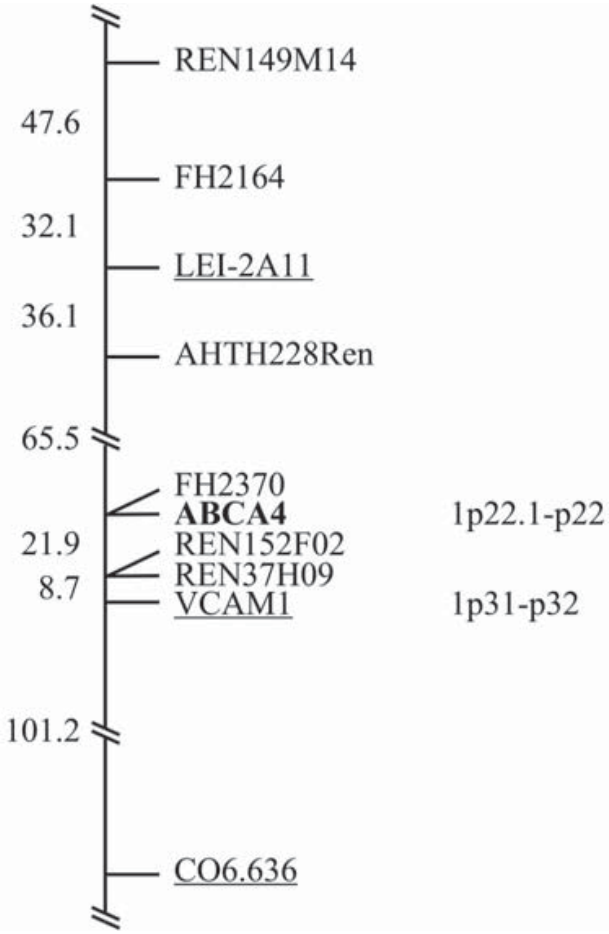

Figure 4. Position of *ABCA4* on CFA6. A whole-genome dog/hamster RH_{3000} panel was used and the order of backbone markers (underlined) was chosen according to previous publication [19]. The map was extended with statistical support >100:1. The map is divided into three linkage groups based on two point linkage, and all distances correspond to cR_{3000} units. Corresponding cytogenetic position of genes on human chromosomes are shown (right panel)

```
                 1   1   1   1   1   1       1   1   1       1   1   1   1   1
                 2   2   2   2   2   3       3   3   3       3   3   3   3   3
                 9   9   9   9   9   0       2   2   2       3   3   3   4   4
                 5   6   7   8   9   0       3   4   5       7   8   9   0   1

HumanABCA4       Glu Asn Val Asn Pro Arg     Gly Ala Pro     Pro Glu Cys Pro Gly
MouseABCA4       ... Gln Ala Gly Leu ...     ... --- Gln     ... ... Asp ... ...
CanineABCA4      ... ... --- --- Leu ...     Glu --- ...     ... ... --- Ala Arg

HumanABCA4       GAA AAC GTC AAC CCC CGA     GGG GCG CCG     CCA GAG TGC CCA GGC
MouseABCA4       ... C.A .C. GGT .T. ..G     ..A --- .AA     ... ... GA. ..C ..T
CanineABCA4      ... ... --- --- .T. ...     .A. --- ..A     ... ... --- G.. C..
```

Figure 3. Partial alignment of human, mouse and canine ABCA4 amino acid and nucleotide sequences. Alignment of ABCA4 from three species showing the position of missing residues in the dog. Amino acid residue numbers are given above the alignments of both protein (top) and nucleotide (bottom) sequence. Positions absent from non-human species are denoted with (-) and numbered in red type.

nas. Single PCR products of the expected length were obtained in every instance suggesting the presence of only one *ABCA4* retinal transcript in both normal and dystrophic retina.

