



Title	Aqueous humor SPARC concentration in canine glaucomas
Author(s)	Kubo, Akira; Miyasho, Taku; Nakamura, Kozo; Ito, Yosuke; Endo, Yusuke; Masuko, Arisa; Hayashi, Miri; Maehara, Seiya; Nakade, Tetsuya
Citation	Japanese Journal of Veterinary Research, 69(4), 205-215
Issue Date	2021-11
DOI	10.14943/jjvr.69.4.205
Doc URL	http://hdl.handle.net/2115/83869
Type	bulletin (article)
Additional Information	There are other files related to this item in HUSCAP. Check the above URL.
File Information	JJVR69-4_205-215_AkiraKubo.pdf (本文)



[Instructions for use](#)

Aqueous humor SPARC concentration in canine glaucomas

Akira Kubo^{1,2)}, Taku Miyasho^{3,*)}, Kozo Nakamura²⁾, Yosuke Ito¹⁾,
Yusuke Endo³⁾, Arisa Masuko²⁾, Miri Hayashi²⁾, Seiya Maehara²⁾
and Tetsuya Nakade²⁾

¹⁾ Veterinary Eye Care Service, 1-1-8 Kita 13-jo-nishi, Kita-ku, Sapporo-shi, Hokkaido 001-0013, Japan

²⁾ Small Animal Clinical Sciences, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyo-dai-Midorimachi, Ebetsu, Hokkaido 069-8501, Japan

³⁾ Laboratory of Animal Biological Responses, Department of Veterinary Science, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyo-dai-Midorimachi, Ebetsu, Hokkaido 069-8501, Japan

Received for publication, March 24, 2021; accepted, November 22, 2021

Abstract

This study was conducted to identify new objective parameters to diagnose and etiologically differentiate canine glaucomas. In the first phase, proteomic analysis was performed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MS) on the aqueous humor of each single eye of five healthy dogs, three dogs with primary glaucoma, and three dogs with secondary glaucoma. Secreted protein acidic and rich in cysteine (SPARC) was identified as a protein that was expressed in all healthy dogs but not in any of glaucomatous dogs. In the second part of the study, we examined SPARC concentrations in the aqueous humor of each single eye of 13 healthy dogs, 14 dogs with primary glaucoma and 13 dogs with secondary glaucoma by enzyme-linked immunosorbent assay. The SPARC concentration was numerically lower with primary glaucoma (0.486 ± 0.047 ng/ml, mean \pm standard error) and numerically higher with secondary glaucoma (0.738 ± 0.089 ng/ml) compared to the control eyes (0.637 ± 0.081 ng/ml). Receiver operating characteristic curve analysis confirmed the ability of the aqueous humor SPARC concentration to differentiate between primary and secondary glaucomas (AUC = 0.72). Thus, 2D-PAGE and MS might be valuable tools for screening of the aqueous humor proteins in canine glaucoma, and the SPARC concentration has a clinical potential in the etiological classification of canine glaucomas.

Key Words: aqueous humor, canine glaucoma, concentration, proteome, SPARC

Introduction

In current veterinary medicine, glaucoma is viewed as a group of neurodegenerative diseases characterized by elevated intraocular pressure (IOP), functional loss and necrosis of

retinal ganglion cells, loss of optic nerve axons, enlargement of the optic cup, and progressive vision loss^{15,19)}. As in humans, it is one of the leading causes of blindness in dogs. Canine glaucomas can be classified etiologically as primary, secondary, or congenital. Primary

* Corresponding author: Taku Miyasho

Laboratory of Animal Biological Responses, Department of Veterinary Science, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyo-dai-Midorimachi, Ebetsu, Hokkaido 069-8501, Japan

FAX: +81(11)388-4882; E-mail: taku-m@rakuno.ac.jp

doi: 10.14943/jjvr.69.4.205

glaucomas develop without any other ocular disorders and are subdivided into open-, narrow- and closed-angle glaucomas based on the drainage angle, and narrow- and closed-angle glaucomas are more commonly diagnosed in canine patients. Secondary glaucomas occur as a result of another ocular disease such as uveitis, lens luxation and intraocular hemorrhage, while congenital glaucomas are rare and develop soon after birth.

In human medicine, diagnosis of glaucoma can now be made in the early stages owing to increased awareness as well as advances in diagnostic devices and technologies. The prognosis is also improving with increased adherence to topical antiglaucoma therapy. The prognosis of canine glaucoma, in contrast, is very poor, because diagnosis is generally made in later stages and largely dependent on IOP measurement and the characteristic ocular findings such as episcleral congestion, diffuse corneal edema, and blepharospasm^{12,13,15}. Therefore, new objective diagnostic parameters are required for early diagnosis and improvement of the vision outcome.

