

Research article

## Screening of the *arrestin* gene in dogs afflicted with generalized progressive retinal atrophy

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### Abstract

**Background:** Intronic DNA sequences of the canine *arrestin* (*SAG*) gene was screened to identify potential disease causing mutations in dogs with generalized progressive retinal atrophy (gPRA). The intronic sequences flanking each of the 16 exons were obtained from clones of a canine genomic library.

**Results:** Using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequence analyses we screened affected and unaffected dogs of 23 breeds with presumed autosomal recessively (*ar*) transmitted gPRA. In the coding region of the *SAG* gene 12 nucleotide exchanges were identified, 5 of which lead to amino acid substitutions (H14C; A111V; A113T; D259T; A379E). 7 other exonic substitutions represent silent polymorphisms (C132C; Q199Q; H225H; V247V; P264P; T288T and L293L). 16 additional sequence variations were observed in intronic regions of different dog breeds.

**Conclusions:** In several breeds, these polymorphisms were found in homozygous state in unaffected and in heterozygous state in affected animals. Consequently these informative substitutions provide evidence to exclude mutations in the *SAG* gene as causing retinal degeneration in 14 of the 23 dog breeds with presumed *ar* transmitted gPRA.

### Background

gPRA is usually inherited as an *ar* blinding disorder with different ages of onset and variable rate of progression observed in more than 100 dog breeds. Typically, gPRA commences with degeneration of the rod photoreceptors. Initial signs include night blindness whereas progression involves the cones and the central vision [1,2]. The human equivalent of canine gPRA is termed retinitis pigmentosa (RP). RP comprises a large and genetically heterogeneous group of blinding disorders. RP may be inherited in an *ar*, dominant, X-linked, digenic or maternal mode [3–7]. Similarly in dogs, at least 4 genes were iden-

tified so far as causing gPRA in 6 breeds. All of these genes encode photoreceptor specific proteins involved in the visual transduction cascade including the  $\beta$ -subunit of the cGMP-specific phosphodiesterase (PDE6B) in Irish Setters and Sloughis [8,9] as well as the  $\alpha$ -subunit of the cGMP-specific phosphodiesterase (PDE6A) in Cardigan Welsh Corgis [10]. A missense mutation was detected in the *PDC* gene that may be associated with photoreceptor dysplasia, a form of gPRA in the Miniature Schnauzer [11]. The X-linked form of PRA in Samoyed and Siberian Husky is caused by mutations in the *RPGR* gene [12]. Recently in English Mastiff dogs an autosomal dominantly transmit-

ted form of gPRA was identified, mimicking human RP. The disease causing mutation is a T4R exchange in the *rhodopsin* (*RHO*) gene [13]. A number of other retinal genes have been excluded as harbouring mutations for gPRA in several dog breeds: *RHO*; [14], *RDS/peripherin* and *ROM-1* [15] as well as the  $\alpha$  - and  $\gamma$ -subunits of *transducin*[16]

and *SAG*[17]. Yet, the *SAG* gene had been analyzed on the exonic level exclusively, *i.e.* by sequencing of cDNA. The human *SAG* gene comprises 16 exons ranging in size between 243 and 10 bp. *SAG* protein (403 amino acids) has been identified only in retinal photoreceptor rods and pinealocytes [18].