High level of interspecies conservation: Pairwise nucleotide diversity was calculated between the human, mouse and canine *ABCA4* sequence homologs. At synonymous sites, the nucleotide diversity (K_ST) between human and mouse *ABCA4* was 0.534±0.025 substitutions/site which is approximately equal to the average for genes compared between these lineages (average K_S=0.566 subs/site derived from a set of 45 genes, [22]). The nucleotide diversity at non-synonymous sites (K_N)=0.059±0.003 revealed the gene has a much lower than average number of coding level substitutions (average K_N=0.125 subs/site, [22]). Pairwise diversity calculations including the cloned canine *ABCA4* sequence revealed lower divergence from the human compared to the mouse gene at both synonymous and non-synonymous sites (K_S=0.356±0.018 canine v human, K_S=0.571±0.026 canine v mouse; K_N=0.046±0.003 canine v human, K_N=0.063±0.004 canine v mouse). Comparison of the predicted amino acid sequence revealed the canine ABCA4 has four missing residues when compared with the human molecule (Figure 3). Each of the four missing residues is located within a 42 amino acid region located within the first intracellular loop. Comparison of interspecies amino acid level diversity reveals this region has accumulated many more changes (K=0.215±0.043; 93 residues) than the average for the entire molecule (K=0.096±0.006; 2266 residues). Interspecies comparison of the 12 transmembrane domain alpha-helical segments revealed few differences (K=0.087±0.018; 242 residues) while no differences were observed within the two ATP binding sites (residues 963-971 and 1972-1980).

Radiation hybrid mapping of canine ABCA4: Human *ABCA4* is located on 1p22.1 in a region estimated to be syntenic with canine chromosome 6 (CFA6) [19]. To reconstitute a portion of the CFA6 RH map and test for linkage with *ABCA4*, primers amplifying nine published canine markers and *ABCA4* exon 27 were tested on the $RH08_{3000}$ canine-hamster RH panel. Figure 4 shows *ABCA4* in tight linkage with marker FH2370 confirming its location on CFA6 (two point LOD=14.0).

ABCA4 sequence from normal and crd retina: The complete 6810 bp coding region was sequenced and compared between a *crd* affected dog (animal 22: *crd/crd*; GenBank accession number AY427777), a non-affected littermate (animal 23: *crd*+ or +/+; GenBank accession number AY427778) and a beagle derived crossbred animal known to be homozygous wildtype for the *crd* mutation (animal P1117: +/+; GenBank accession number AY427779). Alignment revealed an expressed *ABCA4* transcript of the same length from each animal. A total of six point-mutations were identified consisting of three synonymous and three non-synonymous substitutions (Figure 5). Two of the six mutations were observed in