We have previously reported that primary and secondary glaucomas and glaucomas of unknown etiology accounted for 35.5%, 50.4%, and 14.0%, respectively, of all canine glaucomas diagnosed at a single referral ophthalmology center in Japan¹³. The etiological classification and close monitoring are especially important for primary glaucomas, as they initially tend to present as a unilateral condition. It has been reported that if left untreated, primary glaucomas will become bilateral in a median 8 months after the diagnosis of the first eye, but prophylactic topical therapy can delay this process¹⁶. If we can determine whether glaucomas of unknown cause are primary or not, it will greatly help the clinical decision of whether to actively treat the fellow eyes.

The aqueous humor is the clear fluid that fills in the anterior chamber, pupil, and posterior chamber, supplying nutrients to the avascular tissues in the eye while removing their metabolic waste. Its production and drainage are influenced by the anatomical structure of the anterior

segment of the eye as well as neurotransmitters, hormones, prostaglandins, proteins, proteoglycans and many other endogenous factors^{10,15}. Aqueous humor samples can be collected by anterior chamber paracentesis and have been used for laboratory testing, including cytology, bacterial culture, drug susceptibility testing, protein assay, polymerase chain reaction, and antibody titer testing⁸. Proteomic analysis of the aqueous humor has been described about diabetic retinopathy⁴ and open-angle glaucoma in humans^{7,9}, healthy²⁰ and cataract surgery procedures in New Zealand white rabbits²¹.

In the present study, the aqueous humor of healthy and glaucomatous dogs was analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS) in order to identify possible biomarkers that are useful for the diagnosis and etiological differentiation of canine glaucomas. We found that secreted protein acidic and rich in cysteine (SPARC), also called osteonectin, was expressed differently in glaucomatous eyes and compared its concentrations among dogs with normal and glaucomatous eyes.

Materials and Methods

All procedures were performed at the Veterinary Eye Care Service (VECS) and Rakuno Gakuen University (RGU) Animal Medical Center in accordance with the guidelines of the RGU Experimental Animal Research Committee (Approval No. VH21B24 for proteomic study and No. VH17B9 for SPARC measurement) and after obtaining oral consent from animal owners.

Proteomic analysis of canine aqueous humor by 2D-PAGE and MS

Aqueous humor collection from healthy dogs: The aqueous humor was collected from each single eye of five healthy Beagle dogs maintained at RGU (Table 1, No. 1-5). Ophthalmologic evaluation including menace response, pupillary

Table 1. Characteristics of normal and glaucomatous dogs used for proteomic analysis

No.	Breed	Age (years)	Gender	Sampled eye	Ocular disease	IOP (mmHg) ^{a)}	Duration of glaucoma	Vision	Surgery
1	Beagle	1.2	M	OD	None	17	N/A	+	N/A
2	Beagle	1.8	F	OS	None	17	N/A	+	N/A
3	Beagle	6.5	F	OS	None	11	N/A	+	N/A
4	Beagle	7.7	M	OS	None	11	N/A	+	N/A
5	Beagle	6.5	F	OD	None	13	N/A	+	N/A
6	Shiba	8.6	SF	OD	Primary glaucoma	26	19 months	-	ISP
7	Shih Tzu	9.1	F	OS	Primary glaucoma	23	3 months	-	ISP
8	Shih Tzu	3.7	M	OD	Primary glaucoma	69	1 month	-	ISP
9	CKCS	5.7	M	OS	Glaucoma secondary to cataract & retinal detachment	62	23 days	-	ISP
10	MD	10	M	OS	Glaucoma secondary to cataract	39	9 months	-	ISP
11	JRT	6.9	F	OD	Glaucoma secondary to cataract	51	9 days	-	GM

CKCS, Cavalier King Charles Spaniel; F, female; GM, intravitreal gentamicin injection; ISP, intrascleral prosthesis; JRT, Jack Russel Terrier; M, male; MD, Miniature Dachshund; OD, oculus dexter (right eye); OS, oculus sinister (left eye); SF, spayed female.

a) Measured before sample collection.

