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SHORT COMMUNICATION

Exclusion of the *PDE6A* gene for generalised progressive retinal atrophy in 11 breeds of dog

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Summary

The cyclic guanosine monophosphate specific phosphodiesterase (cGMP-specific PDE) is a key enzyme in the phototransduction cascade of the vertebrate retina. This enzyme consists of two catalytic α and β subunits, two identical inhibitory γ subunits as well as a δ subunit. Mutations in *PDE6A* and the *PDE6B* genes lead to autosomal recessive (ar) forms of retinitis pigmentosa (RP) in human and to the homologous disease in dogs, designated generalised progressive retinal atrophy (gPRA). We investigated the *PDE6A* gene in 13 gPRA-affected dog breeds including healthy animals, obligate gPRA carriers and gPRA-affected dogs. In the coding region of *PDE6A* only a rare sequence variation (G103A; Asp35Asn) was found in exon 1 of two healthy Tibet Terriers and one affected Cocker Spaniel. Using single-stranded conformation polymorphism (SSCP) analyses we detected several sequence variations in eight of the *PDE6A* introns in different investigated breeds. Most informative for excluding the *PDE6A* gene as a cause for gPRA was a polymorphic microsatellite ($(GT)_{10}CG(GT)_2CG(GT)_{12}$) in intron 14 and four sequence variations in intron 18 for almost all breeds investigated. The sequence variations of *PDE6A* did not segregate together with gPRA in 11 breeds. Since diseased animals were heterozygous for the polymorphisms, the *PDE6A* gene is unlikely to harbour the critical mutation causing gPRA in the following breeds: Chesapeake Bay Retriever, Entlebucher Sennenhund, Labrador Retriever, Tibet Mastiff, Dachshund (long-and wire-haired), Tibetan Terrier, Miniature Poodle, Australian Cattle Dog, Cocker Spaniel, Saarloos/Wolfshound, Sloughi.

Keywords: generalised progressive retinal atrophy, retinits pigmentosa, PDE6A, SSCP, microsatellite, dog

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The critical effector enzyme of the visual cascade is cGMP PDE, a heterotetrameric protein attached to the disc membrane. PDE hydrolyses 3′,5′-cGMP to 5′-GMP. PDE6A and PDE6B are catalytic subunits, that are complexed as dimers in rods while in cones PDEA homodimers exist. Both, the *PDE6A* and *PDE6B* genes show mutations leading to RP in humans (Danciger *et al.* 1995; Huang *et al.* 1995; McLaughlin *et al.* 1993). RP represents a genetically heterogeneous progressive photoreceptor degeneration in man which always ends in blindness. The disease is transmitted according to different modes of inheritance: autosomal dominant (adRP) and recessive (arRP), X-linked (XLRP), digenic as well as maternal. The homologous group of photoreceptor degeneration is designated as gPRA in dogs. gPRA is mainly inherited in an autosomal recessive manner, except for a single X-linked form (XLPRA) in Siberian Huskies (Acland *et al.* 1994; Clements *et al.* 1996). So far, the genetic origin of two forms of gPRA has been resolved: (1) the rod cone dysplasia 1 in Irish Setters is caused by a transition in codon 807 of the *PDE6B* gene (Suber *et al.* 1993) and (2) rod cone dysplasia 3 in Cardigan Welsh Corgi relates to a 1-bp deletion in codon 616 of the *PDE6A* gene (Petersen-Jones *et al.* 1999). The location of the canine *PDE6A* gene is unknown; the corresponding human gene maps to chromosome 5q31·2–34 (Pittler *et al.* 1990). We investigated the *PDE6A* gene as a candidate causing retinal degeneration in different dog breeds affected with gPRA (Table 1). For mutation screening, we used canine exon (Kylma *et al.* 1997) and intron sequences of *PDE6A* gene (Petersen-Jones *et al.* 1999, see accession numbers in Table 3; Dekomien accession number AJ251206).

