

Analysis of *PDE6D* and *PDE6G* genes for generalised progressive retinal atrophy (gPRA) mutations in dogs

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Abstract – The δ and γ subunits of the *cGMP-phosphodiesterase* (*PDE6D*, *PDE6G*) genes were screened in order to identify mutations causing generalised progressive retinal atrophy (gPRA) in dogs. In the *PDE6D* gene, single nucleotide polymorphisms (SNP) were observed in exon 4, in introns 2 and 3 and in the 3' untranslated region (UTR) of different dog breeds. In the coding region of the *PDE6G* gene, exclusively healthy Labrador Retrievers showed an A \rightarrow G transition in exon 4 without amino acid exchange. SNP were also observed in introns 1 and 2 in different dog breeds. The different SNP were used as intragenic markers to investigate the involvement of both genes in gPRA. The informative substitutions allowed us to exclude mutations in the *PDE6D* and *PDE6G* genes as causing retinal degeneration in 15 of the 22 dog breeds with presumed autosomal recessively transmitted (ar) gPRA.

***cGMP-phosphodiesterase* / canine / generalised progressive retinal atrophy / SNP / retinitis pigmentosa / SSCP**

1. INTRODUCTION

Rod *cGMP-phosphodiesterase* (PDE) is the G-protein-activated effector enzyme that regulates the level of cGMP in vertebrate photoreceptor cells [3, 13]. Rod cGMP PDE is generally viewed as a protein composed of catalytic α and β subunits, two identical inhibitory γ subunits [30] and a δ subunit. Respective DNA sequences were recently identified in men, mice, cows and dogs [15, 20, 21]. The exact function of the δ subunit is still not known, since *in vitro* it does not affect the catalytic activity of PDE. Loss of γ subunits entails reduced hydrolytic activity and leads to an increased PDE activity [32].

Defects in genes encoding PDE subunits have been associated with retinal disease in humans and several animal models [5, 6, 16, 20, 22, 26, 31, 32]. For autosomal recessively transmitted (ar), generalised progressive retinal atrophy

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(gPRA), the most common hereditary form in dogs, mutations have been identified in the β subunit of the PDE (*PDE6B*) gene in Irish Setters and Sloughis [12, 31] and in the α subunit (*PDE6A*) gene in Cardigan Welsh Corgis [25].

Retinitis pigmentosa (RP) in man is the homologous disease to gPRA in dogs. Ar transmitted forms of RP have been mapped to the δ subunit (*PDE6D*; 2q35–36; RP26) and to the γ subunit genes in man (*PDE6G*; 17q25 RP17; RetNet: <http://www.sph.uth.tmc.edu/Retnet/>). On the basis of reciprocal chromosome painting the canine *PDE6D* gene is, therefore, predicted to map to CFA 25 [7] and the *PDE6G* gene to CFA 9 [36], the homologous chromosomal regions in dogs. These genes were recently excluded for rod-cone dysplasia 2 (*rcd2*) in collies [34], but mutations in these genes could cause gPRA in other breeds. Therefore, these genes were investigated as candidate genes for gPRA in 22 different breeds including gPRA affected dogs.

2. MATERIALS AND METHODS

2.1. Animals

The blood of 808 dogs from 22 different breeds including 114 gPRA-affected animals (see Tab. I) was received from the owners in cooperation with breeding organisations. The blood of most dogs was obtained from different regions of Germany. In addition, several Saarloos Wolfdogs (Sa), Schapendoes (SD), Sloughi (Sl) and Tibetan Terriers (TT) originated from the Netherlands (Sa, SD), Switzerland (Sl, TT), Sweden (Sl) and the USA (Sl). By observing the cases of PRA in the pedigrees, the breeders have assumed ar inheritance in the following breeds (personal communications): Australian Cattle Dog, Collie, Dachshund, Engl. Cocker Spaniel, Entlebuch Cattle dog, Irish Setter, Labrador Retriever, Miniature Poodle, Saarloos Wolfdog, Schapendoes, Sloughi and Tibetan Terrier. Experienced veterinarians confirmed the gPRA status of affected and unaffected dogs by ophthalmoscopy.