light reflex, dazzle reflex, IOP measurement, slit-lamp biomicroscopy, fundic examination, and gonioscopy confirmed the absence of ocular conditions that may affect the IOP (e.g., glaucoma, lens subluxation, severe vitreous prolapse into the anterior chamber, and advanced cataract). After immobilization and sedation with intravenous midazolam (0.15 mg/kg; Fuji Pharma, Toyama, Japan), butorphanol (0.025 mg/kg; Vetorphale, Meiji Seika Pharma, Tokyo, Japan), and medetomidine (0.01 mg/kg; Domitor, ZENOAQ, Fukushima, Japan) followed by topical anesthesia with 0.4% oxybuprocaine (Benoxil, Santen, Osaka, Japan), aqueous centesis was performed using a 27-gauge needle directed into the anterior chamber through the limbus. After sample collection, atipamezole (0.05 mg/kg; Antisedan, ZENOAQ) was administered intravenously to reverse the sedation. After the procedure, animals were treated with 0.3% topical

ofloxacin (Pharxacin, Kyorin Rimedio, Toyama, Japan) three to four times daily for a week to prevent bacterial infection and oral tepoxalin (10 mg/kg; Zubrin, Intervet, Tokyo, Japan) once daily for a week to prevent uveitis. Aqueous samples were stored at -80°C until assayed.

Aqueous humor collection from dogs with glaucoma: All glaucoma cases were diagnosed with ophthalmic examination including menace response, pupillary light reflex, dazzle reflex, IOP measurement, slit-lamp biomicroscopy, fundic examination, and gonioscopy.

The aqueous humor was collected at VECS from each single eye of three dogs diagnosed with primary glaucoma and three dogs diagnosed with secondary glaucoma under general anesthesia before surgery. The same ophthalmologic evaluation was performed before the procedure, and the same sampling methods were used as described for control dogs. Aqueous

Table 2. Characteristics of healthy and glaucomatous dogs used for SPARC ELISA analysis

No.	Breed	Age (years)	Gender	Eye	Ocular disease	IOP (mmHg) ^{a)}	Duration of glaucoma (days)	Vision	Surgery
1	Beagle	9.2	F	OD	None	7	N/A	+	N/A
2	Beagle	8.8	M	OS	None	17	N/A	+	N/A
3	Beagle	9.4	F	OD	None	13	N/A	+	N/A
4	Beagle	9.3	F	OS	None	16	N/A	+	N/A
5	Beagle	7.1	M	OD	None	17	N/A	+	N/A
6	Beagle	7.1	M	OS	None	12	N/A	+	N/A
7	Beagle	7.1	F	OS	None	9	N/A	+	N/A
8	Beagle	7.1	M	OD	None	14	N/A	+	N/A
9	Beagle	7.1	M	OD	None	15	N/A	+	N/A
10	Beagle	3.9	F	OD	None	19	N/A	+	N/A
11	Beagle	3.8	F	OS	None	19	N/A	+	N/A
12	Beagle	3.8	M	OD	None	22	N/A	+	N/A
13	Beagle	3.8	M	OS	None	16	N/A	+	N/A
14	Shiba	9.9	SF	OD	Primary glaucoma	84	2	+	AGV
15	ACS	13.5	CM	OD	Primary glaucoma	66	18	-	GM
16	Pomeranian	7	CM	OS	Primary glaucoma	40	541	-	ISP
17	Shiba	8.3	SF	OD	Primary glaucoma	67	44	-	ISP
18	Shiba	12.2	CM	OS	Primary glaucoma	85	93	-	ISP
19	Shiba	10	CM	OS	Primary glaucoma	71	606	-	ISP
20	MD	8.6	SF	OD	Primary glaucoma	14	200	-	EC
21	ACS	5.7	CM	OS	Primary glaucoma	60	111	-	EC
22	Shih Tzu	7.7	SF	OS	Primary glaucoma	20	33	+	GM
23	Shiba	7.4	SF	OS	Primary glaucoma	74	Unknown	-	ISP
24	Brussels Griffon	12.7	CM	OS	Primary glaucoma	38	24	+	AGV
25	Shiba	11.5	F	OS	Primary glaucoma	61	71	-	EC
26	Beagle	15.7	SF	OS	Primary glaucoma	71	262	-	GM
27	MD	13.9	SF	OD	Primary glaucoma	48	19	-	ISP
28	Toy Poodle	10.7	SF	OS	Secondary glaucoma from cataract & lens subluxation	77	59	-	ISP
29	Mix	10.1	M	OS	Secondary glaucoma from cataract & retinal detachment	76	16	-	ISP
30	Toy Poodle	11.8	SF	OS	Secondary glaucoma from cataract	45	24	-	ISP
31	ACS	11.5	CM	OS	Secondary glaucoma from cataract surgery	86	395	-	GM
32	MD	11.1	CM	OS	Secondary glaucoma from cataract	27	315	-	ISP
33	MD	14.5	CM	OS	Secondary glaucoma from cataract	53	9	-	ISP