Two hundred and thirty-one dogs from 13 different breeds including 59 gPRA-affected individuals were studied. DNA samples from four Chesapeake Bay Retrievers and 10 Labrador Retrievers were obtained from the Animal Health Trust Suffolk, UK (Table 1). Genomic DNA was extracted from peripheral blood according to standard protocols (Miller *et al.* 1988). For each dog coding and intron sequences were amplified (Table 2) by PCR in

Table 1. Dog breeds examined

Breed (abbreviation)	Total number of dogs	gPRA-affected dogs
Chesapeake Bay Retriever (CBR)	4	2
Entlebucher Sennenhund (ES)	3	1
Labrador Retriever (LR)	45	5
Tibet Mastiff (TM)	3	2
Dachshund long-and wire-haired (D)	51	18
Tibetan Terrier (TT)	32	3
Miniature Poodle (MP)	29	18
Australian Cattle Dog (AC)	8	2
Yorkshire Terrier (Y)	3	1
Cocker Spaniel (C)	2	2
Collie (Co)	2	1
Saarloos/Wolfhound (Sa)	46	3
Sloughi (Sl)	3	1
Total	231	59

a thermocycler (Biometra, Goettingen, Germany) from genomic DNA. PCRs were performed in 96-well microtiter plates (Thermowell Costar, Corning, NY). Each well contained 50 ng DNA in 10 μl 100 mM Tris (pH 8·3), 500 mM KCl, 1 U *Taq*I Polymerase (Gencraft, Münster, Germany), 0·2 mmol of each NTP, 0·4 mM of each primer and varying concentrations of $MgCl_2$ (Table 2). For SSCP and microsatellite analysis, 10 mCi/ml of [α^{32}P] dCTP was included in the PCR. A 'touchdown' PCR procedure was applied: initial denaturation step (5 min at 95 °C), two initial cycles at 6 and 3 °C above the annealing temperature (Table 2), 25 cycles of 95 °C (1 min), annealing temperature elongation at 72 °C (30 s) and a final elongation step at 72 °C (5 min). SSCP samples were treated according to Dekomien *et al.* (1998). PCR products were digested depending on the length of the fragments with different restriction enzymes (Table 3). PCRs (3 μl) were denatured with 7 μl of loading buffer (95% deionised formamide, 10 mM NaOH, 20 mM EDTA, 0·06% (w/v) xylene cyanol and 0·06% (w/v) bromophenol blue). The samples were heated to 95 °C for 5 min and snap cooled on ice. Aliquots (3 μl) of the single stranded fragments were separated through two sets of 6% polyacrylamide (acrylamide/bisacrylamide: 19/1) gels, one set with 10% glycerol, another with 5% glycerol and 1 M urea. Gels were run with 1× TBE buffer at 30–50 W for 4–6 h at 4 °C. For analyses of the microsatellite in intron 14, 5 μl PCR product was denatured in 5 μl loading buffer at 95 °C, and separated on 6% polyacrylamide (acrylamide/bisacrylamide: 19/1) gels containing 8 M urea, and 1× TBE (60 W, RT). All gels were dried and analysed by autoradiography. Sequencing reactions were run on an automatic DNA sequencer (Applied Biosystems 373A, Forster City, CA) and analysed with the corresponding software.

