Screening *ADAMTS10* in Dog Populations Supports Gly661Arg as the Glaucoma-Causing Variant in Beagles

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Purpose. Previously, we mapped the disease locus in the beagle model of autosomal recessive primary open angle glaucoma (POAG) to a 4-Mb interval on chromosome 20, and identified a Gly661Arg variant in *ADAMTS10* as the candidate disease-causing variant. The purpose of this study was to test the hypothesis that the Gly661Arg variant of *ADAMTS10* causes glaucoma by genotyping dogs of various breeds affected and unaffected by primary glaucoma.

Methods. Dogs of various breeds, affected or unaffected with primary glaucoma, were genotyped for the Gly661Arg variant of *ADAMTS10*, as well as 7 other nonsynonymous single nucleotide polymorphisms (SNPs) in other genes in the beagle POAG locus that segregate with disease. Alternate allele frequencies were calculated with 95% confidence intervals and comparisons made to expected allele frequency relative to disease prevalence or between cases and controls.

Results. For the nonsynonymous SNPs other than the *ADAMTS10* variant, control dogs were identified that were homozygous for the alternative alleles, ruling out those variants as causative. None of the nonsynonymous SNPs were found associated with primary glaucoma in American cocker spaniels. The Gly661Arg variant of *ADAMTS10* was the only variant with minor allele frequency consistent with the prevalence of primary glaucoma in the general beagle population. The only dog found homozygous for the Gly661Arg variant of *ADAMTS10* was an affected beagle, unrelated to the POAG colony.

Conclusions. These findings support the Gly661Arg mutation of *ADAMTS10* as the likely cause of POAG in beagles. (*Invest Ophthalmol Vis Sci.* 2013;54:1881–1886) DOI:10.1167/iovs.12-10796

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Supported by NEI Grant EY020894 (RWK), a Departmental Unrestricted Award from Research to Prevent Blindness, Inc., and Vanderbilt Vision Research Center (P30EY008126).

Submitted for publication August 17, 2012; revised November 28, 2012; accepted February 8, 2013.

Disclosure: **J. Kuchtey**, None; **J. Kunkel**, None; **D. Esson**, None; **J.S. Sapienza**, None; **D.A. Ward**, None; **C.E. Plummer**, None; **K.N. Gelatt**, None; **R.W. Kuchtey**, None

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The genetic locus for POAG inherited as an autosomal recessive trait within a colony of beagles was previously identified on canine chromosome 20.[1] Sequencing the entire locus revealed eight variants that segregated with disease and caused amino acid substitutions (Fig. 1), all of which were single nucleotide polymorphisms (SNPs). One of these variants found in *ADAMTS10* results in the substitution in the ADAMTS10 protein (NCBI GI: 73986982) of an arginine for a highly conserved glycine residue at amino acid position 661 (Gly661Arg), a change predicted to have deleterious effects on protein function.[1] Genotyping the eight nonsynonymous SNPs in 48 control beagles showed that the Gly661Arg variant of *ADAMTS10* had the lowest minor allele frequency (MAF). The Gly661Arg variant of *ADAMTS10* was identified as the likely cause of POAG in the beagle colony because it was the only rare nonsynonymous variant that caused a highly nonconservative amino acid substitution in a highly conserved region of a gene.

Extensive study of the POAG beagle colony has allowed for detailed characterization of the clinical course of canine POAG. In the POAG beagle colony, the initial manifestation of disease is reduced facility of aqueous humor outflow.[2,3] Affected dogs develop elevated intraocular pressure (IOP) with open iridocorneal angles, as determined by gonioscopy and histology, and subsequently develop glaucomatous optic nerve damage.[2,4,5] Following prolonged IOP elevation, the globes become enlarged and the lens zonules become weakened and disinserted, leading to luxation of the lens and narrowing of the iridocorneal angle.[2] Since reduced facility of aqueous humor outflow precedes all other clinical signs in the POAG beagles, the causative locus is most likely involved in increased outflow resistance and the resultant elevation of IOP.