**Table 1: Characteristics of dog breeds examined**

Breed (abbreviation)	Number of dogs	Diagnosis	Onset forms of gPRA	Age distribution (year) <sup>f</sup>
Afghan Hound (AW)	1	gPRA-affected	late <sup>b</sup>	6
	4	normal		2–12
Australian Cattle dogs (AC)	2	gPRA-affected	late <sup>a</sup>	10
	19	normal		2–4
Berger des Pyrénées (BDP)	1	gPRA-affected	mid-onset <sup>b</sup>	5
	42	normal		1–10
Berner Sennenhund (BS)	1	gPRA-affected	late <sup>b</sup>	10
Bolognese (Bo)	1	gPRA-affected	late <sup>b</sup>	9
Collie (Co)	3	gPRA-affected	early <sup>cd</sup>	4–8
	1	normal		8
Curly Coated Retriever (CCR)	1	gPRA-affected	late <sup>b</sup>	6
Dachshund (wire; D)	20	gPRA-affected	variable <sup>b</sup>	1–13
	49	normal		6–13
Engl. Cocker Spaniel (ECS)	6	gPRA-affected	late <sup>cd</sup>	3–11
	6	normal		6–14
English Springer Spaniel (SP)	1	gPRA-affected		7
Entlebucher Mountain Dog (EM)	17	gPRA-affected	late <sup>e</sup>	5–13
	10	normal		1–7
Giant Schnauzer (GS)	1	gPRA-affected	late <sup>b</sup>	10
Golden Retriever (GR)	2	gPRA-affected	late <sup>b</sup>	5–10
	8	normal		3–6
Irish Setter (IRS)	2	gPRA-affected	early <sup>cd</sup> /late <sup>b</sup>	0.6–7
	1	normal		3–13
Labrador Retriever (LR)	5	gPRA-affected	late <sup>cd</sup>	8–12
	140	normal		3–13
Miniature poodle (MP)	28	gPRA-affected	late <sup>cd</sup>	5–12
	15	normal		1–12
Newfoundland Dog (NF)	1	gPRA-affected	mid-onset <sup>b</sup>	3
Polski Owczarek Nizinny (PON)	1	gPRA-affected	late <sup>b</sup>	9
Rottweiler (Ro)	1	gPRA-affected	late <sup>b</sup>	3
Saarloos/Wolfhond (Sa)	7	gPRA-affected	late <sup>b</sup>	7–9
	118	normal		2–10
Schapendoes (SD)	3	gPRA-affected	early <sup>d</sup>	2–6
	7	normal		3–6
Sloughi (SI)	5	gPRA-affected	mid-onset <sup>b</sup>	2
	183	normal		0.1–12
Tibetan Terrier (TT)	3	gPRA-affected	mid-onset <sup>cd</sup>	7–8
	93	normal		2–10

<sup>a</sup>[34]. <sup>b</sup> owners report/certificate of eye examination. <sup>c</sup> classifications of the different onset forms of gPRA in the reviews [1] and [2]. <sup>d</sup> online information among PRA Today [<http://www.sheepdog.com/diseases/practical/>]. <sup>e</sup>[35]. <sup>f</sup> at the time when blood was taken for DNA analysis.

SAG belongs to a family of inhibitory proteins that bind to tyrosine-phosphorylated receptors, thereby blocking their interaction with G-proteins and effectively terminating the signalling chain. In the phototransduction cascade, SAG and rhodopsin kinase (RHOK) act together in the recovery phase of RHO. After photoactivation, RHOK phosphorylates photoexcited RHO which is then blocked by SAG binding thus inhibiting its ability to interact with transducin [19,20]. The existence of stable complexes between RHO and its regulatory protein SAG were demonstrated to be responsible for retinal degeneration in

several mutations in *Drosophila* [21]. Accumulation of these complexes triggers apoptotic cell death showing that retinal degeneration requires the endocytic machinery (*op. cit.*). Interestingly, loss of function in the SAG gene causes an inherited Oguchi disease in Japanese, a variant of congenital stationary night blindness [22,23]. Apparently the mutation causing Oguchi disease can also lead to arRP in Japanese families [24]. Here we report on the identification of intronic sequences and mutation screening of the canine photoreceptor-specific SAG gene in 19 different dog breeds.