34	Shih Tzu	13.5	SF	OD	Secondary glaucoma from cataract surgery	26	236	-	ISP
35	ACS	9.1	SF	OD	Secondary glaucoma from cataract surgery	51	40	-	ISP
36	Shih Tzu	5.6	CM	OD	Secondary glaucoma from cataract & retinal detachment	36	7	-	EC
37	Shih Tzu	11.5	SF	OD	Secondary glaucoma from retinal detachment	22	unknown	-	ISP
38	Golden Retriever	12.6	CM	OD	Secondary glaucoma from pigmentary uveitis	50	140	-	ISP
39	ACS	9.7	F	OS	Secondary glaucoma from cataract	37	115	-	ISP
40	Shih Tzu	10.8	F	OS	Secondary glaucoma from retinal detachment	19	2554	-	ISP

ACS, American Cocker Spaniel, AGV, Ahmed glaucoma valve implant; CM, castrated male; EC, enucleation; F, female; GM, intravitreal gentamicin injection; ISP, intrascleral prosthesis; M, male; MD, Miniature Dachshund; OD, oculus dexter (right eye); OS, oculus sinister (left eye); S F, spayed female.

a) Measured before sample collection.

samples were stored at -80°C until assayed. Signalment, ophthalmic examination findings, and classification of glaucoma (and primary ocular diseases if secondary), and type of surgery performed are summarized in Table 1.

2D-PAGE: Aqueous samples were concentrated and desalted at 4°C using Amicon Ultra filters (Millipore, Billerica, MA, USA), and protein concentration was determined using 2-D Quant Kit (GE Healthcare, Buckinghamshire, UK). Samples containing $8\ \mu\text{g}$ of total protein were mixed with a rehydration buffer [8 M urea, 2% CHAPS, 0.5% carrier ampholyte (BioLyte 3/10, Bio-Rad, Hercules, CA, USA), 18 mM dithiothreitol, and bromophenol blue; final volume of $125\ \mu\text{l}/\text{sample}$], loaded onto an immobilized pH gradient polyacrylamide gel (ReadyStrip IPG Strips, 7 cm, pH 3-10 nonlinear, Bio-Rad), and rehydrated for 14 hr at 20°C . The first-dimension isoelectric focusing (IEF) was performed using the Protean IEF Cell Electrophoresis System (Bio-Rad) at a rapid 250 V for 1 hr, linear 4000 V

for 2 hr, and rapid 4000 V for 30,000 Vhr. After IEF, IPG strips were equilibrated for 30 min in an equilibration buffer containing 64.8 mM dithiothreitol and alkylated for 30 min in the second equilibration buffer containing 135 mM iodoacetamide. The second-dimension sodium dodecyl sulfate (SDS)-PAGE was performed according to the methods described by Laemmli¹⁴, with minor modifications, at a contrast current of 5 mA at 4°C until the bromophenol blue dye front reached the bottom of the gel. After SDS-PAGE, gels were stained with silver (Dodeca Silver Stain Kit, Bio-Rad) and Coomassie brilliant blue (CBB).

In-gel digestion and protein identification: Protein spots that were differentially expressed between healthy and glaucomatous dogs and stained with both CBB and silver were excised from silver-stained gels and destained in a mixture of 50 mM $\text{NH}_4\text{HCO}_3/50\%$ methanol. Gel plugs were washed in ultrapurified water (Milli-Q, Millipore), dehydrated with 50 mM $\text{NH}_4\text{HCO}_3/50\%$ acetonitrile and then with 100%

acetonitrile. For protein digestion, a digestion buffer [50 ng of porcine trypsin (Promega, Madison, WI, USA) dissolved in 5 μ l of 50 mM NH_4HCO_3] and 5 μ l of 100 mM Tris-HCl (pH8.8) were added to each gel plug and incubated at 37°C for 15 hr. Resulting peptides were extracted first with 50 μ l of 50% acetonitrile/0.1% trifluoroacetic acid (TFA) and then with 90% acetonitrile/0.1% TFA and vacuum centrifuged to reduce the volume to 20 μ l.

For matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS (Autoflex, Bruker Daltonics, Bremen, Germany), samples were further extracted with 50% acetonitrile/0.1% TFA and then 90% acetonitrile/0.1% TFA, cleaned up with sample preparation pipette tips (ZipTip, Millipore), applied to a MALDI target plate, overlaid with 0.5 μ l of 25% α -cyano-4-hydroxycinnamic acid (Bruker Daltonics), 50% acetonitrile, and 0.1% TFA, mixed on the target plate, and analyzed. The MS data were used to identify peptide sequences using the MASCOT search engine (<http://www.matrixscience.com>). Database search results were manually checked by comparing them with the nominal mass and the calculated pI value.

