Mapping of the Disease Locus and Identification of ADAMTS10 As a Candidate Gene in a Canine Model of Primary Open Angle Glaucoma

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Abstract

Primary open angle glaucoma (POAG) is a leading cause of blindness worldwide, with elevated intraocular pressure as an important risk factor. Increased resistance to outflow of aqueous humor through the trabecular meshwork causes elevated intraocular pressure, but the specific mechanisms are unknown. In this study, we used genome-wide SNP arrays to map the disease gene in a colony of Beagle dogs with inherited POAG to within a single 4 Mb locus on canine chromosome 20. The Beagle POAG locus is syntenic to a previously mapped human quantitative trait locus for intraocular pressure on human chromosome 19. Sequence capture and next-generation sequencing of the entire canine POAG locus revealed a total of 2,692 SNPs segregating with disease. Of the disease-segregating SNPs, 54 were within exons, 8 of which result in amino acid substitutions. The strongest candidate variant causes a glycine to arginine substitution in a highly conserved region of the metalloproteinase ADAMTS10. Western blotting revealed ADAMTS10 protein is preferentially expressed in the trabecular meshwork, supporting an effect of the variant specific to aqueous humor outflow. The Gly661Arg variant in ADAMTS10 found in the POAG Beagles suggests that altered processing of extracellular matrix and/or defects in microfilibr structure or function may be involved in raising intraocular pressure, offering specific biochemical targets for future research and treatment strategies.

Introduction

Elevated intraocular pressure is a strong risk factor for glaucoma development and progression [1]. In POAG, increased resistance to outflow of aqueous humor through the trabecular meshwork is the cause of elevated intraocular pressure [2]. Currently, the only proven treatments for POAG patients involve reduction of intraocular pressure by inhibiting aqueous humor production, or bypassing the diseased trabecular meshwork. The mechanisms of increased resistance to aqueous humor outflow are not well-understood [2], but may involve changes in extracellular matrix composition of the trabecular meshwork [3].

Linkage studies have identified a number of POAG loci [4]. So far only three genes have been shown to be associated with POAG [4], but they account for only a small fraction of POAG cases, and none have shed much light on the disease process. Although genome-wide association studies could be a powerful tool to establish more POAG loci, this requires recruitment of a large number of patients. Moreover, causal association between sequence variants and disease can be difficult to establish in human studies. In this study, we have used a canine model to identify a candidate POAG gene, which has the advantage of availability of tissues from normal and affected dogs as well as future gene rescue experiments to investigate the pathogenic mechanisms of the gene variant.

A colony of Beagle dogs established in 1972 [5], which is a well-characterized and naturally occurring animal model of POAG, was used for this study. For POAG-affected dogs in this colony, increases in intraocular pressure begin at 8 to 16 months of age, due to increased resistance to outflow of aqueous humor [6], despite normal appearing open iridocorneal angles. As with POAG in humans, optic nerve cupping, loss of optic nerve axons [7] and vision loss occur in affected Beagles following slowly progressing and sustained elevations of intraocular pressure, if left untreated. Multigenerational breeding experiments have shown that POAG in the Beagle colony is inherited as an autosomal recessive trait [8].

Domestication of the dog from wolves and recent breed creations have resulted in extensive linkage disequilibrium and
Author Summary

Primary open angle glaucoma (POAG) is a leading cause of vision loss and blindness affecting tens of millions of people. Ocular hypertension is a strong risk factor for the disease and the only effective target of treatment. Ocular hypertension results from increased resistance to outflow of aqueous humor through the trabecular meshwork, a specialized filtration tissue consisting of alternating layers of cells and connective tissue, but the specific reasons for the increased resistance are not known. The animal model for human POAG used in this study was a colony of Beagle dogs that carry an inherited form of the disease in which ocular hypertension is the primary manifestation. We have found a variant in ADAMTS10 that belongs to a family of genes that contribute to formation of extracellular matrix and may itself be involved in formation of elastic microfiber structures. We found that the ADAMTS10 protein is expressed at particularly high levels in the trabecular meshwork. The candidate variant in ADAMTS10 found in the POAG-affected Beagles suggests that altered processing of connective tissue and/or elastic microfiber defects may be involved in raising eye pressure, offering specific biochemical targets for future research and treatment strategies.