2.2. Isolation of DNA and polymerase chain reaction (PCR)

DNA was extracted from the peripheral blood according to standard protocols [23]. Genomic DNA from each affected dog as well as representative healthy dogs and obligatory carriers was screened for mutations. Parts of the *PDE6D* and *PDE6G* genes were amplified by PCR in a thermocycler (Biometra, Goettingen, Germany). PCR were performed in 96-well microtiter plates (Thermowell Costar Corning, NY). Each well contained 50 ng DNA in a 10 μ L reaction volume 100 mM Tris (pH 8.3), 500 mM KCl, 1 U *Taq* Polymerase (Genecraft, Münster, Germany), 0.2 mM of each dNTP, 0.4 mM of each primer and varying concentrations of $MgCl_2$ (see Tab. II). For SSCP analysis, 0.06 μ L of [$\alpha^{32}P$] dCTP (10 mCi \cdot mL $^{-1}$) was included in the PCR.

Table I. Characteristics of dog breeds examined.

Breed (abbreviation)	Number of dogs	Diagnosis	Onset forms of gPRA	Age distribution (year) ^f
Australian Cattle Dog (AC)	2	gPRA-affected	late ^a	10
	19	normal		2–4
Pyrenean Sheepdog (BDP)	1	gPRA-affected	mid-onset ^b	5
	42	normal		1–10
Bernese Mountain Dog (BMD)	1	gPRA-affected	late ^b	10
Bolognese (Bo)	1	gPRA-affected	late ^b	9
Collie (Co)	3	gPRA-affected	early ^{cd}	4–8
	1	normal		8
Dachshund (wire; D)	20	gPRA-affected	variable ^b	1–13
	49	normal		6–13
English Cocker Spaniel (ECS)	6	gPRA-affected	late ^{cd}	3–11
	6	normal		6–14
Entlebuch Cattle dog (EC)	17	gPRA-affected	late ^e	5–13
	10	normal		1–7
Golden Retriever (GR)	2	gPRA-affected	late ^b	5–10
	8	normal		3–6
Irish Setter (IRS)	2	gPRA-affected	early ^{cd} /late ^b	0.6–7
	1	normal		3–13
Labrador Retriever (LR)	5	gPRA-affected	late ^{cd}	8–12
	139	normal		3–13
Miniature Poodle (MP)	28	gPRA-affected	late ^{cd}	5–12
	15	normal		1–12
Newfoundland (NF)	1	gPRA-affected	mid-onset ^b	3
Polish Lowland Sheepdog (PON)	1	gPRA-affected	late ^b	9
Rottweiler (Ro)	1	gPRA-affected	late ^b	3
Saarloos Wolf dog (Sa)	7	gPRA-affected	late ^b	7–9
	118	normal		2–10
Scottish Terrier (ScT)	1	gPRA-affected	late ^b	6
Schapendoes (SD)	3	gPRA-affected	early ^d	2–6
	7	normal		3–6
Sloughi (Sl)	5	gPRA-affected	mid-onset ^b	2
	183	normal		0.1–12
Tibetan Mastiff (TM)	2	gPRA-affected		
	1	normal		
Tibetan Terrier (TT)	3	gPRA-affected	mid-onset ^{cd}	7–8
	93	normal		2–10

^a [19]; ^b owners report/certificate of eye examination; ^c classifications of the different onset forms of gPRA in the reviews ([9] and [24]); ^d online information among PRA Today (<http://www.sheepdog.com/diseases/prac/clinical>); ^e [29]; ^f at the time when blood was taken for DNA analysis.

Table II. Primers and conditions for PCR amplification of the canine *PDE6D* and *PDE6G* genes for mutation screening.
(continued on the next page)

Primer	Location (bp) ^a	Forward primer reverse primer (5'-3')	PCR conditions [T-°C/ MgCl ₂ -mM]	PCR amplicon length (bp)	Restriction enzymes for SSCP analyses
<i>PDE6D</i> ^b					
1 F	1	CCGTC TCGGAGGCTCCGC	55/1	109	-
1a R	109 ^d	ACAGTTTGAAGCCCTTCAGG			
1a-2 F	551	TCGTGGCACTTAGCAGATAG	54/1	218	<i>TruI</i>
E1-2 R	768	CACCAGGGACAGACAAAGTC			
1b F	690	AGAAATTGGATGAACCTCCGG	59/1.5	213	<i>XmnI</i>
1 R	901	ACAACAACACATGCTGTG			
1-12 F	1354	CTGTAGCTATCTCTGTGACT	55/1	190	<i>DdeI</i>
E 2 R	1540	CCTTCTAGGCAITGCCCTTT			
E 2 F	1412	AGCCCGTGTTCCTCCCAAGAAA	60/1	907	<i>MnII</i> + <i>XmnI</i>
E 3 R	2318	ACGTTAGCACACTGGCGG			
E 3 F	2210	GAATGGTCTTCGAGTTTGG	52/2	249	<i>XbaI</i>
3 R	2458	ACAGAAGTCAGTAACT			
4 F	4110	GGTTCTAAGTGGGTGCATGT	52/2 ^e	674	<i>MboII</i>
4 R	4783	CATTATGTAATAATAATATCAGTC			

Table II. Continued.