Measurement of aqueous humor SPARC concentrations

Aqueous humor collection from healthy dogs: The aqueous humor was collected from each single eye of 13 healthy Beagle dogs maintained at RGU (Table 2, No. 1-13; 7 males and 6 females, 3.8-9.4 (median 7.1) years old). All procedures and ophthalmologic examination were performed as described for the proteomic study, except that dogs received oral carprofen (4.4 mg/kg; Rimadyle, Zoetis JAPAN, Tokyo, Japan) once daily for three days instead of tepoxalin. Aqueous samples were stored at -80°C until assayed.

Aqueous humor collection from dogs with glaucoma: Signalment, ophthalmologic findings, classification of glaucoma, duration of glaucoma and surgical procedure in glaucomatous dogs are shown in Table 2. The aqueous humor was collected

at VECS from each single eye of 14 dogs diagnosed with primary glaucoma (Table 2, No. 14-27; 6 males and 8 females, 5.7-15.7 (median 10.0) years old) and 13 dogs diagnosed with secondary glaucoma (Table 2, No. 28-40; 6 males and 7 females, 5.6-14.5 (median 11.1) years old) under general anesthesia before surgery. All procedures and ophthalmologic examination were performed as described for the normal dogs, and aqueous samples were stored at -80°C until assayed. Surgical techniques used included Ahmed glaucoma valve implantation (2 eyes), intrascleral prosthesis (6 eyes), enucleation (3 eyes), and intravitreal gentamicin injection (3 eyes) for primary glaucoma and intrascleral prosthesis (11 eyes), enucleation (1 eye), and intravitreal gentamicin injection (1 eye) for secondary glaucoma. The causes of secondary glaucoma were cataract (7 eyes), retinal detachment (4 eyes), cataract surgery (2 eyes), lens subluxation (1 eye), and pigmentary uveitis (1 eye).

Enzyme-linked immunosorbent assay (ELISA): Canine SPARC ELISA Kit (Bluegene, Shanghai, China) was used to determine SPARC concentrations in the aqueous humor. This kit is based on monoclonal antibody technology specific for canine SPARC. Aqueous humor samples were centrifuged at 1000 \times g at 4°C for 15 min, and 100 μ l were added to wells in duplicate. Calibration standards (0.0, 1.0, 2.5, 5.0, 10, and 25 ng/ml) and a blank (PBS) were also assayed in duplicate (100 μ l each). To each well, 50 μ l of the enzyme conjugate were added. The plate was shaken for 30 sec, sealed and incubated for 1 hr at 37°C. Wells were washed with 350 μ l of working Wash Solution 5 times, and 50 μ l of Substrate A and 50 μ l of Substrate B were added to each well. The plate was shaken for 30 sec, sealed and incubated for 15 min at 37°C. The reaction was terminated by adding 50 μ l of Stop Solution. The optical density at 450 nm was measured using a microplate reader (Ultramark, Bio-Rad). The standard curve of SPARC ELISA is shown in Supplemental data 1.

Statistical analysis

Statistical analysis was performed using

Table 3. Protein identification result by MALDI-TOF MS and MASCOT searching in this study

Protein identification result ^{a)}	Score ^{b)}	pI ^{c)}	% Sequence coverage ^{d)}	Nominal mass ^{e)}
SPARC precursor	85	4.67	27	35390

a) Proteins identified by Mascot search. b) The proteins score is $-10^3 \log(P)$, where P represents the probability that the observed match is a random event. Protein scores greater than 66 are significant ($P < 0.05$). c) The isoelectric point expected for a protein with the sequence returned by the database search. d) Refers to the percent of the protein sequence identified by mass spectra. e) The nominal mass is obtained by summing the integer masses of the most abundant naturally occurring stable isotopes of the elements constituting the protein.

Table 4. Receiver-operating curve analysis for detection and classification of canine glaucoma.

	Normal vs. primary glaucoma	Normal vs. secondary glaucoma	Primary vs. secondary glaucoma
Cutoff value (ng/ml)	0.478	0.585	0.574
AUC	0.58	0.63	0.72
95% CI	0.359-0.797	0.407-0.849	0.528-0.908
Sensitivity (%)	54.4	76.9	66.7
Specificity (%)	75	58.3	76.9
P value	0.2717	0.3078	0.0265

AUC, area under the receiver-operating curve; CI, confidence interval. P value is the probability that the observed AUC is found when AUC is 0.5 (null hypothesis: AUC = 0.5, Chi squared test)

BellCurve for Excel (Social Survey Research Information, Tokyo, Japan) and JMP Pro 15 (SAS Institute Inc., Cary, NC, USA). Male/female ratios were analyzed by contingency table analysis. Because aqueous humor SPARC concentrations determined by ELISA were normally distributed (by D'Agostino's test for skewness, Kolmogorov-Smirnov test, and Shapiro-Wilk test using Q-Q and normal P-P plots), they were expressed as means and standard errors of the mean (SEM), and the mean values were analyzed for significant intergroup difference by one-way factorial analysis of variance. The diagnostic ability of aqueous humor SPARC concentration was examined by receiver operating characteristic (ROC) curve analysis. The level of significance was set at less than 5%.