Results

To map the POAG locus, we genotyped 19 affected and 10 carrier dogs from the POAG Beagle colony using version 2 of the Affymetrix Canine Genome SNP array. Since the colony has been maintained primarily by affected to affected breeding, with periodic introduction of unrelated normal Beagles (Figure 1), we hypothesized that the disease allele would be contained within an extensive haplotype block homozygous for affected and heterozygous for carrier dogs. Therefore, we identified SNPs that fulfilled the zygosity criterion, defined as being both homozygous for all affected dogs and heterozygous for all carriers. Regions of homozygosity for all affected dogs were common for all chromosomes, as expected for the highly inbred pedigree. However, only Chromosome 20 contained SNPs homozygous for all carriers (Figure 2A), consisting of 41 consecutive SNPs covering 4.7 Mb. Of those 41 SNPs, 27 consecutive SNPs were also homozygous for all affected dogs, satisfying the zygosity criterion (Figure 2B and Figure S1). Haplotype analysis of the region revealed informative recombination events within the pedigree that defined a 4 Mb locus in which all carriers were heterozygous and all affected dogs homozygous for the affected haplotype (Figure 2C).

In addition to applying the zygosity criterion, two-point and multipoint parametric linkage analyses of the pedigree genotype data were performed. Initial power calculations predicted that with the available pedigree, a single locus could be identified with a LOD score of 2.67. With genome-wide two-point analysis, regions with LOD score >2 were found on chromosomes 5, 15 and 20 (Figure 3A). Follow-up multipoint linkage analysis reduced the LOD score of the chromosome 5 region to below 0.5, excluding this as a candidate locus (Figure 3B). For chromosome 15, multipoint analysis did not reduce the LOD score (Figure 3C). However, haplotype analysis revealed a pattern of inheritance discordant with phenotype (Figure 4), excluding chromosome 15. The distal end of chromosome 20 had a two-point LOD score of 2.42 and a multipoint LOD score of 2.70 (Figure 3D), consistent with initial power calculations. This region identified by linkage analysis coincided with the 4 Mb locus identified by the zygosity criterion.

Comparison of the 4 Mb POAG locus in dog with the human genome revealed shared synteny within a segment of human chromosome 19, previously identified as a quantitative trait locus for intraocular pressure in humans [13] (Figure 5A). The order and number of genes within the POAG locus on the canine chromosome are highly conserved in the human syntenic region (Figure 5B). Since increased intraocular pressure is the initial manifestation of disease in the POAG Beagles, synteny with the human intraocular pressure locus offers compelling biological support that the 4 Mb region contains the disease-causing genetic variant.

To identify the disease gene, the entire 4 Mb POAG locus in an affected and a carrier dog, as well as a normal dog from the colony (dogs 3, 9 and 11, Figure 1) was isolated by microarray-based sequence capture and then sequenced with the Illumina Genome Analyzer. Alignment of the sequences to the reference canine genome revealed 2,692 sequence variants segregating with disease
homozygous for the affected dog and heterozygous for the carrier dog, with the additional criterion that the normal dog is not homozygous for the same allele as the affected dog. Of the segregating variants, 54 were located within coding regions of canine genes identified by the human protein alignment track of the UCSC genome browser (http://genome.ucsc.edu). Of the 54 variants within coding regions, 8 resulted in non-synonymous amino acid substitutions in 7 genes. Among those 8 variants, based on BLOSUM62 score for amino acid substitution and mammalian conservation score from the vertebrate multiz alignment and conservation track of the UCSC human genome browser, the best candidate variant was at position 56,097,365 of chromosome 20 (canFam build 2) from a G in the reference sequence, to an A in the affected dog. This variant was confirmed by conventional Sanger sequencing of affected, carrier and normal dogs from the POAG colony (Figure 6A). To determine the frequency of the disease allele (56097365 A) in the normal Beagle population, 48 Beagles not affected by glaucoma and not related to the colony were sequenced. Only one of the unaffected dogs was found heterozygous for the disease allele, the rest were homozygous for the normal allele, suggesting a disease allele frequency of ~1% in Beagles.

The 56097365 G->A variant is within exon 17 of ADAMTS10, a member of the disintegrin and metalloproteinase with thrombospondin motifs family of secreted proteases involved in formation of the extracellular matrix [14–16]. The variant results in a Gly->Arg substitution at position 661 within the protein sequence (NCBI accession XP_854320). The glycine at position 661 is completely conserved in 38 vertebrate species (7 representative species shown, Figure 6B). The Gly661Arg substitution was predicted to have a deleterious effect on protein function by the prediction programs SIFT [17] and SNPs3D [18] and occurs within the cysteine-rich domain (Figure 6C), which may be involved in regulation of protease activity [19].