Primer	Location (bp) ^a	Forward primer reverse primer (5'-3')	PCR conditions [T-°C/ MgCl ₂ -mM]	PCR amplicon length (bp)	Restriction enzymes for SSCP analyses
<i>PDE6G</i> ^c					
UTR-A1	1	GAGCACACCCGTGACCCT	57/0.5 ^e	440	<i>AvaI</i>
UTR-B1	440	CCGGCTGCTCTGGCCCCCT			
UTR-A2	496	ACCACCTGGGCTGGGGA	57/1 ^e	887	<i>PvuII</i>
UTR-B2	1283	CTGGAACCAGGAGACCCAGG			
UTR-MA2	669	GGTGGTGCCTGGGCATCT	60/1.5	149	-
UTR-MB2	801	ACCCTGCTCAAGGGCAA			
I-2A	1196	CTGCCGTGACCCAGGTGGA	53/2	416	<i>HinfI/MseI</i>
I-2B	1611	CCCAATTCTGGGTAGCC			
I-3A	1782	CCTGTGTCCCCCGCATGCA	58/1	153	-
I-3B	1934	CGGGAGAGTTGGGGGATC			
I-4A	2181	CTCTGGGCGTGGACAACA	58/1	205	-
I-4B	2385	GGCACCCGGAGCAGGGGA			
E-2A	1398	TTCTCTGCCAACCCCTGGCC	58/2.5 ^e	537	<i>AvaII</i>
I-3B	1934	CGGGAGAGTTGGGGGATC			
I-3A	1782	CCTGTGTCCCCCGCATGCA	57/1.5	551	<i>BstHKAI</i>
E-4B	2332	TGGGTCAGGCTCTGGGCG			

^a Nucleotide position to which the 5' end of the primer (bold face) hybridises; ^b PCR primer sequences selected from the canine *PDE6D* gene (EMBL accession numbers AJ427395 and AJ427396); ^c PCR primers on the basis of the canine *PDE6G* gene ([35]; EMBL accession number CF49360) for mutation analysis; ^d including the putative splice donor site; ^e addition of 5% formamide.

For genomic mutation analysis the following PCR procedure was applied: an initial denaturation step (5 min at 95 °C), 10 initial cycles 1 °C above the annealing temperature (see Tab. II), 22–25 cycles of 95 °C (30 s), annealing temperature (30 s), elongation at 72 °C (30 s) and a final elongation step at 72 °C (3 min).

2.3. Cloning and identification of exon/intron junctions of intron 1 of the *PDE6D* gene

Parts of the *PDE6D* gene were cloned from a genomic canine λ -DNA library (λ FIX[®] II Library; host: *E. coli* XL1-Blu MRA (P2) Stratagene, La Jolla, CA, USA) according to the Stratagene standard protocol. Recombinant λ DNA was fixed to Hybond[™]-N Nylon membranes (Amersham, Buckinghamshire, UK) and UV-crosslinked (1' 70 mJ · cm⁻²). The library was screened using PCR amplicates from exon 2 corresponding to nucleotide positions 109–321 of the canine *PDE6D* gene (EMBL accession number AF113996). These probes were labelled using [α ³²P] dATP and the Megaprime Labelling System (Amersham, Buckinghamshire, UK). Hybridisations were performed as described [8] and hybridising clones were isolated and plaque purified [28]. Exon 1 of the *PDE6D* gene was not identified in the clones. To search for sequences of intron 1 of the *PDE6D* gene, the isolated λ clones were digested with *Hind*III and subcloned in pBluescript[®] II+ phagemid (Stratagene, La Jolla; [27]). Parts of the cloned intron 1 were amplified by PCR using the T7 primer (for the λ phage) and an exonic primer specific for exon 2 in order to characterize the intron-exon boundary of the *PDE6D* gene (EMBL accession number AJ427396). For annealing temperatures see Table II. Long-range PCR using the Elongase enzyme mix (GIBCO BRL, Karlsruhe, Germany) was performed from genomic DNA in order to identify the splice donor site of intron 1 according to the recommendations of the manufacturer. Sequencing reactions of 2–3 clones including exon 2, introns 1 and 2 were carried out by the dideoxy-chain termination method using the Big Dye Terminator (Perkin-Elmer, Norwalk, CT, USA) according to the manufacturer's instructions. All sequencing reactions were run on an automated DNA sequencer (Applied Biosystems 373 XL, Foster City, USA) and analysed using the corresponding software.