Results

Proteomic analysis of canine aqueous humor by 2D-PAGE and MALDI-TOF

All glaucomatous dogs showed similar 2D-PAGE patterns. One spot on the 2D-PAGE gel was detected in all control dogs but not in any of glaucomatous dogs (Fig. 1). This protein was identified as SPARC by a MALDI-TOF MS and MASCOT search (Table 3). There was no difference in the 2D-PAGE pattern between primary and secondary glaucomatous eyes.

SPARC concentration in canine aqueous humor with or without glaucoma

There was no significant difference in the male/female ratio between the healthy and

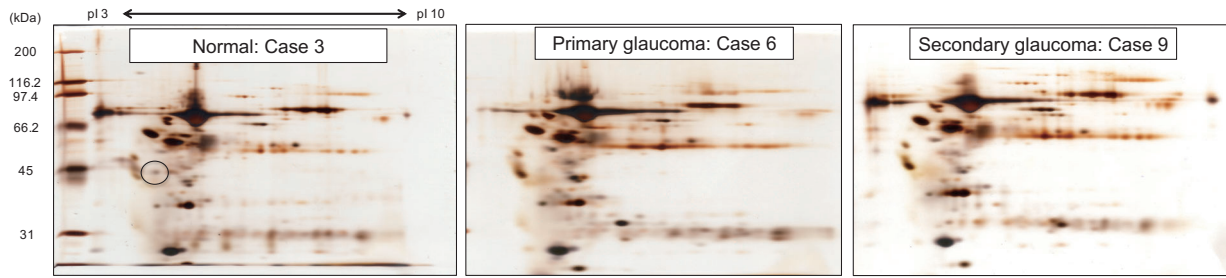


Fig. 1. Two-dimensional electrophoresis patterns of the aqueous humor of normal dogs and dogs with primary and secondary glaucoma.

The circle indicates a protein spot that was observed only in control dogs but not in glaucomatous dogs. See Table 1 for case numbers.

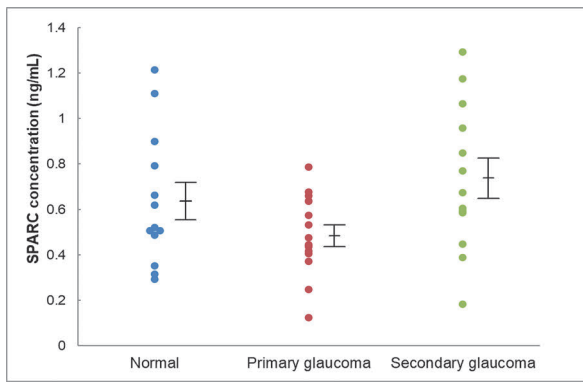


Fig. 2. SPARC concentrations in the aqueous humor of normal dogs and dogs with primary glaucoma and secondary glaucoma.

The middle horizontal line indicates the mean in each group, while the error bars indicate the standard error of the mean.

glaucomatous groups ($P = 0.8435$).

A dot plot of aqueous humor SPARC concentration is shown in Fig. 2. The mean \pm SEM of SPARC concentration was 0.637 ± 0.081 ng/ml in normal dogs, 0.486 ± 0.047 ng/ml in dogs with primary glaucoma, and 0.738 ± 0.089 ng/ml in dogs with secondary glaucoma. Compared to healthy dogs, dogs with primary glaucoma had a lower mean value, while dogs with secondary glaucoma had a higher mean value. These differences, however, were not statistically significant.

The ROC analysis of diagnostic performance of SPARC concentration is shown in Table 4. The SPARC concentration had an ability for differential classification between primary and secondary glaucomas as indicated by the greater area under the receiver-operating curve (AUC = 0.72, $P = 0.0265$).

Discussion

SPARC has been studied as a screening marker, and circulating and urinary SPARCs have been shown to increase with progression and after recurrence in lung, urinary bladder and pancreatic cancer of humans^{1,6,18}. For ocular conditions, the expression of SPARC has been studied in the aqueous humor of human primary angle closure glaucoma⁵) and SPARC-deficient mice¹¹). To our knowledge, our study is the first proteomic analysis of the canine aqueous humor to indicate the possible involvement of SPARC in the disease state.