The hypothesis that the Gly661Arg variant of *ADAMTS10* causes POAG in the beagle colony is supported by the pattern of tissue expression and the biological function of *ADAMTS10*. ADAMTS10 protein is expressed abundantly in the trabecular meshwork,[1] consistent with a role for *ADAMTS10* in aqueous humor outflow. Functionally, ADAMTS10 is a secreted matrix metalloproteinase which has been proposed to be involved in the structure and function of microfibrils.[6–9] In addition to providing elastic support in tissues such as blood vessels and skin, microfibrils are the primary reservoir of latent transforming growth factor beta (TGF-β).[10–12] In diseases associated with microfibril defects, such as Marfan syndrome, TGF-β signaling is hyperactivated and TGF-β concentration is elevated.[13,14] Defective microfibrils could provide a mechanistic explanation for the well-established elevation of TGF-β concentration in the aqueous humor of human glaucoma patients.[15,16] Discovery of the Gly661Arg variant of *ADAMTS10* in beagle POAG led us to form the hypothesis that disruption of microfibril structure or function may be an underlying mechanism of increased resistance to aqueous humor outflow in POAG.[1] Since the defective microfibrils hypothesis offers a fundamental mechanism of glaucoma pathogenesis, we sought further validation

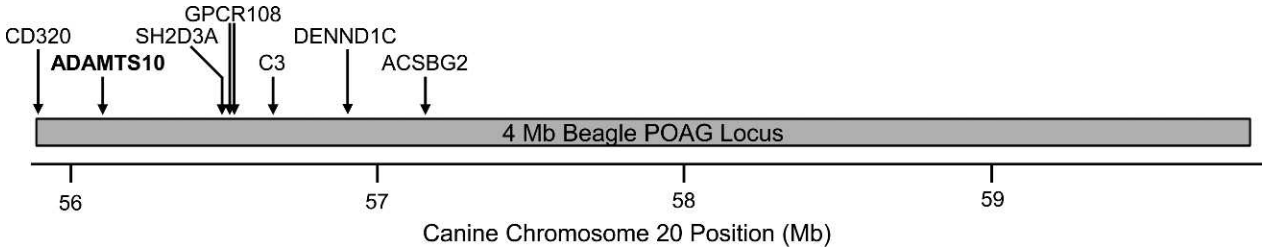

Figure 1. Nonsynonymous SNPs segregating with disease in the beagle glaucoma colony. The locations of the eight nonsynonymous SNPs that segregate with disease in the POAG beagle colony are indicated by *arrows* labeled according to the gene in which the SNPs are located. The nonsynonymous SNPs are clustered in a region of approximately 1.2 Mb within the 4-Mb beagle glaucoma locus, represented by the *gray bar*, below which the location on canine chromosome 20 is indicated, based on the CanFam 2 reference canine genome. Two SNPs were found in *GPCR108*.

of the *ADAMTS10* variant as causative for glaucoma in this study.

Many breeds of dogs are susceptible to primary glaucoma, defined as elevated IOP without other causative ocular disease. Although the course of glaucoma in affected beagles bred for research has been well established, in clinic settings nearly all dogs present at late stages of the disease and most often suffer from significant vision loss. The high prevalence of primary glaucoma in many breeds suggests a genetic component.[17,18] To test our hypothesis that the Gly661Arg variant of *ADAMTS10* causes glaucoma, we genotyped this SNP, and the other seven nonsynonymous SNPs that segregated with disease in the POAG colony, in dogs of various breeds, both those affected and those unaffected by primary glaucoma.

Materials and Methods

Animals and Samples

Subject dogs were examined by board certified veterinary ophthalmologists. Affected dogs were diagnosed with primary glaucoma, defined as elevated IOP in the absence of antecedent ocular disease. Control dogs were at least 2 years of age and were unaffected by glaucoma or other ocular disease, with the exception of cataracts. Blood samples from dogs were obtained by standard venipuncture, in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. DNA extractions from whole blood were performed on commercial equipment (Gentra Systems AutoPure robot; Qiagen, Inc., Valencia, CA) using a purification kit (Puregene; Qiagen, Inc.) by the DNA services core of the Vanderbilt Center for Human Genetics Research.