**Table 2: Primers for the characterization of the SAG gene**

Exon	Forward primer, reverse primer (5'-3')	Exon (#) and size (bp)	Intron size (bp)	splice donor site (gt), splice acceptor site (ag)
1-2	GGGCAACCCTGTCCAGG ACACCCTGGGGTCTGTGTC	(1) 156	836	CACAAGgtacatg ttcccagCTTGCT
2-3	AGTGAAAGAAGCTACCAGGGA ACCCACAGGCTCTACTTGT	(2) 132	~ 5500	AAGTCGgtaagtgg cctatagGTGACC
3-4	AGGTGACCATCTACTTGGG AAAGTTCTTTCCTAGCACTAAG <sup>a</sup>	(3) 61	~ 2600	CTGTGGgtaagtgg ggtttagATGGTA
4-5	AGATGGTATCGTGTGGTGG ACCGTGAGCAGGAAGGG	(4) 45	~ 1800	AGAAAGgtaagaca cctccagTGATATGT
5-6	AGTGATGTCTCTCTGACCTG ACCTTCCCCATGTCTTGCG	(5) 194	~ 2000	CTCACGgtgggtg cccacagTTCCTG
6-7	CAAGTTTTACACACTGAGTGC <sup>a</sup> TCGCAGGCCACAGAGGAGAAG <sup>a</sup>	(6) 60	~ 1300	GGGAAGgtagtag actgcagTGCTGTG
7-8	GTGCTGTGGGGTCGACTTT CAGCACCCACAGCAGATTG <sup>a</sup>	(7) 77	~ 1500	CAAGAAgtaagagt tctgcagGAGCTCC
8-9	AGGAGGTCCGTGCGTTTTAC CTAATGCCTTTAATCTTCTTC	(8) 136	905	AAAGAGgtgagcca ttttcagATCTATT
9-10	CCTAGAAAGCCATGAGATTA <sup>a</sup> AAGCCAAGCATCCTACTTCC	(9) 85	~ 1500	CATTAGgtagaac tctgcagTGGAACA
10-11	TGGAACAAGTGGCCAACGTT <sup>a</sup> ACATGGTGCTGCAAGG	(10) 73	~ 2500	AACACAgtagtag cctacagAGAAAAA
11-12	GGAAGTGGTGGCTTTATG <sup>a</sup> ACCCTGACACCGTGAGCTTC	(11) 138	~ 3200	CACCATgtgagtag tgagcagAATAAAG
12-13	TGAGGGATGTGTTTCATCTAG <sup>a</sup> TTCATAACACCTCTGAGCTAC <sup>a</sup>	(12) 78	1390	GTCAGGgtgagtag ttcctagCTTTCTG
13-14	CTGGAGGTGAGCTCTCCCA GTGCTCAGGGACCACTAG	(13) 24	~ 1400	CTCCAGgtagcct ttccagTGAAGTG
14-15	AGTGAAGTGGCAACTGAGGT AAGCCGAAGACTGGGAGC <sup>a</sup>	(14) 56	~ 3000	ACCCAGgtcagtag cttacagCTACGGC
15-16	CCTGCTCACGATTCTCTTC GTCCAGGTCGACCGACCT	(15) 16 (16) 237	426	GGAAAGgtgagccc tcttttagTTTTCA

PCR amplification of the canine SAG gene: primers for the identification of exon/intron boundaries and for the determination of intronic lengths of the canine genomic SAG clones (exon sequences are shown in upper case) <sup>a</sup>the intronic sequences of the SAG gene received the EMBL accession numbers AJ426068-AJ426078

## Results and Discussion

### Genomic organization of the SAG gene

The screening of the canine genomic library with probes for exons 2, 5 and 16 led to the isolation of seven DNA clones, each containing different parts of the SAG gene. This gene contains 16 exons with the 5'UTR split into exons 1 (156 bp) and in 2 (57 bp). The 3'UTR is comprised in exon 16 (137 bp). The coding region is 1215 bp long. Most introns were longer than 1.5 kb (Table 2). Compared to the human SAG gene the position of intron 1, which is in the 5'UTR, is 23 bp further upstream in the dog. This means the canine exon 1 is 23 bp shorter and exon 2 23 bp longer than the equivalent human exons. In dogs exon 15 is 6 bp longer and exon 16 6 bp shorter than the equivalent exons in human. Therefore, the predicted protein in both species are 405 aa long and have a similarity of 89.8%. The intron sizes in man and dog differ, leading to gene sizes of ~ 35 kb in dog and ~ 40 kb in man. The splice donor and acceptor sites follow the GT/AG rule (Table 2). Canine-specific, tRNA-derived short interspersed nucleotide elements (SINE; [25]) were identified in introns 1, 3 and 14 and additional repetitive elements in introns 2, 3 and 14. The human SAG gene maps to chromosome 2q37.1. On the basis of reciprocal chromosome painting [26], the canine gene is, therefore, predicted to map to CFA 28 or 33, the homologous chromosomal regions in dogs.