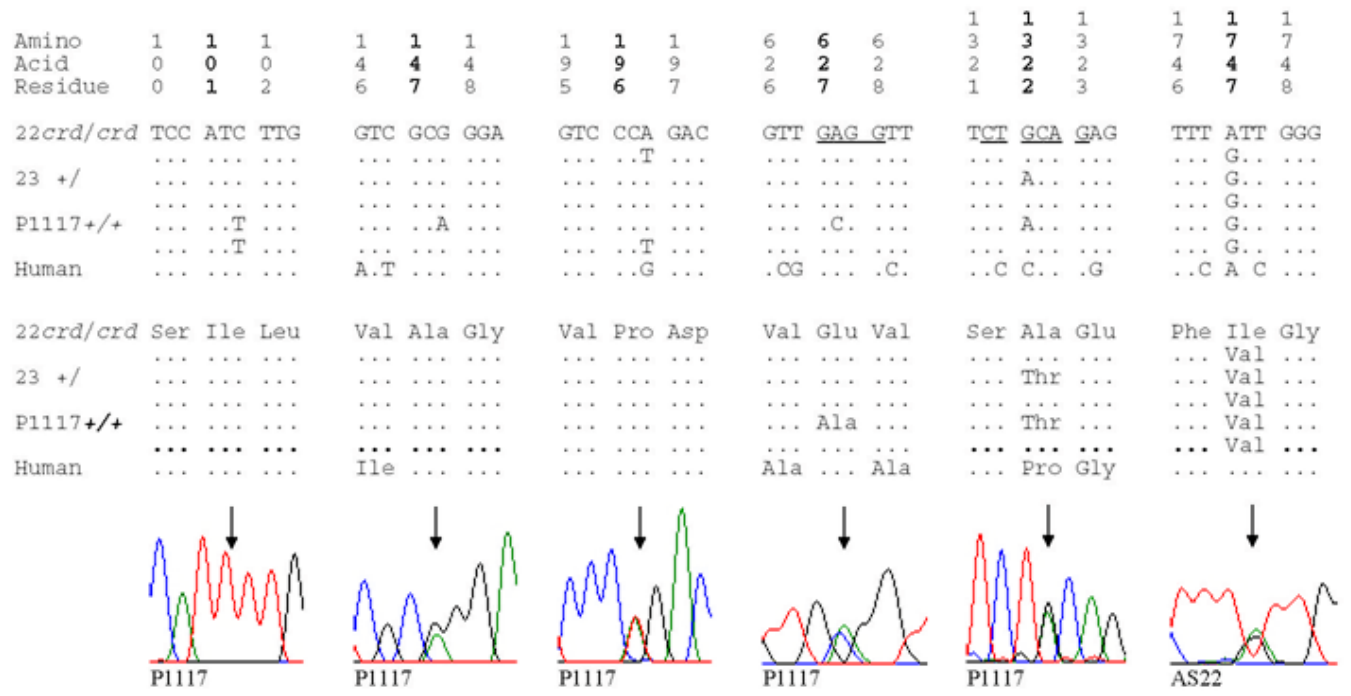

Figure 5. Nucleotide and amino acid substitutions identified within the canine ABCA4 gene. Nucleotide and amino acid sequence variations are shown for both chromosomes for each of 3 animals (AS22 is Animal 22, genotype crd/crd; AS23 is Animal 23, genotype +/; Animal P1117, genotype +/+) and compared to the corresponding human sequence. Six polymorphisms (within codons 101, 147, 196, 627, 1322 and 1747, as identified in the top label) are present among the canine sequences. Complete sequence (nucleotide in top set, amino acid in bottom set) is only shown for one chromosome of AS22 (top row of each set), for other data rows only the variant allele is shown (dots represent conserved sequence identities). Chromatograms (bottom figures) demonstrate sequence traces from one animal per polymorphism (red is T, blue is C, black is G, green is A), with arrows indicating the polymorphic site. Thus, P1117 is TT at the polymorphic site in codon 101, AG in 147, AT in 196, AC in 626, and AG in 1322; and AS22 is AG in 1747.

the heterozygous state within the affected animal 22 while another three were observed only within the homozygous wildtype animal P1117 (Figure 5). The three non-synonymous substitutions result in amino acid replacements Glu627Ala, Thr1322Ala, and Val1747Ile, none of which have to date been implicated in the pathogenesis of retinal disease in other species.

Association analysis in crd1 and crd2 pedigrees: In the absence of clearly pathogenic exonic sequence variants, association analysis was conducted to investigate the segregation of the identified *ABCA4* polymorphisms within the resource pedigrees. Restriction endonuclease based diagnostic PCR assays were designed to determine the nucleotide sequence at both the Glu627Ala and Thr1322Ala mutations. Genotypic data from these two *ABCA4* polymorphisms and two CFA6 microsatellites (CFA6-2A11 and C06.636) permitted construction and examination of chromosomal haplotypes within the disease pedigrees. Assuming a recessive mode of inheritance, symptomatic F2 animals are predicted to possess the same haplotype combination as their affected grandparent for the chromosomal region containing the casual mutation. Inspection of the CFA6 haplotypes present within a number of F2 animals showed this was not the case (Figure 6). The affected F2 animal 27, for example, inherited a chromosomal haplotype [180-C-A-160 from markers CFA6-2A11, Glu627Ala, Thr1322Ala, C06.636] from its homozygous normal grandmother (animal N3). Similar analyses of a further seven affected and eight non-affected F2 animals revealed a total of eight animals which were discordant between their CFA6 haplotypes and their cone-rod dystrophic phenotype (animals 17, 18, 20, 27, 40, 41 55, 56). This clearly excludes *ABCA4* and the surrounding CFA6 region as causal to the disease originating from both founding animals.