2.4. PCR-SSCP and DNA sequence analyses of the *PDE6D* and *PDE6G* genes

Primers were created for mutation screening of intron 1 after DNA sequence analysis of the genomic *PDE6D* clones and genomic sequences of the *PDE6D* and *PDE6G* genes (CF49360; see Tab. II). SSCP samples were treated as described [10, 11]. PCR products were digested dependent on the lengths of the fragments [17] with different restriction enzymes (see Tab. II). The sequence

variations in the *PDE6D* gene were investigated in intron 3 (with *MnII*; *XmaI*) and exon 5 (with *HaeIII*) using restriction fragment length polymorphism (RFLP) analysis. Three μL of the PCR 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. Three μL aliquots of the single-stranded fragments were separated through two sets of 6% polyacrylamide (acrylamide/bisacrylamide: 19/1) gels, one set containing 10% glycerol, and the other containing 5% glycerol and 1 M urea. The gels were run with 1X TBE buffer at 50–55 W for 4–6 h at 4 °C. All gels were dried and subjected to autoradiography over night. All DNA samples with band shifts evidenced by SSCP electrophoresis were purified and cycle sequenced as described above.

3. RESULTS AND DISCUSSION

3.1. Identification of intron 1 in the canine *PDE6D* gene

It was demonstrated that the human *PDE6D* gene comprises five exons [21] vs. four exons in dogs [35]. Since the described “exon 1” of the *PDE6D* gene of dogs could not be amplified from genomic DNA, an additional intron was also assumed in dogs. Therefore, three genomic DNA clones with parts of the *PDE6D* gene were isolated from a λ -DNA library. Yet the 5' part of “exon 1” was always lacking in these clones. Comparisons with the recently published human genomic DNA (EMBL accession number AC073476) showed an intron 1 size of 41 877 base pairs (bp). Therefore, the canine intron 1 may well exceed clonable sizes in λ -phages. Also, the exact size of intron 1 could not be determined *via* long range PCR of genomic DNA. Parts of intron 1 were sequenced after subcloning of the inserts of the λ -phages and PCR (splice acceptor site intron1/exon2: atattgatc**ag**AAATTGGATGAA).

3.2. Mutation analysis

All coding exons of the *PDE6D* and *PDE6G* genes were investigated by PCR-SSCP analysis including splice donor and acceptor sites as well as adjacent intronic sequences except for 20 bp (primer sequence) of exon 1 and the splice donor site of intron 1 in the *PDE6D* gene. The DNA of 22 dog breeds including 114 gPRA-affected animals are covered in this study. For six of these breeds either the causative gPRA mutations (Irish Setter [31], and Sloughi [12]) or linked markers for the progressive rod cone degeneration (*prcd*) form of gPRA are already known (Australian Cattle Dog, English Cocker Spaniel, Labrador Retriever, Miniature Poodle; patented by OptiGen, USA). The *PDE6G* gene is located near the *prcd* region, but is excluded as a cause for RP 17 in man [4], the homologous gPRA form in these

breeds [1]. The other 16 dog breeds were included in the analysis, because all polymorphisms identified in these six breeds could then be excluded as a causative mutation for gPRA in the remaining breeds. A second gPRA form may exist in Irish Setters since one affected Setter showed late manifestation of gPRA symptoms without the typical *PDE6B* mutation. Because of similar phenotypic heterogeneity also in Miniature poodles, two forms of gPRA are possible (see <http://www.optigen.com>).

3.2.1. *PDE6D* gene

In the coding region of the *PDE6D* gene, no polymorphisms were identified. To identify intragenic SNP markers for the exclusion of the *PDE6D* gene as a cause for gPRA, the 3' UTR and intron 3 were screened completely. Sequencing of the canine *PDE6D* gene revealed several differences to the published data [35]: in intron 3 five exchanges and in the 3'UTR a single sequence variation were identified in all genomic DNA. Furthermore SNP were observed in intron 2 (874A → T), 3 (1808A → G; 2166T → A) and the untranslated exon 5 (4439C → T; 4483T → C; 4664C → T) of different dog breeds in the *PDE6D* gene (Tab. III).