Genotyping

SNP genotyping was carried out by amplification of genomic sequence surrounding SNPs using PCR primers designed with Primer 3 software (available in the public domain, http://frodo.wi.mit.edu/) and based on the canine reference genome (canFam2) followed by Sanger sequencing by the Vanderbilt DNA Sequencing Facility using a 96-capillary ABI 3730xl DNA Analyzer (Life Technologies Corp., Carlsbad, CA). DNA Sequence data was analyzed using DNA sequencing software (Sequencher v. 4.8; Gene Codes Corp., Ann Arbor, MI).

Statistics

Statistical significance of MAF differences between affected and control American cocker spaniels was evaluated using a 2-tailed Fisher exact test calculated using software available online (in the public domain, http://www.langsrud.com/fisher.htm), with Bonferroni correction for multiple comparisons.

Results

To test the hypothesis that the Gly661Arg variant of *ADAMTS10* causes primary glaucoma, we genotyped affected and unaffected dogs for the *ADAMTS10* variant and for the other seven nonsynonymous SNPs that segregated with disease in the autosomal recessive beagle POAG locus (Fig. 1). Identification of control dogs homozygous for a variant allele would argue against that allele as causative. Additionally, the frequency of the alternative allele must be consistent with the disease prevalence. For beagles, with a prevalence of primary glaucoma of approximately 1%,[17] the alternative allele frequency cannot be more than 10%, by Hardy-Weinberg equilibrium. Conversely, finding a dog affected with primary glaucoma that is homozygous for the alternative allele would support that allele as causative.

Beagles

In our original study,[1] we genotyped the eight nonsynonymous SNPs (Fig. 1) from the beagle POAG locus in a collection of 48 unaffected beagles, primarily obtained from two large colonies from animal supply companies (Figs. 2A, 2B). Within the two supplier colonies, one control dog was found homozygous for the alternative alleles in both SNPs in *GPCR108* (Fig. 2A); one dog was found homozygous for one of the alternative alleles in a *GPCR108* SNP (Fig. 2B); and another dog was found homozygous for the alternative allele in *CD320* (Fig. 2B). Homozygosity of the alternative alleles in these control beagles argues against the *GPCR108* and *CD320* SNPs as causative. Minor allele frequencies in the supplier colony populations were consistent with *GPCR108* and *CD320* SNPs not being causative of primary glaucoma in beagles, though the other variants could not be ruled out, as the 95% confidence intervals (CI) for their minor allele frequencies fell below the upper threshold of 10% (Fig. 2D).

To increase the genetic diversity of our control population, we genotyped an additional 15 older beagles from veterinary clinics that were also examined by veterinary ophthalmologists and found to be unaffected by glaucoma (Fig. 2C). For all variants except the one in *ADAMTS10*, examples of control dogs homozygous for the alternative allele were found in this general beagle population. This essentially rules out all variants as causative for primary glaucoma in beagles, except for the *ADAMTS10* variant. The minor allele frequencies of the variants also rule out all variants other than *ADAMTS10* (Fig. 2D). Only one of the control beagles was found heterozygous for the *ADAMTS10* variant, giving a minor allele frequency in the clinic population of 3.1%, well below the upper limit of 10% for a causative allele.