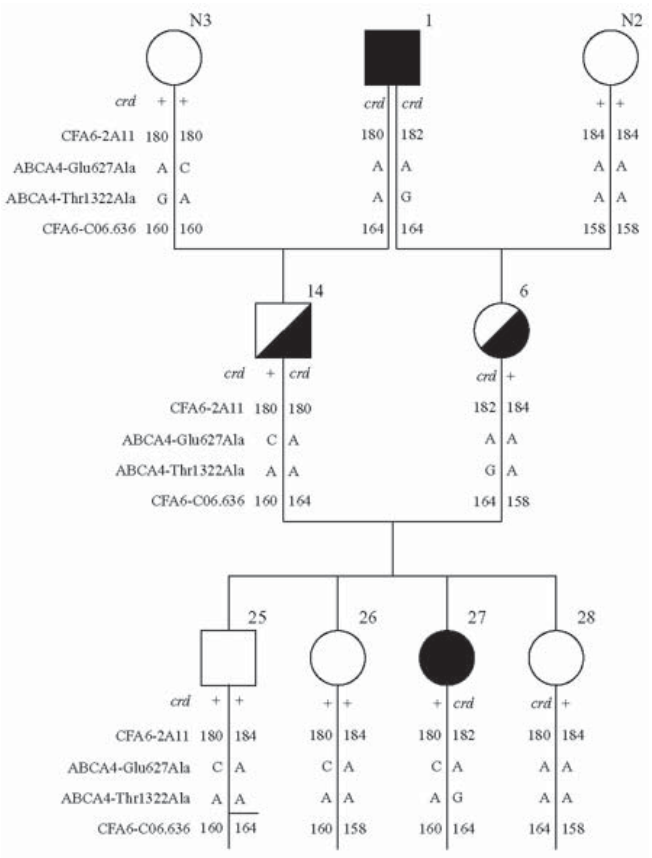

Figure 6. Association data for CFA6 polymorphisms in a pedigree segregating a canine cone-rod dystrophy. Association data for CFA6 polymorphisms within a subset of pedigrees segregating canine cone-rod dystrophy. Haplotypes derived from two CFA6 microsatellites (2A11 and C06.636) and two exonic *ABCA4* point substitutions are shown below each animal. The horizontal line indicates a recombination event observed in animal 25.

Association analysis with canine PRA: Two identified *ABCA4* polymorphisms were also genotyped in a set of PRA affected and non-affected animals from 11 additional dog breeds. Table 2 shows that affected members in nine of the eleven breeds were heterozygous at one or both of the polymorphic positions. One of the remaining two breeds (akita) has affected members homozygous for different haplotypes while the second breed (Glen of Imaal terrier) has PRA affected animals sharing the same homozygous genotype with phenotypically normal animals. Examination of the haplotypes arising from the two polymorphic positions revealed all four theoretically possible genotypic combinations were observed in the set of 67 dogs. The two SNPs are tightly linked but not in complete linkage disequilibrium (D'=0.844).

DISCUSSION

The development of animal models of ocular disease is required for the testing and evaluation of treatment strategies relevant to human. Investigation within canine populations has identified a number of disease models, and the present studies aimed to document and investigate a retinal phenotype observed within a population of pit bull terrier derived animals. The disease in adult dogs is broadly similar to other early onset hereditary retinal degenerations in the dog, but the differences seen in younger dogs are likely to be more significant. In particular, compared to rod-cone dysplasia type 1 (*rcd1*), for example, the early severe loss of cone function as assessed both by ERG and clinical assessment of visual function is striking. In *rcd1* the primary cell biologic defect affects rods, and cones only seem to die as a consequence of the loss of rods. This is also generally held to be true for other forms of PRA and for RP. In the present disease, however, cone dysfunction is at least synchronous with the rod dysfunction, and is the main cause of the early clinical manifestations. No other form of PRA exhibits dayblindness as early as do these affected dogs, and no other form of PRA exhibits such early, rapid, and severe loss of cone ERG function both in absolute terms and relative to the loss of rod function. We therefore classify their disease as an early onset cone rod dystrophy.

Because morphologic examination of affected eyes has so far been from dogs over 6 months of age, we have only limited insight into the natural history of either disease or the differences between *crd1* and *crd2*, except that both are extremely fast and severe. Further studies of much younger af-

fected tissues will be necessary to better understand these diseases.

Breedings designed to test allelism of the disease(s) affecting the two founders produced ten offspring all of which proved non-affected, by criteria that reliably demonstrate disease in inbred affected descendants of either founder. This unexpectedly established that the phenotype in these dogs represents not one new disease but two different, non-allelic diseases. That is, the diseases in the two founders derive from non-allelic mutations. For mapping the disease locus in these pedigrees, and evaluation of candidate genes, it is thus critical to evaluate each arm of the pedigree independently. For these reasons we refer to the locus and disease derived from Animal 3 as *crd1*, and from Animal 1 as *crd2*, for cone rod dystrophy type 1 and 2, respectively. In young animals, it is possible to distinguish *crd1* from *crd2*, as cone ERG function in the former is apparently absent at the earliest age tested, but in the latter, although severely reduced, it is recordable. In retrospect this difference was present between the ERGs recorded from animals 73 and 78 used in the complementation test (data not shown), but at the time was thought to be within the range of variation of what was expected to be a single disease. This difference disappears in older dogs as the diseases progress.