By initial screening using 2D-PAGE and MALDI-TOF MS, we found that SPARC was downregulated in canine primary and secondary glaucomas. To investigate further, we used an ELISA-based assay to determine aqueous humor SPARC concentrations in a larger number of samples, and the greater area under the ROC confirmed that the aqueous humor SPARC level was useful for differentiation between primary and secondary glaucomas. Hence, the SPARC concentration does not seem to have the ability to diagnose primary or secondary glaucoma but has a clinical potential for the etiological classification of canine glaucoma.

The results of our proteomic analysis, which showed downregulation of SPARC in both primary and secondary glaucomas, were different from those obtained by ELISA, in which the SPARC concentration was elevated in secondary

glaucomas. This discrepancy is likely due to the total protein content that was adjusted equally across all samples for the proteomic analysis. In addition, dogs with primary and secondary glaucomas had numerically lower and higher SPARC concentrations, respectively, than healthy dogs, but these differences were not statistically significant. Further studies are necessary to examine the influence of total protein content and to improve the statistical power using a larger sample number.

In dogs, primary glaucomas are generally breed-related and hereditary and often affect both eyes^{15,19}. In many dogs, the condition is initially unilateral and then progresses to a bilateral disorder. Clinically, it is important to monitor unaffected fellow eyes regularly to detect the earliest signs of IOP elevation and initiate prophylactic treatment to prevent bilateral blindness. In fact, prophylactic treatment has been shown to significantly delay the onset of glaucoma in these eyes¹⁶. Thus, the decision to initiate prophylactic treatment depends heavily on the etiological classification of glaucoma. However, concurrent or secondary lens luxation and retinal detachment observed in chronic cases of primary glaucomas often complicate the differentiation of primary glaucoma from secondary glaucoma¹³. The SPARC concentration may play a key role in the etiological classification of these complicated, chronic cases, providing important information for future therapeutic plans.

SPARC is involved in the repair and remodeling of damaged tissues^{2,3}. For ocular conditions, SPARC and type I collagen are markedly upregulated in people with primary angle closure glaucoma, indicating the possible role of SPARC in the development of this condition⁵. SPARC overexpression increases IOP in perfused cadaveric human anterior segments resulting from a qualitative change the juxtacanalicular extracellular matrix¹⁷. In SPARC-deficient mice, IOP was lower than that of wild-type mice¹¹. Although the mechanism of the SPARC concentration difference between primary

and secondary glaucomatous dogs could not be determined in the present study, we speculate that the antecedent ocular condition have caused uveitis, which in turn activates the tissue remodeling mechanism, resulting in increased SPARC expression in dogs with secondary glaucoma. On the other hand, the decreased SPARC concentration in dogs with primary glaucoma contradicts the results of the human and murine studies. Although it is not possible to speculate the reason from the available data, different pathogenetic pathways may be involved in the development of glaucoma in dogs, humans, and other animals. Alternatively, the lower SPARC expression in canine primary glaucomas may suggest underlying impairment in tissue repair, which can generate resistance in the aqueous humor outflow tract. It is of great interest to further investigate how the SPARC concentration is regulated in healthy and glaucomatous eyes and how SPARC can morphologically, molecularly and functionally affect the ocular tissues.

The present study has limitations. First, multiple breeds of dogs were represented in the glaucoma population in this study, whereas control dogs were all Beagles. Matching breed types or inclusion of breeds predisposed to glaucoma in the control group would confirm the validity of the data, but sampling of the aqueous humor from client-owned, healthy dogs is considered unethical, as aqueous centesis is a physiologically and psychologically invasive procedure. Future studies are needed to evaluate whether blood and other noninvasive samples are useful for SPARC measurement. Second, most of the aqueous humor samples were obtained from dogs with advanced glaucoma. It is necessary to include samples at earlier stages in future studies.

Supplemental data

Supplemental data associated with this article can be found, in the online version, at <https://doi.org/10.14943/jjvr.69.4.205>