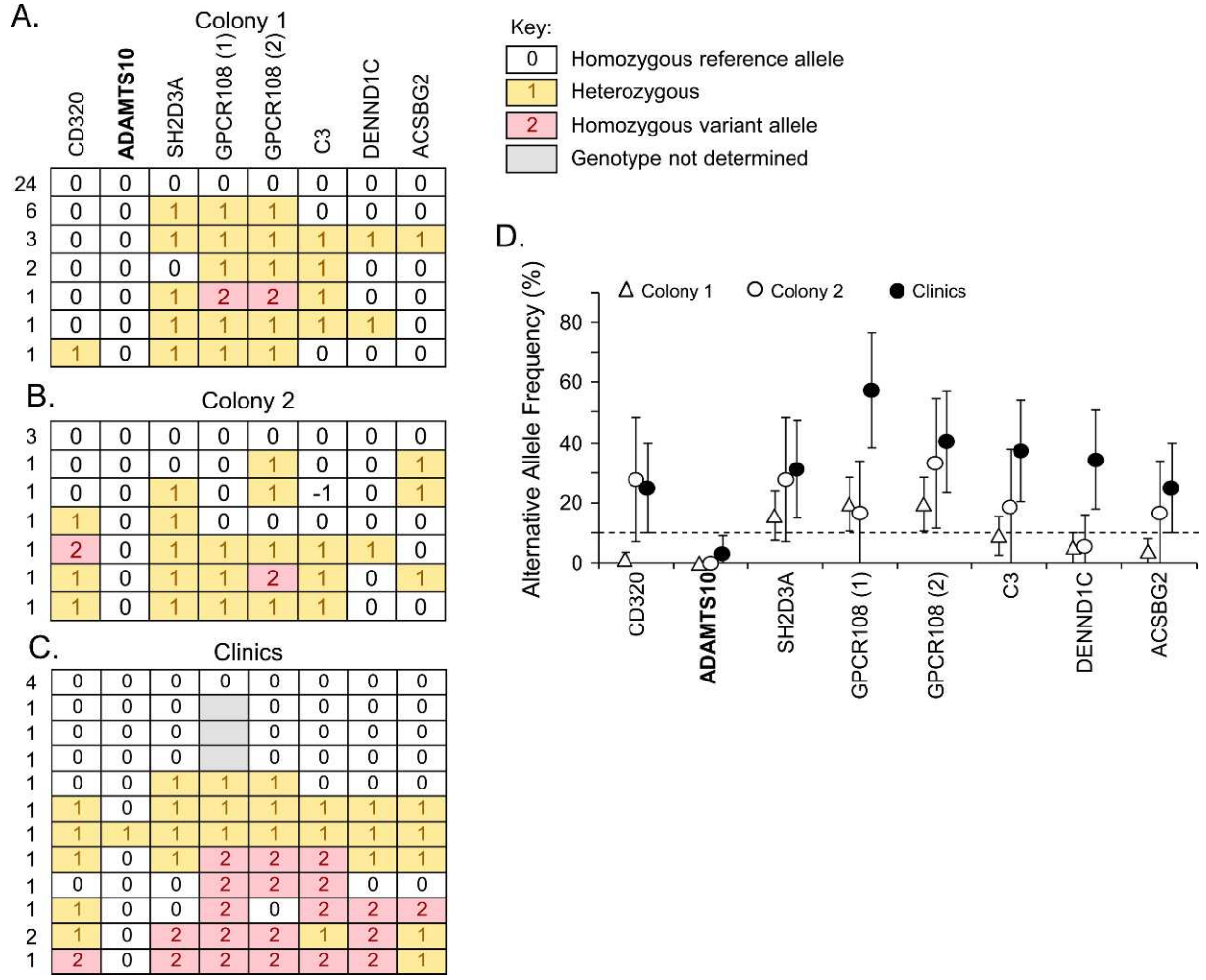

Figure 2. Beagles genotyped for the nonsynonymous SNPs in the beagle glaucoma locus. Beagles not affected by glaucoma from two different animal supplier colonies (**A**, **B**) and from veterinary clinics (**C**) were genotyped for the eight nonsynonymous SNPs in the beagle glaucoma locus that segregated with disease in the beagle glaucoma colony. The key for genotype calls is shown (**A**). Each *horizontal row* represents the haplotype of one or more dogs, with number of dogs represented to the *left of the row* (**A**–**C**). The gene containing each variant is indicated *above* (**A**). In GPCR108, there are two nonsynonymous SNPs, GPCR108 (1) and GPCR108 (2). Minor allele frequencies for the variants are shown for the supplier colonies and clinic samples (**D**) with *error bars* representing +/− 95% CIs. The alternative allele frequency upper threshold of 10% for a putative disease allele in beagles is indicated by the *dashed line* (**D**).

Control Dogs of Various Breeds

The eight nonsynonymous SNPs were also genotyped in a collection of 28 control dogs of 19 different breeds (Fig. 3). Dogs homozygous for the alternative alleles in *SH2D3A*, *GPCR108*, *C3*, and *ACSBG2* were identified in this set, further ruling these out as causative. The alternative allele in *ADAMTS10* was not ruled out since none of the 28 control dogs were found homozygous for the alternative allele.