The *PDE6A* gene has 22 exons. In addition to exons 1–4 and 10–22, the introns 2, 11, 15, 16 and 18 were also screened completely for sequence variations. The short exons 5–9 were not examined, because we detected many informative sequence variations in the other parts of the *PDE6A* gene which allowed indirect gene analysis. Altogether we evaluated the DNA of 231 dogs from 13 breeds (Table 1) including 59 gPRA affected dogs. SSCP and microsatellite analyses of genomic DNA revealed several sequence polymorphisms among the dogs. PCR products for each unique SSCP or microsatellite patterns were sequenced in order to identify the underlying DNA variation. The sequences are summarised in Table 3. In exon 1 of the *PDE6A* gene we identified an infrequent substitution (G103A) that causes an Asp35Asn exchange in two unaffected control Tibetan Terriers and an affected Cocker Spaniel. Sequence variants with respect to the published sequences (Kylma *et al.* 1997; Petersen-Jones *et al.* 1998; EMBL accession number AJ251206) were observed in both unaffected and gPRA-affected dogs (Table 3). Thus polymorphisms within the PDE6A gene did not discriminate between affected and unaffected dogs. Furthermore, with the exception of Collie and Yorkshire Terrier all sequence variants were observed in the heterozygous state in affected dogs. The affected Collie was homozygous for intron 14 and 18 variants. The Yorkshire Terrier was homozygous for intron 11, 14, 15 and 18 variants. Therefore, these intronic sequence variations aided to exclude the *PDE6A* gene indirectly as causative for the gPRA in Chesapeake Bay Retrievers, Entlebucher Sennenhunds, Labrador Retrievers, Tibet Mastiffs, Dachshunds, Tibetan Terriers, Mini-

Table 2. Primers for PCR amplifications of individual exons (and introns)

Exon	Forward primer 5′→3′	Accession numbers/ location	Reverse primer 5′→3′	Accession numbers/ location	PCR conditions (T)/[$MgCl_2$]/[DMSO]/ [formamide]
1	1·1 ATGGGTGAGGTGACAGCAG	Z68340/94–112	1·2 CTCTTCTGTGTTGACGACGT	Z68340/548–567	59 °C/1 mM/–/3%
2–3	2-IF GATGAGCATTTCTGTGACTTTG	AJ233678/1–22	3-IR CTGGCCACGCCGAGTCT	AJ233678/969–985	56 °C/2 mM/–/–
4	4-IF TCTGTCTTGGAGTTATTGTGAA	AJ251206/33–54	4·2 CTTCTGCTTGGTCATGTCTAA	Z68340/931–951	57 °C/2 mM/–/–
10	10·1 TCTTTGGCTCAATTCCTGGG	Z68340/1357–1376	10-IR CACCTGTCTCTGCCCTCAT	AJ233685/427–445	54 °C/1 mM/–/–
11–12	11-IF TCTAGACCTCAGATAGATAAA	AJ233686/217–238	12·2 CTCCTGAGGAATGTGAAATTT	Z68340/1693–1713	54 °C/4 mM/5%/–
13	13-IF CAAAGTACACTCACACGTTA	AJ233687/103–123	13·2 CACCAGCAAGGAGAACATG	Z68340/1803–1801	54 °C/1 mM/–/–
14	14·1 ACCGGAAAGCTGAAGCGATA	Z68340/1822–1841	14·2 TTCATCTGGTAGAGATTGTT	Z68340/1912–31	52 °C/2 mM/–/–
15	15-IF AGGACTGGGTGAGGATGATA	AJ233689/310–330	15-IR CACTCAAGCTTCTTGTGAGAA	AJ233689/532–550	54 °C/2 mM/–/–
15–16	15-IF AGGACTGGGTGAGGATGATA	AJ233689/310–330	16·2 TTGAAATACAGGGCGAGGTC	Z68340/2101–2120	54 °C/2 mM/–/–
16–17	16·1 AGCCTGAATATCTTTCAAAAC	Z68340/2020–2040	17·2 ATAACAATTTCCTTCCGTGTC	Z68340/2208–2228	54 °C/3 mM/–/–
18–19	18·1* AGGGCCATGATGATGACCG	AJ233690/1–17	19-IR GGTAAGATCACATGCACCTAT	AJ233690/501–522	57 °C/2 mM/–/–
20	20-IF AACAAGAATGCTTCATAGGT	AJ233691/126–145	20-IR TCCTGAGGACCAAATGCCTT	AJ233691/431–450	54 °C/2 mM/–/–
21	21-IF GGTTGCCAAAGGAGGGTAA	AJ233692/2–20	21·2 TGGCTGTCTGCTGCTTCTG	Z68340/2575–2593	54 °C/2 mM/–/–
22	22-IF CCTTAGTTTGCAACTTGGTCTA	AJ233693/430–451	22UTR CAAATCCGATTACTATTTTAC	Z68340/2770–2791	53 °C/3 mM/–/–

*The primer was elongated to include the conserved splice site sequence 'AG'.