### Mutation screening by PCR-SSCP analysis

The SAG gene was screened for mutations in 23 breeds, including all gPRA-affected, selected healthy dogs as well as obligate carriers. The 16 exons were analysed by PCR-SSCP including all intronic splice signal sequences as well as the UTRs (*i.e.* complete cDNA plus >3070 bp of the introns). In the coding region of the SAG gene, 5 polymorphisms were identified that result in altered amino acid coding (H14C, A111V, A113T, D259T and A379E), and 7 silent polymorphisms were identified (C132C, Q199Q, H225H, V247V, P264P, T288T and L293L; Table 4). In addition, 13 sequence variations were identified in 9 introns of gPRA affected and unaffected animals (Table 4). Several gPRA-affected dogs in 14 of the 23 breeds were heterozygous for one of the aforementioned polymorphisms (Table 4). In 6 of these 14 dog breeds the major cause of gPRA has meanwhile been determined. Direct DNA tests are possible for Irish Setters and Sloughis [8,9]. Indirect tests for progressive rod cone degeneration (*prcd*) were recently offered for Australian cattle dogs, English Cocker Spaniels, Labrador Retrievers and Miniature poodles (patented by OptiGen, USA). These dog breeds were included as controls to characterise the identified polymorphisms to exclude linkage for causal gPRA mutations. A second gPRA form may exist in Irish Setter because one affected Setter shows a late form of gPRA without the typical *PDE6B* mutation. Because of the clinical signs, also in

Miniature poodles two types of gPRA are possible (OptiGen).

## Conclusions

None of the amino acid changes identified here in dogs correspond to residues that are mutated in known RP, nor are they known to be important for binding activated dephosphorylated RHO [22,23,27]. As detailed above, Oguchi disease and some forms of arRP is caused by the deletion in codon 309 in Japanese. None of the aa exchanges in the dog breeds investigated here correspond with this region. Nevertheless, these novel sequence variations can be used as intragenic markers for segregation analyses with ar gPRA. The breeding history, small population sizes and gPRA abundance in the investigated breeds point together to few meiotic events in which intragenic recombinations could have occurred between an unidentified mutation in the SAG locus in gPRA dogs and the polymorphisms investigated here. Given ar transmission our typing results suggest that the sequence variations in the SAG gene are not causative for gPRA in the following 14 dog breeds: AC, BDP, Bo, BS, ECS, D, IRS, GR, MP, NF, PON, Sa, SD and Sl. In 6 of these dog breeds only one gPRA affected animal was available for mutation analysis (Table 1). For these breeds the exclusion of the SAG gene is not definitive, since the possibility of false diagnosis is not ruled out completely. Nevertheless, gPRA-affected AW, CCR, SP, LR, Ro and TT show homozygous sequence variation patterns and 3 dog breeds (Co, EM, GS) did not harbour any sequence variations. Therefore, the SAG gene cannot be excluded as a cause for gPRA in these breeds, especially because of mutations in the elusive regulatory regions for gene expression.

## Materials and Methods

### Animals

Blood from 810 dogs of 23 different breeds, including 113 gPRA-affected animals (Table 1) was collected with the permission of the owners and in cooperation with breeding organisations. Experienced veterinarians confirmed the gPRA status of healthy and affected dogs by ophthalmoscopy.