American Cocker Spaniels

A collection of 16 affected and 26 control American cocker spaniels was genotyped for the eight nonsynonymous SNPs and minor allele frequencies determined (Fig. 4). The *ADAMTS10* variant allele was not found in any of the American cocker spaniels, suggesting involvement of other genes. The alternative alleles for the other nonsynonymous SNPs were common, with no significant difference between affected and control groups, ruling out these SNPs as causative of primary glaucoma in American cocker spaniels.

Various Breeds Affected with Primary Glaucoma

Since dog breeds are highly related genetically, we next investigated whether the *ADAMTS10* variant might be found in dogs of various breeds affected with primary glaucoma. All eight nonsynonymous SNPs were genotyped in seven affected basset hounds (Fig. 5A) and a collection of 10 affected dogs from various breeds, including Shiba Inu, Shih Tzu, Chihuahua, Australian Cattle Dog, Jack Russell Terrier, Jindo, Siberian Husky, and Yorkshire Terrier (Fig. 5B). The *ADAMTS10* variant was not found in any of these affected dogs, suggesting other genes are involved.

A beagle not related to the POAG beagle colony was found with primary glaucoma. This beagle was the only dog identified as homozygous for the *ADAMTS10* variant allele (Fig. 5C), strongly supporting this variant as causative for primary glaucoma in beagles. This affected beagle was also homozygous for the variant alleles across the 8-SNP locus formed by the nonsynonymous SNPs, suggesting a shared haplotype with the beagle glaucoma colony.

CD320	**ADAMTS10**	SH2D3A	GPCR108 (1)	GPCR108 (2)	C3	DENND1C	ACSBG2	
0	0	0	0	0	0		0	Bassett Hound
0	0	1	0	1	0	0	2	Bassett Hound
0	0	0	0	0	0	0	1	Boston Terrier
0	0	0	2	2	2	0	2	Boston Terrier
0		0	0	0	0	0	0	Boxer
0	0	0	0	0	0	0	0	Cock-A-Poo
0	0	0	0	0	0	0	0	French Bulldog
0	0	2	1	1	0	0	0	German Shepherd
0	0	1	0	0	0	0	0	German Shepherd
0	0	0	0	0	0	0	0	Golden Retriever
0	0	1	1	1	1	0	0	Great Dane
0	0	1	1	1	0	0	1	Jack Russell Terrier
0	0	1	0	0	0	0	0	Jack Russell Terrier
0	0	0	1	1	0	0	0	Jack Russell Terrier
0	0	2	1	2		0	1	Lhasa Apso
0	0	2	1	1	0		1	Maltese
0	0	0	1	1	0	0	0	Mini Poodle
0	0	0	0	0	0	0	0	Mini Schnauzer
0		0	0	1	0	0	0	Mixed Breed
0	0	1	1	1	1	0	0	Mixed Breed
0	0		1	1	1	0	0	Mixed Breed
0	0	0		0	0	0	0	Poodle
0	0	0	0	1			2	Poodle
0	0	0	0	0	0		2	Pug
0	0	2	0	0	0	0	0	Retriever
1	0	0		1	0	1	1	Siberian Husky
0	0	0	0	1	0	0	0	Yorkshire Terrier
0	0	0	0	1	0	0	1	Yorkshire Terrier

Figure 3. Control dogs of various breeds genotyped for the nonsynonymous SNPs in the beagle glaucoma locus. Dogs of various breeds, not affected by glaucoma, were genotyped for the eight nonsynonymous SNPs that segregated with disease in the beagle glaucoma colony. Each *horizontal row* represents the haplotype of an individual dog. The gene containing each variant is indicated above. Genotypes are displayed as in Figure 2.