Western blot analysis of protein extracts from tissues dissected from normal dog eyes showed high expression of ADAMTS10 protein in the trabecular meshwork, relative to other eye tissues examined (Figure 7). ADAMTS10 was also expressed in the cornea, and to a much less extent in the iris, ciliary body and optic nerve (Figure 7).

Structural modeling was performed using crystal structure of ADAMTS13 [20] to predict the structures of normal and Gly661Arg ADAMTS10 proteins. In the predicted fold of ADAMTS10, Gly661 is located within a tight turn (Figure 8A), suggesting a glycine may be required at this position for proper folding. Gly661 is predicted to be buried in the structure within the interface between the C_4 and T_1 domains (Figure 8B). Substitution of arginine for glycine at position 661 would be sterically unfavorable, with the longer charged side chain of arginine extending into the T_1 domain (Figure 8C), suggesting that the Gly661Arg change would likely disrupt normal ADAMTS10 structure.
of four independent experiments, mutated ADAMTS10 decayed equal to the negative inverse of the slope of the best fit line. In each were plotted vs.

Calculation of two-point LOD scores revealed regions of interest, data. Defined as having LOD score below 1 (B). Multipoint LOD scores for the two regions of linkage analysis reduced the LOD score of the chromosome 5 region of analyses are shown for chromosomes 5, 15 and 20 (B–D). Multipoint two-point (black lines) and follow-up multipoint (red lines) linkage

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A

B

C

D

Figure 3. Genome-wide linkage analysis of SNP genotyping data. Calculation of two-point LOD scores revealed regions of interest, defined as having LOD score >2, on chromosomes 5, 15 and 20 (A). Two-point (black lines) and follow-up multipoint (red lines) linkage analyses are shown for chromosomes 5, 15 and 20 (B–D). Multipoint linkage analysis reduced the LOD score of the chromosome 5 region of interest to below 1 (B). Multipoint LOD scores for the two regions of interest (R1 and R2) of chromosome 15 were similar or higher than the two-point scores (C). The region on the distal end of chromosome 20 with two-point LOD score >2 had multipoint score of 2.70, and corresponds to the 4 Mb locus identified by the zygosity criterion. doi:10.1371/journal.pgen.1001306.g003

To investigate possible effects of the Gly661Arg substitution on ADAMTS10 protein stability, the protein half-lives for normal and mutated ADAMTS10 were determined. Since ADAMTS10 produced by trabecular meshwork cells would be secreted into aqueous humor, half-lives were determined in the presence of aqueous humor. In vitro transcribed normal and mutated ADAMTS10 protein labeled with biotinylated lysine was incubated in aqueous humor for various time periods and the amount of ADAMTS10 remaining at each time point was determined by Western blotting with fluorescently labeled streptavidin. The Gly661Arg mutant appeared to decay more rapidly than did normal ADAMTS10 (Figure 9). The Log2 of the band intensities were plotted vs. time to determine the protein half-life, which is equal to the negative inverse of the slope of the best fit line. In each of four independent experiments, mutated ADAMTS10 decayed more rapidly than did normal ADAMTS10 (261+/−29.5 vs. 601+/−219.7 min., mean +/- SD, half-lives for mutated and normal, respectively, significantly different, p<0.05). The slopes of the lines fit to data from all four experiments, combined by normalizing band intensities to the initial time point, were significantly different (p<0.0001) and correspond to half lives of 253.9 min. for mutated and 636.9 min. for normal ADAMTS10 (Figure 9C). These results suggest that mutated ADAMTS10 decays more rapidly, with a protein half-life ~40% that of normal.

Discussion

By application of the zygosity criterion, linkage and haplotype analyses, we were able to map the Beagle POAG locus to a single 4 Mb region on chromosome 20. This canine POAG locus is syntenic with a region on human chromosome 19 within a quantitative trait locus for regulation of intraocular pressure identified by a genome-wide scan of 486 families [13]. Since ocular hypertension occurs early in the disease process in the Beagles, synteny with the intraocular pressure locus gives biological support to the genetic identification of the POAG locus and suggests that the disease gene directly participates in intraocular pressure regulation. Furthermore, synteny with the human locus suggests that the disease gene found in the Beagles may also be disrupted in human glaucoma patients.

Using affected and unaffected dogs of other breeds to fine map the disease locus has proven to be an effective approach in other canine genetic studies [10]. However, because clinical identification of POAG cases in dogs is rare, this approach would be challenging and further would require the assumption that the POAG locus in Beagles is shared with affected dogs of other breeds. Alternatively, refining the locus by further breeding within the colony to allow for informative recombinations would be time consuming and costly since definitive diagnosis cannot be made until two years of age.