### Isolation of canine DNA and PCR

DNA was extracted from peripheral blood according to standard protocols [28]. Portions of the SAG gene were amplified by PCR in a thermocycler (Biometra, Goettingen, Germany) from the inserts of the  $\lambda$  phages in order to obtain intronic sequences. Genomic DNAs from all gPRA-affected, obligate carrier and gPRA-unaffected dogs were screened for sequence variations. PCRs were performed in 96-well microtiter plates (Thermowell Costar Corning, NY). Each well contained 50 ng DNA in 10  $\mu$ l reaction volume (100 mM Tris [pH 8.3], 500 mM KCl, 1 U Taq Polymerase [Genecraft, Münster, Germany], 0.2 mmol of

**Table 3: Primer sequences used for mutation analysis of individual exons/introns of the canine SAG gene**

Primer	Location	Forward primer, reverse primer (5'-3') <sup>a</sup>	PCR conditions [T-°C/MgCl <sub>2</sub> -mM]	PCR amplicon length (bp)	Restriction enzymes for SSCP analyses
UTR-IF	exon 1	GGGCAACCCTGTCCAGGT	54/1.0	699	<i>NlaIII/RsaI</i>
UTR-IaR	intron 1	TCTATCATGACGGGACGCCT			
UTR-IaF	intron 1	GAAAATGATATTTGCAAAGCAG	50/1.0 <sup>b</sup>	283	<i>TruI</i>
UTR-IaR	intron 1	TCTATCATGACGGGACGCCT			
1-IF	intron 1	CTAATGGGCACACAGCATCTC	53/1.0	249	<i>PvuIII; NlaIII<sup>c</sup></i>
2-I2R	intron 2	TTCTGTTAAGCCACTCACTTC			
3-IF	intron 2	TGTTTTTATCTAACACTGACTACTTC	48/1.0 <sup>b</sup>	224	<i>NlaIII</i>
3-IR	intron 3	AAATAACAAAGTAGCAGCTGTC			
4-IF	intron 3	AACTGCAGATAAATATATGAAG	52/1.0	189	<i>AluI</i>
4-IR	intron 4	AAAGTTCTTTCCTAGCACTAAG			
5-IF	intron 4	GGTTACCCCATGTTCACTTG	56/1.0	343	<i>MnII</i>
5-IR	intron 5	GCTCCTGGTCACACTGCAAG			
6-IF	intron 5	CAAGTTTTACACTGAGTGC	55/1.0	213	<i>AluI</i>
6-IR	intron 6	ATTTTCCCAGAGAAAAGGCTA			
7-IF	intron 6	CGGGAAGGGAGGTGCTGA	58/1.0	233	<i>RsaI<sup>c</sup></i>
7-IR	intron 7	TCGCAGGCCACAGAGGAGAAG			
8-IF	intron 7	ATCACAGCGTGAGTACGGGGAG	55/1.0	288	<i>RsaI; PstI<sup>c</sup></i>
8-IR	intron 8	CAGCACCCACAGCAGATTG			
9-IF	intron 8	CCTAGAAAGCCATGAGATTAA	55/1.5	214	<i>TruI; StyI<sup>c</sup></i>
9-IR	intron 9	GACCAGACTGAGAAATCTAG			
10-IF	intron 9	GGCACCATGCACATGCGTG	58/1.0	235	<i>AluI</i>
10-IR	intron 10	AAGCCAAGCATCCTACTTCC			
11-IF	intron 10	GACTGATGGTGGCTTTATG	58/1.0	266	<i>BsuRI</i>
11-IR	intron 11	AGCAGACCAGCACCTCCTC			
12-IF	intron 11	TGAGGGATGTGTTTCATCTAG	55/1.0	183	-
12-IR	intron 12	GCCCATGGTGGTGGCTCTTG			
13-IF	intron 12	CATGCTTGGGACATGTCCAC	64/1.0	209	<i>MvaI</i>
13-IR	intron 13	TTCATAACACCTCTGAGCTAC			
14-IF	intron 13	CTCTGCAGCCACAGCCCTTC	50/2.0	229	<i>Avall</i>
14-IR	intron 14	GTGCTCAGGGACCACTAG			
15-IF	intron 14	CCTGCTCACGATTCTCTTTC	58/1.0	244	<i>HphI</i>
15-IR	intron 15	AAGCCGAAGACTGGGAGC			
16-IF	intron 15	GATCGGTCCCTTGTGCA	52/2.0 <sup>b</sup>	347	<i>AluI</i>
16-ER	Exon 16	CACAGCTGAACAGACAAACTT			