Discussion

Genotyping affected and unaffected dogs of various breeds proved to be an effective method for testing the hypothesis that the Gly661Arg variant of *ADAMTS10* is the cause of inherited disease in the POAG beagle colony. The candidate disease allele had to satisfy the criterion that unaffected dogs could not be homozygous for the disease allele, since the disease is inherited as an autosomal recessive trait in the beagle POAG colony. The candidate disease allele also had to satisfy the criterion that its frequency in beagles was lower than the upper threshold of 10%, based on Hardy-Weinberg equilibrium, disease prevalence of 1% and autosomal recessive inheritance. The upper threshold is conservatively high because it assumes that a single genetic variant accounts for all beagle primary glaucoma, which is unlikely. Failure to satisfy the two criteria was strong evidence against a variant as being causative.

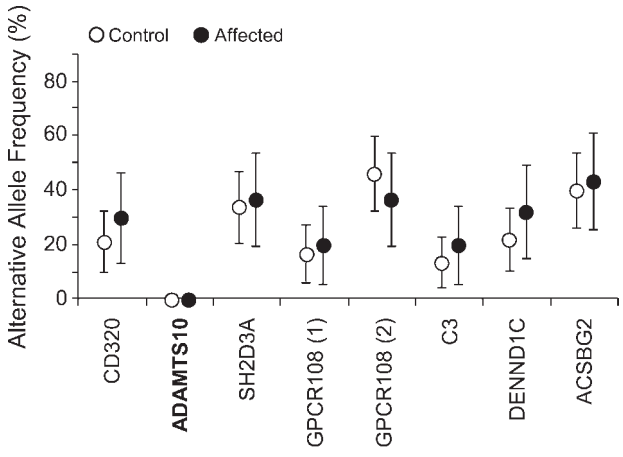

Figure 4. American cocker spaniels affected and unaffected by primary glaucoma genotyped for the nonsynonymous SNPs in the beagle glaucoma locus. Minor allele frequencies for the variants are shown for 26 unaffected (*open symbols*) and 16 affected (*closed symbols*) American cocker spaniels. *Error bars* represent +/− 95% CIs.

A. Bassett Hounds

CD320	**ADAMTS10**	SH2D3A	GPCR108 (1)	GPCR108 (2)	C3	DENND1C	ACSBG2
0	0	0	0	0	0	0	0
0	0	0	0	0	0		0
0	0	0	1	1		0	1
0	0	0	1	1		0	0
0	0	0	1	1		0	1
0	0	2	1	1		0	1
0	0	2	0	0	0	0	1

B. Various Breeds

1	0	1	1	1	0	0	0	Australian Shepherd
0	0	0	0	1		0	1	Chihuahua
1	0	2	0	0	0	0	0	Jack Russell Terrier
0	0	1	0	0	2	0	0	Jindo
0	0	0	0	0	2		0	Shiba Inu
0	0	0	0	0	0	0	0	Shiba Inu
0	0	1	2	2	0	0	1	Shih Tzu
0	0	1	0	0	0	0	0	Shih Tzu
0	0	1	0	0	0	2	0	Siberian Husky
0	0	2		0	0	0	0	Yorkshire Terrier

C. Beagle

2	2	2	2	2	2	2	2

Figure 5. Affected dogs of various breeds genotyped for the nonsynonymous SNPs in the beagle glaucoma locus. Bassett Hounds (**A**), dogs of various breeds (**B**), and a beagle (**C**), affected by primary glaucoma, were genotyped for the eight nonsynonymous SNPs in the beagle glaucoma locus. Each *horizontal row* represents the haplotype of an individual dog. The gene containing each variant is indicated *above*. Genotypes are displayed as in Figure 2.

In addition to the *ADAMTS10* variant, some of the nonsynonymous SNPs were functionally compelling but could not previously be ruled out on the basis of allele frequency. For example, complement factor *C3* is functionally plausible, since there is evidence that complement could play a role in glaucoma.[20] *DENND1C* is a GDP-GTP exchange factor for rab13,[21] which coordinates assembly of tight junctions,[22] which may play a role in aqueous humor outflow resistance.[23] However, in the current study, complement factor *C3*, *DENND1C* and the other nonsynonymous SNPs other than that found in *ADAMTS10* were ruled out because control beagles were found homozygous for the alternative alleles. Identification of control dogs of various breeds homozygous for nonsynonymous SNPs further argues against these variants as glaucoma-causing, though it is possible that breed-specific