References

- 1) Andriani F, Landoni E, Mensah M, Facchinetti F, Miceli R, Tagliabue E, Giussani M, Callari M, Cecco LD, Colombo MP, Roz L, Pastrino U, Sozzi G. Diagnostic role of circulating extracellular matrix-related proteins in non-small cell lung cancer. *BMC Cancer* 18, 899, 2018.
- 2) Bornstein P, Sage EH. Matricellular proteins: extracellular modulators of cell function. *Curr Opin Cell Biol* 14(5), 608-616, 2002.
- 3) Brekken RA, Sage EH. SPARC, a matricellular protein: at the crossroads of cell-matrix communication. *Matrix Biol* 19(8), 816-827, 2001.
- 4) Chiang SY, Tsai ML, Wang CY, Chen A, Chou YC, Hsia CW, Wu YF, Chen HM, Huang TH, Chen PH, Liu HT, Shui HA. Proteomic analysis and identification of aqueous humor proteins with a pathophysiological role in diabetic retinopathy. *J Proteomics* 75(10), 2950-2959, 2012.
- 5) Chua J, Seet LF, Jiang YZ, Su R, Htoon HM, Charlton A, Aung T, Wong TT. Increased SPARC expression in primary angle closure glaucoma iris. *Mol Vis* 14, 1886-1892, 2008.
- 6) Critselis E, Rava M, Marquez M, Lygirou V, Chatzicharalambous D, Liapi P, Lichtinghagen R, Brand K, Cecchini L, Vlahou A, Malats N, Zoidakis J. Diagnostic and prognostic performance of secreted protein acidic and rich in cysteine (SPARC) assay for detecting primary and recurrent urinary bladder cancer. *Proteomics Clin Appl* 13(2), e1800148, 2019.
- 7) Duan X, Xue P, Wang N, Dong Z, Lu Q, Yang F. Proteomic analysis of aqueous humor from patients with primary open angle glaucoma. *Mol Vision* 16, 2839-2846, 2010.
- 8) Featherstone HJ, Heinrich CL. Ophthalmic examination and diagnostics. In: *Veterinary Ophthalmology*, 5th ed. Gelatt KN, Gilger BC, Kern TJ eds. Blackwell Publishing, Ames, pp. 533-613, 2013.
- 9) Grus FH, Joachim SC, Sandmann S, Thiel S, Bruns K, Lackner KJ, Pfeiffer N. Transthyretin and complex protein pattern in aqueous humor of patients with primary open-angle glaucoma. *Mol Vision* 14, 1437-1445, 2008.
- 10) Gum GG, MacKay EO. Physiology of the eye. In: *Veterinary Ophthalmology*, 5th ed. Gelatt KN, Gilger BC, Kern TJ eds. Blackwell Publishing, Ames, pp. 171-207, 2013.
- 11) Haddadin RI, Oh DJ, Kang MH, Filippopoulos T, Gupta M, Hart L, Sage EH, Rhee DJ. SPARC-null mice exhibit lower intraocular pressures. *Invest Ophthalmol Vis Sci* 50(8), 3771-3777, 2009.
- 12) Itoh Y, Umeda Y, Wakaiki S, Saito A. Study of vision maintenance and duration for consultation with veterinary clinic in canine glaucomatous case. *Journal of the Japan Veterinary Medical Association* 63, 281-285, 2010.
- 13) Kubo A, Ito Y, Masuko A, Maehara S, Miyasho T, Nakade T. Vision outcome with antiglaucoma therapy and prognostic factors in canine glaucoma: A 6-year retrospective study in Japan. *Jpn J Vet Res* 67(1), 93-102, 2019.
- 14) Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685, 1970.
- 15) Miller PE. The glaucomas. In: *Slatter's Fundamentals of Veterinary Ophthalmology*, 5th ed. Maggs DJ, Miller PE, Ofri R. Elsevier Saunders, St. Louis. pp. 257-271, 2013.
- 16) Miller PE, Schmidt GM, Vainisi SJ, Swanson JF, Herrmann MK. The efficacy of topical prophylactic antiglaucoma therapy in primary closed angle glaucoma in dogs: A multicenter clinical trial. *J Am Anim Hosp Assoc* 36(5), 431-438, 2000.
- 17) Oh DJ, Kang MH, Ooi YH, Choi KR, Sage EH, Rhee DJ. Overexpression of SPARC in human trabecular meshwork increases intraocular pressure and alters extracellular matrix. *Invest Ophthalmol Vis Sci* 54, 3309-3319,

- 2013.
- 18) Papapanagiotou A, Sgourakis G, Karkoulas K, Raptis D, Parkin E, Brotzakis P, Panchal S, Papavassiliou AG. Osteonectin as a screening marker for pancreatic cancer: A prospective study. *J Int Med Res* 46(7), 2769-2779, 2018.
 - 19) Plummer CE, Regnier A, Gelatt KN. The canine glaucoma. In: *Veterinary Ophthalmology*, 5th ed. Gelatt KN, Gilger BC, Kern TJ eds. Blackwell Publishing, Ames, pp. 1050-1145, 2013.
 - 20) Stastna M, Behrens A, McDonnell PJ, Van Eyk JE. Analysis of protein composition of rabbit aqueous humor following two different cataract surgery incision procedures using 2-DE and LC-MS/MS. *Proteome Sci* 9(1), 8, 2011.
 - 21) Stastna M, Behrens A, Noguera G, Herretes S, McDonnell P, Van Eyk, JE. Proteomics of the aqueous humor in healthy New Zealand rabbits. *Proteomics* 7(23), 4358-4375, 2007.