<sup>a</sup>See EMBL accession numbers AJ426068-AJ426078 <sup>b</sup>with 5% formamide <sup>c</sup>with RLFP analysis

each NTP, 0.4 mM of each primer and varying concentrations of MgCl<sub>2</sub> [Table 3]). For SSCP analysis, 0.06 µl of [<sup>32</sup>P] dCTP (10 mCi/ml) was included in the PCR. Parts of the λ phage inserts were amplified in a one step PCR (annealing temperatures in Table 3). For genomic mutation analysis PCR conditions included initial denaturation (5 min at 95°C), the 10 initial cycles 1°C above the annealing temperature (Table 3), 22–25 cycles of 95°C (30 s), annealing temperature (30 s), elongation at 72°C (40 s) and a final elongation step at 72°C (3 min).

#### Cloning and identification of exon/intron junctions

Clones containing the SAG gene were isolated from a genomic canine λ-DNA library (λ FIX<sup>®</sup>II Library; host: *E. coli* XL1-Blu MRA (P2) Stratagene, La Jolla, Ca, USA) accord-

ing the manufacturer's protocol. Recombinant λ DNA was fixed to Hybond<sup>™</sup>-N Nylon membranes (Amersham, Buckinghamshire, UK) and UV-crosslinked (1' 70 J/cm<sup>2</sup>). The library was screened with probes prepared from PCR products corresponding to portions of exons 2, 5 and 16 (nucleotides 909–1157 of EMBL accession number CFA426068, nucleotides 395–588, and 1333–1579 of EMBL accession number X98460, respectively). These probes were labelled using [<sup>32</sup>P] dATP and the Megaprime Labelling System (Amersham, Buckinghamshire, UK). Hybridisations were performed at 65°C in 0.5 M sodium phosphate buffer (pH 7.2)/7% sodium dodecyl sulfate [29]. After hybridisation the filters were washed twice for 30 min each in 2× SSC/1% SDS, once for 15 min with 0.2× SSC/1%SDS at 65°C and for 30 min with 6×SSC at

**Table 4: SAG sequence variations and amino acid exchanges in respective dog breeds**

Location	Sequence variation*	Amino acid exchange	Sequence variations in breed(s) <sup>a</sup> In affected and gPRA unaffected dogs	gPRA affected dogs in heterozygous state
Intron 1	IVS1+393T→C	-	BDP, BS, GR, IRS, Ro <sup>b</sup> , TT	BDP, GR,
Exon 2	UTR-5A→G	-	Bo, BS, GR <sup>c</sup> , BDP, IRS, SD <sup>c</sup> , SI, TT <sup>b</sup>	Bo, GR, SD
	c255A→G	H14C	SI	SI
Exon 5	c526C→T	A111V	Sa <sup>c</sup>	Sa
	c531G→A	A113T	Sa <sup>c</sup>	Sa
Intron 5	IVS5-30C→T	-	ECS, MP, Ro <sup>b</sup> , Sa <sup>c</sup>	ECS, MP, Sa
Exon 6	c610C→T	C132C	GR	GR
Intron 7	IVS7+10C→T	-	IRS <sup>b</sup> , GR, SP <sup>c</sup>	D, IRS, GR
	IVS7+52Ins.G	-	GR <sup>c</sup>	GR
	IVS7-4Del.G	-	CCR <sup>b</sup> D, GR, IRS, MP, SD <sup>c</sup> , SI <sup>c</sup> , SP <sup>b</sup> , TT <sup>c</sup>	D, GR, IRS, SD
Exon 8	c811G→A	Q199Q	GR	GR
Intron 8	IVS8+8A→G	-	D, ECS <sup>c</sup> , GR <sup>c</sup> , LR <sup>c</sup> , Sa	D, ECS, GR, Sa
Exon 9	c874T→C	H225H	Bo, BDP, CCR <sup>b</sup> , ECS, D, GR <sup>c</sup> , IRS, MP, NF, Ro <sup>b</sup> , Sa, SI <sup>b</sup> , TT <sup>bc</sup>	Bo, ECS, D, GR, IRS, MP, NF, Sa
Exon 10	c949G→T	V247V	IRS, GR	GR
	c983G→T	D259T	AC <sup>b</sup>	AC
	c1000A→G	P264P	AC, LR, IRS <sup>b</sup> , GR	GR
Intron 10	IVS10-18G→C	-	GR, IRS, BDP	GR
	IVS10-33C→T	-	GR, IRS	GR
Exon 11	c1063G→A	T288T	BS, BDP, GR, IRS <sup>b</sup> , Sa	BS, GR, Sa
	c1076C→T	L293L	BS, BDP, GR <sup>c</sup> , Sa, TT	BS, Sa
Intron 11	IVS11-51Ins.TT	-	Sa, IRS <sup>b</sup>	Sa
Intron 13	IVS13+66C→G	-	Sa, IRS <sup>b</sup>	Sa
Intron 14	IVS14-45Del.A	-	PON	PON
Intron 15	IVS15+14C→T	-	Sa	-
	IVS15+86T→A	-	Sa	-
	IVS15-45Ins.C	-	PON, AW <sup>bc</sup> , SP <sup>b</sup>	PON
Exon 16	c1344C→A	A379E	IRS <sup>b</sup> , ECS <sup>b</sup> , GR <sup>c</sup> , PON, Sa <sup>bc</sup> , TT <sup>b</sup>	ECS, GR