The number of genes or loci currently identified with CRD in humans is relatively few, compared to those for RP. Only four, *ABCA4* (HSA) [10,11,23-25], CORD8 (HSA1q12-q24) [26], CORD9 (HSA8p11) [27], and *RPGRIP1* (14q11) [28], have so far been incriminated in nonsyndromic autosomal recessive CRD. Of these 4 loci, *ABCA4* appears to account for a plurality of known autosomal recessive CRD patients [11,23,24]. As a first step towards identifying the genes involved in *crd1* and *crd2*, *ABCA4* was therefore selected to be tested as a candidate gene.

Retinal mRNA was used as the template to sequence all 50 exons of the wildtype canine *ABCA4* homolog. This allowed pairwise comparison of the nucleotide diversity with both human and mouse *ABCA4* and revealed a gene with higher than average synonymous but lower than average non-synonymous divergence. This likely reflects a gene which holds a non-redundant metabolic function as amino acid level substitutions are heavily selected against. This is consistent with the large number of identified pathogenic substitutions identified within human populations. Translation of the obtained sequence and alignment with human ABCA4 revealed a canine protein with four less residues. Each of the four missing amino acids are clustered in a region which has accumulated many more interspecies substitutions than the average for the entire protein, suggesting an ABCA4 region of lowered functional significance. By contrast, those regions identified as critical for function including the two ATP nucleotide binding sites and transporter signature motifs [29] were almost completely conserved.

To evaluate the role of *ABCA4* in the cone-rod dystrophy disease described herein, comparative sequence analysis was conducted for all 50 exons expressed in an affected (animal 22), a non-affected littermate (animal 23) and a homozygous wildtype colony animal (P1117). Three non-synonymous substitutions were identified however none appear to contribute to canine *crd*. A Glu627Ala substitution was observed in the heterozygous state only within the normal animal P1117 and is not among the growing list of 176 ABCA4 substitution mutations associated with human retinal disease (Human Gene Mutations Database). A Thr1322Ala substitution is homozygous within the affected animal (22) and heterozygous in the others, however Thr1322 is neither conserved within human ABCA4 or its substitution implicated with human disease. In addition, replacement of Thr1322 with a non-polar residue (Ala) introduces an amino acid highly similar to Pro1322 found in human ABCA4 indicating it is likely nonpathogenic in the dog. The third non-synonymous substitution (Val1747 Ile) is heterozygous in the affected animal and replacement of Val1747 with Ile reconstitutes the amino acid present within the human molecule.

The presence of heterozygous positions, when detected within affected individuals, has been used as evidence to exclude candidate genes from involvement in autosomal recessive canine PRA [30,31]. Testing in this study revealed heterozygosity at *ABCA4* exonic variants within a limited sample of affected individuals from nine of the eleven breeds examined. While this argues against the involvement of *ABCA4* with PRA in these breeds, it does not by itself constitute exclusion. Pairwise LD between the two *ABCA4* exonic SNPs showed they are tightly linked but not in complete disequilibrium (the genotype at one does not perfectly predict the genotype at the other). Exons 13 and 27 are separated by only 25 kB in the human homolog which indicates a neutral polymorphism, even when located within the same gene and closely linked to the mutation, may not be theoretically sufficient for exclusion. A second impediment is that disease heterogeneity may be more common in dog breeds than is widely recognized. Thus, a causal gene might be falsely excluded if a disease resulted from compound heteroallelism and exclusion was based solely on the presence of heterozygosity. Nonetheless, it is considered unlikely that *ABCA4* is a valid candidate gene for the forms of PRA in the breeds examined.

Analysis of polymorphism within even a small pedigree, as undertaken here for *crd1* and *crd2*, however, ensures exclusion of causal sequence variants in promoter or other regulatory elements physically located with the gene under investigation. We therefore confidently conclude that *ABCA4* can be excluded as the gene causing either *crd1* or *crd2*. This leaves open the question of which genes do cause these diseases. Because the canine retina developes postnatally, rather than prenatally as in humans, it can be argued that one or both of these 2 diseases might correspond to one of the apparently earlier onset human diseases classified as Leber Congenital Amaurosis. This would expand the list of candidate genes that might be worth evaluating individually before undertaking a genome wide screen. Such studies are currently underway.

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