Table III. *PDE6D* and *PDE6G* sequence variations and heterozygous patterns in gPRA-affected dogs.

Gene	Location	Sequence variation	Amino acid exchange	Breed(s) ^a
<i>PDE6D</i> ^b	Intron 2	847A → T	–	LR, NF
<i>PDE6D</i>	Intron 3	1808A → G	–	Bo, BMD, Co, EC, LR, MP, NF, Ro, Sa, SD
		2166T → A	–	BMD, EC, LR, Ro
<i>PDE6D</i>	3'UTR Exon 5	4439C → T	–	SD
		4483T → C	–	AC, BMD, Co, ECS, EC, LR, MP, Ro, SI
		4664C → T	–	AC, D, Co, ECS, EC, IRS, LR, MP, Ro, SD, SI
<i>PDE6G</i> ^c	Intron 1	744G → A	–	AC, BMD, Co, D, ECS, EC, GR, IRS, LR, MP, Ro, Sa, ScT, SD, TT
<i>PDE6G</i>	Intron 2	1662C → T	–	ECS, EC, LR, Sa, SD
		1694G → A	–	ECS, EC, LR, Sa, SD
<i>PDE6G</i>	Exon 4	2285G → A	(L78L)	(LR) [*]

^a For abbreviations see Table I; ^b position of SNP of the *PDE6D* gene refer to EMBL accession number AJ427396; ^c SNP of the *PDE6G* gene refer to EMBL accession number CF49360; * heterozygous sequence variation in healthy Labrador Retrievers.

3.2.2. *PDE6G* gene

In the coding region of the *PDE6G* gene (exon 4) a “silent” sequence variation was identified at position 2285 (G → A) in healthy Labrador retrievers. The additional PCR-SSCP analysis of the complete 5′UTR, parts of the 3′UTR and the two introns revealed informative SNP in intron 1, (position 744, G → A) and in intron 2 (position 1662; C → T; position 1694, G → A; see Tab. III).

3.3. Exclusion of *PDE6D* and *PDE6G* genes for ar transmitted gPRA

The identified intronic SNP were found in the heterozygous and homozygous states in gPRA affected and unaffected dogs of different breeds (see Tab. III). The breeding history, small population sizes and gPRA abundance in the investigated breeds point together to a few meiotic events in which intragenic recombinations could have occurred between any unidentified mutation in the *PDE6* loci and the SNP investigated here. Although the complete promoters and all introns of the *PDE6D* and *PDE6G* genes could not be included in the SSCP analyses, the observed sequence variations can be used as intragenic markers for excluding the *PDE6D* and *PDE6G* genes as causing the ar transmitted eye disease. gPRA is most commonly inherited as an ar transmitted trait although in two dog breeds it is sex linked (Samojed and Siberian Husky [2]) and in one there is autosomal dominant (Mastiff) inheritance [18]. By assuming ar inheritance, we excluded *PDE6D* as a candidate gene for gPRA *via* intragenic SNP in 15 breeds: Australian Cattle Dog, Bernese Mountain Dog, Bolognese, Collie, Entlebuch Cattle dog, Dachshund, English Cocker Spaniel, Irish Setter, Labrador Retriever, Miniature Poodle, Newfoundland, Rottweiler, Saarloos Wolf dog, Schapendoes and Sloughi. Similarly, in 15 breeds the *PDE6G* gene was excluded for the assumed ar gPRA in the Australian Cattle Dog, Bernese Mountain Dog, Collie, Dachshund, English Cocker Spaniel, Entlebuch Cattle dog, Golden Retriever, Irish Setter, Labrador Retriever, Miniature Poodle, Rottweiler, Saarloos Wolf dog, Scottish Terrier, Schapendoes and Tibetan Terrier. Some dog breeds are only represented by one gPRA affected individual (Tab. I). For these breeds the exclusion of the *PDE6D* and *PDE6G* genes is not definitive, since the possibility of false clinical diagnosis is not ruled out completely. Fortunately, the identified SNP in the *PDE6D* and *PDE6G* genes occurred in several breeds. Therefore, it is possible to use these markers in further studies [33].

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