<sup>a</sup> For abbreviations see Table 1 <sup>b</sup> Homozygous state in gPRA affected dogs <sup>c</sup> Homozygous state in unaffected dogs \* (EMBL accession number for cDNA: X98460; EMBL accession numbers for genomic DNA: AJ426068-AJ426078)

room temperature. The filters were exposed to phosphorimager screens (STORM 860) and evaluated with the programs STORM Scanner Control and Image Quant (Molecular Dynamics). Hybridising clones were isolated and plaque purified as described [30]. The approximate insert sizes of the different clones were estimated with exon primers via PCR (see conditions described above using ~0.2 ng phage DNA, 2 mM MgCl<sub>2</sub> and annealing temperature of 54°C in the PCR).

Exon/intron boundaries were analysed by comparison of canine mRNA ([17], EMBL accession number X98460) with 16 genomic sequences of the human SAG gene (5'-flanking region and exon 1 [18]; EMBL accession number X12453); exons 2-16 ([31]; EMBL accession numbers U70963-U70976) using the program Blast Search (NCBI [http://www.ncbi.nlm.nih.gov/blast]). Intronic sizes were estimated by overlapping PCR including parts of neighbouring exons. PCR products were extracted from 1.5%

agarose gels using the Easy Pure extraction kit (Biozym, Germany) and sequenced with intron-overlapping primers (Table 2). Sequencing reactions from 2-3 clones were carried out by the dideoxy-chain termination method using the BDT (Perkin-Elmer, Norwalk, CT) 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 ABI Prism™ 373XL.

#### PCR-SSCP and DNA sequence analysis

Positions of intronic primers which were used for mutation screening were selected after DNA sequence analysis of the genomic SAG clones (Table 3). SSCP samples were treated as previously described [16,32]. PCR products were digested dependent on the lengths of the fragments [33] with different restriction enzymes (Table 3). Using restriction length fragment polymorphism (RLFP) analysis the sequence variants in exon 2 (*NlaIII*), intron 7 (*RsaI*),

exon 8 (*Pst*I) and exon 9 (*Sty*I) were investigated. 3 µl of the PCRs 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. 3 µl aliquots of the single-stranded fragments were separated in 2 sets of 6% polyacrylamide (acrylamide/bisacrylamide: 19/1) gels, one set containing 10% glycerol, another containing 5% glycerol and 1 M urea. Gels were run with 1× TBE buffer at 50–55 W for 4–6 h at 4°C. All gels were dried and subjected to autoradiography over night. Selected DNA samples with band shifts evidenced by SSCP electrophoresis were purified and cycle sequenced as described above.

### Authors' contributions

Author 1 carried out the molecular genetic analyses, sequence alignments and drafted the manuscript during her predoctoral studies supervised by the second senior author.

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