To overcome these limitations, we obtained high quality sequence information for the entire 4 Mb locus by sequence capture and next-generation sequencing. Using this approach, 2,692 single nucleotide variants that segregated with disease were identified, 54 of which were within exons, 8 of which were nonsynonomous. Since POAG in the POAG Beagle colony is autosomal recessive with 100% penetrance, we focused on nonsynonomous changes because these are likely to have strong functional effects. However, synonymous changes in coding regions or variants outside coding regions could have pathogenic effects and cannot be ruled out. In addition, our sequence capture and sequence analysis rely on the quality of the reference canine genome and therefore our approach could miss variants due to errors in the reference genome assembly or annotation.

Among the 8 nonsynonomous variants segregating with disease, the strongest candidate identified was a single base pair change in the affected dogs that results in a non-conservative amino acid substitution in a region of ADAMTS10 that is highly conserved in vertebrate species. In POAG-affected dogs, an arginine is substituted for a glycine at amino acid position 661 which is an invariant amino acid in ADAMTS10 in 38 species, from lamprey to human. Consistent with a highly penetrant rare disease allele, the frequency of the variant in ADAMTS10 estimated from genotyping 48 unrelated normal Beagles was 1%.

ADAMTS10 is a member of a family of secreted metalloproteases [14,16]. All ADAMTS family members share a common structural organization including a metalloproteinase domain followed by a disintegrin-like module, a thrombospondin repeat unit, a cysteine-rich domain and a spacer region (see Figure 6C).
Diversity within the ADAMTS family largely arises from structural differences in ancillary domains of the carboxy-terminal half of the proteins. The Gly661Arg variant found in this study is within the cysteine-rich domain of ADAMTS10 and is predicted to disrupt protein function by the amino acid substitution prediction programs SIFT [17] and SNPs3D [18]. Consistent with this, our homology modeling of the ADAMTS10 structure suggests that the Gly661 residue is located within a tight turn and is buried within the interface between the cysteine rich and thrombospondin repeat domains. The long polar side chain of arginine substituted at this position is predicted to disrupt the normal protein fold. Consistent with disruption of normal protein folding, we found that the Gly661Arg form of ADAMTS10 is less stable, with a protein half-life ~40% that of normal ADAMTS10. Although we cannot be certain if the reticulocyte lysate-based in vitro transcription and translation system produced normally folded protein, this system has been used to produce functional secreted proteins such as metalloproteinases [21], neutrophil elastase [22] and myocilin [23]. Any effects of the in vitro system on folding would be experienced by both the normal and mutated proteins in our assays. Our data show that the mutated form of ADAMTS10 has a shortened half-life, consistent with our homology modeling which suggested that the Gly661Arg substitution would disrupt interactions at the interface of two domains.

Figure 4. Haplotype analysis of chromosome 15 regions R1 and R2. Although region R1 (A) and R2 (B) had multipoint LOD scores >2, as shown in Figure 3C, their inheritance patterns were discordant with disease status, ruling out these regions as containing the disease allele. The minimal informative pedigree is shown, with dog numbers and symbols corresponding to the pedigree shown in Figure 1. The chromosomal positions for the SNPs defining the haplotypes are shown in Figure S2. doi:10.1371/journal.pgen.1001306.g004

Figure 5. The canine POAG locus is syntenic with a human quantitative trait locus for intraocular pressure. The 4 Mb POAG locus (solid blue rectangle) found in the Beagle colony maps to a 6.5 Mb region (solid red rectangle) on the short arm of human chromosome 19, and within a 20 cM quantitative trait locus for intraocular pressure in humans located near microsatellite marker D19S586 (purple symbol) (A). The number and order of genes within the canine POAG locus and the syntenic region of human chromosome 19 are well conserved (B). Canine genes, blue circles, human genes, red circles. Orthologous genes are connected by gray lines. The canine chromosome 20 locus extends from base pair positions 55,881,144 to 59,844,869, corresponding to human chromosome 19 base pair positions 2,389,784 to 8,841,863. Direction of increasing base pair number of the reference sequence is indicated by red and blue arrows (B). At the centromeric end of the canine locus, a 0.3 Mb portion of the locus is inverted with respect to the human chromosome (B). The figure is drawn to scale, with scale bars of 5 Mb (A) and 0.5 Mb (B) shown. doi:10.1371/journal.pgen.1001306.g005
Clinical evidence for the importance of the cysteine rich domain in ADAMTS function comes from patients with thrombocytopenic purpura (OMIM #274150) who have autoantibodies recognizing the cysteine-rich domain of ADAMTS13, causing reduced proteolytic activity of ADAMTS13 in vivo and in vitro [24,25]. Structural studies and deletion analysis have established that the cysteine-rich domain plays a vital role in regulation of protease activity or substrate recognition for ADAMTS family proteins [20,25]. In addition, alignment of the cysteine-rich domains of all 19 human ADAMTS family members and 5 related ADAMTSL proteins by Akiyama et al. [20], revealed that Gly661 of ADAMTS10 is an invariant amino acid. Such stringent evolutionary conservation of this glycine residue, across 38 vertebrate species and within 24 protein superfamily members, supports the hypothesis that the arginine substitution would have a detrimental effect on ADAMTS10 function.