Table 3. *PDE6A* sequence variations (heterozygous pattern) in gPRA-affected dogs in respective breeds (for abbreviations see Table 1)

Exon–intron	Sequence variation*	Fragment length (bp)	Restriction	Breed†
Exon1	G103A→D35N	*488 bp*: 14 135 231	*Eco*57I	C
Intron2	Intron2 + 53 C→T	*985 bp*: 273, 125, 9, 45, 236, 181	*Mse*I	D, AC, CBR
Intron10	Intron10 + 51 T→C	*239 bp*: 128, 111	*Sau*3AI	CBR
Intron11	Intron11 + 82 insC	*493 bp*: 125, 117, 251	*Sau*3AI	MP, D, TT
Intron14‡	Microsatellite–11del $(GT)_1$[1]; –11del $(GT)_3$[2]; –36 + –42 C→A[3]; –63 del $(GT)_1$[4];	*486 bp*: 139, 96, 92, 159	*Alu*I	MP[1+3], D[1], CBR[2+4], Sl[1], TM[2+4]
Intron15	Intron15 + 36 G→A	*486 bp*: 139, 96, 92, 159	*Alu*I	MP, D, CBR, Sl, TM, Sa
Intron16	Intron16 + 41 6 bp del	*626 bp*: 221, 268, 100, 37	*Alu*I, *Rsa*I	TM, D
Intron18§	Intron18 + 55 C→T; intron18 + 65 T→C; intron18 + 88 A→C; intron18 + 121 G→C	*501 bp*: 46, 54, 165, 126, 110	*Mva*I	MP, D, CBR, ES, C, TM, Sa, TT, LR
Intron20	Intron20 + 29 G→A	*326 bp*: 200, 126	*Hae*III	MP
Intron20	Intron20 + 56 A→C	*326 bp*: 200, 126	*Hae*III	CBR, Sa

*Sequence variations are expressed with reference to the published sequence (Kylma *et al.* 1997; Petersen-Jones *et al.* 1998; EMBL accession number AJ251206).
†The sequence variants were observed in the breeds listed.
‡Numbers in superscript identify observed variations in the different breeds.
§Haplotypes in intron 18 were either C–T–A–G or T–C–C–C in the respective dog breeds.

ature Poodles, Australian Cattle dogs, Cocker Spaniels, Saarloos/Wolfhounds and Sloughis. The founder effect can be exploited for genetic analysis: animals affected by autosomal recessively transmitted traits are expected to be homozygous not only for the disease causing mutation but also for tightly linked nonpathogenic sequence variations in the same gene. Therefore, in affected animals the following facts provide good evidence for excluding the candidate gene in question as being implicated in the retinal disorder in a definite breed: (1) heterozygosity for any sequence variations or (2) homozygosity patterns for two different alleles of the same polymorphism. For the Collie, the gPRA-affected dogs were typed homozygous for the detected sequence variants in intron 14 and 18 and the Yorkshire Terrier in intron 11, 14, 15 and 18. Therefore a disease causing mutation in the *PDE6A* gene could not be excluded in these breeds. gPRA in the Collie is characterised by similar biochemical and histopathological changes as in Cardigan Welsh Corgis (Petersen-Jones *et al.* 1999) and Irish Setters (Suber *et al.* 1993). However, the typical 1-bp deletion in codon 616 of Cardigan Welsh Corgis could be excluded in the gPRA affected dogs of the aforementioned two breeds in our study. Recently, Wang *et al.* (1999) excluded a mutation in the *PDE6 A* gene as a cause for rcd2 in collies. Further investigations will have to clarify which mutations are decisive for gPRA in Yorkshire Terrier.

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