differences could result in variable penetrance of a disease allele. The Gly661Arg variant of *ADAMTS10* was the only SNP that satisfied the criterion that no control dogs were found homozygous for the variant allele. *ADAMTS10* was also the only variant with a minor allele frequency below the upper limit of 10% in beagles and therefore the only plausible candidate among the eight nonsynonymous SNPs. The criteria of no homozygosity in controls and sufficiently low allele frequency were effective at eliminating the presumably false-positive candidate SNPs and support the Gly661Arg variant of *ADAMTS10* as causative for disease in the beagle POAG colony.

For breeds other than beagle, no evidence was found for involvement of *ADAMTS10* in primary glaucoma. The *ADAMTS10* variant was not found in a total of 43 affected dogs of breeds other than beagle, including 26 American Cocker Spaniels, seven Basset Hounds and 10 dogs of several other breeds. While the sample sizes are too small to rule out *ADAMTS10* as causative, these data suggest that primary glaucoma in dogs is likely to be genetically complex, involving other loci in addition to *ADAMTS10*.

While a genetic basis for canine primary glaucoma is likely because of the high prevalence in many breeds, differences in age of onset, sex ratios, and inheritance patterns suggest distinct disease processes and involvement of multiple genes. Clinically, primary glaucoma in dogs most often presents with narrowed or closed angles, marked by dysplasia of the pectinate ligament structures, with complete angle closure and collapse of ciliary cleft typically seen after prolonged elevation of IOP. However, studies of POAG in the beagle have documented angle closure secondary to prolonged ocular hypertension, likely related to enlargement of the globe. Some dogs presenting to clinics at late stages of the disease, as is common with canine patients, may previously have had open angles with elevated IOP.

A colony of Basset Hounds with inherited glaucoma has been reported recently with the disease well-characterized as primary angle-closure glaucoma.[24] Absence of the *ADAMTS10* variant in the basset hounds affected with primary glaucoma would be consistent with a disease process different from POAG in beagles, a notion corroborated by the invariably dysgenic iridocorneal angles in these dogs. Although most American cocker spaniels present with severely dysgenic iridocorneal angles, primary glaucoma with open angles was reported in 1968 in this breed.[25] In this study, three Cocker Spaniels bred from glaucomatous dogs were investigated. These Cocker Spaniels had POAG, with elevated IOP and reduced outflow facility, but normal-appearing and open angles by gonioscopy and postmortem histology. Lack of the Gly661Arg variant of *ADAMTS10* in affected American cocker spaniels in the present study is consistent with either multiple genes involved in canine POAG or in most cases a distinct disease process.

A limitation of this study is that although seven of the eight variants have been effectively ruled out, it does not provide definitive evidence that the Gly661Arg variant is causal. Future studies either preventing glaucoma in dogs from the POAG beagle colony by expression of the normal form of the gene, or by inducing glaucoma by ablation of *ADAMTS10* in mice would provide more conclusive data that the Gly661Arg variant of *ADAMTS10* is causative.

The strongest evidence supporting the hypothesis that Gly661Arg variant of *ADAMTS10* causes glaucoma is the identification of an affected beagle homozygous for the variant. At 5 years of age, this dog was carefully examined by a veterinary ophthalmologist (CEP) and found to have open iridocorneal angles and elevated IOP in both eyes. A total of 63 control beagles were sequenced, which is a relatively large number for dog breeds which are isolated populations with very limited genetic diversity relative to humans.[26] A single control beagle was identified heterozygous for Gly661Arg, giving a MAF of 0.78%. By Hardy-Weinberg equilibrium, fewer than 1 in 1600 beagles would be homozygous for Gly661Arg. Though additional cases would strengthen our conclusions, our identification of a beagle that is both homozygous for this rare allele and affected by a disease with low prevalence supports the *ADAMTS10* variant as causative. This support for the Gly661Arg variant of *ADAMTS10* in beagle POAG is consistent with our more general hypothesis that disruption of microfibril structure or function may be an underlying mechanism of increased resistance to aqueous humor outflow in POAG.

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