Unlike the three POAG genes identified thus far in humans (MYOC, WDR36 and OPTN) [4], the ADAMTS10 variant identified in this study has obvious functional implications, supporting ADAMTS10 as a strong candidate gene. A role for metalloproteases in ocular hypertension has long been suggested by numerous in vitro studies [26]. Changes in the amount or composition of extracellular matrix within the trabecular meshwork have been hypothesized to contribute to ocular hypertension by increasing resistance to outflow of aqueous humor through the trabecular meshwork [3]. Although the specific substrate for ADAMTS10 is unknown, other ADAMTS family members are known to participate in collagen processing and proteoglycan degradation. ADAMTS10 is likely to function in some capacity in regulation of extracellular matrix and therefore disruption of its function could lead to POAG by increasing resistance to aqueous humor outflow through the trabecular meshwork. Several ADAMTS family members have been investigated as candidates for regulating outflow resistance, and it has been shown that perfusion of anterior segment organ cultures with ADAMTS4 increases outflow facility [27]. The faster decay of the Gly661Arg ADAMTS10 would likely reduce the amount of ADAMTS10 available, which could possibly result in increased resistance to aqueous humor outflow. Future studies with anterior segment organ cultures perfused with normal and mutated ADAMTS10 could test this hypothesis.

The Beagles of the POAG colony are phenotypically normal and without systemic abnormalities other than POAG in the affected dogs. Our Western blotting results showed that ADAMTS10 is expressed at high levels within the trabecular meshwork as compared to other eye tissues, which would be consistent with an effect of the Gly661Arg ADAMTS10 variant specific to aqueous humor outflow.

Mutations in ADAMTS10 have been identified in human patients with autosomal recessive Weill-Marchesani syndrome (WMS) [28,29], a rare disease with systemic features including short stature and stubby hands and feet (OMIM #277600). A mutation in type I fibrillin has also been found in autosomal dominant WMS [30], which is clinically indistinguishable from the autosomal recessive form [31], suggesting a functional link between ADAMTS10 and type I fibrillin. WMS belongs to a group of rare connective tissue disorders, including Marfan syndrome (OMIM #154700), for which causative mutations in type I fibrillin, a major constituent of microfibrils [32], have been found. While glaucoma is common in WMS patients [31], the mechanism is not well-studied, due to the extremely small patient population. The prevalence of glaucoma in Marfan syndrome patients is higher than in the general population [33]. Clinically,
Figure 8. Structural modeling predicts disruption of the normal protein fold by the Gly661Arg variant of ADAMTS10. The structure of normal (A and B) and Gly661Arg mutated (C) ADAMTS10 was predicted by homology modeling using the amino acid sequence of ADAMTS10 and the crystal structure of ADAMTS13 [20], which includes the disintegrin-like domain (D), the thrombospondin-1 type-1 repeat domain (T1), the amino-terminal portion of the cysteine-rich domain (CA), the carboxy-terminal portion of the cysteine-rich domain (CB) and the spacer domain (S). Rotated views (90°) of the entire predicted structure for ADAMTS10 are shown, with the portion of backbone corresponding to Gly661 in the CA domain colored black (A). The boxed portion in A is expanded to show the substitution site for normal (B) and mutated (C) ADAMTS10, with selected amino acid side chains shown.

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Figure 9. Gly661Arg-mutated ADAMTS10 decays more rapidly than does normal ADAMTS10. In vitro transcribed normal (A and B) and Gly661Arg mutated (B and red squares in C) ADAMTS10 labeled with biotinylated lysine residues was incubated in aqueous humor for various times at 37°C. A band of ~130 kDa was detected by Western blotting with fluorescently labeled streptavidin, as shown in a representative experiment for both normal (A) and mutated (B) ADAMTS10 (incubation times, in minutes, shown below lanes). The slopes of the lines fit to data from all four experiments combined by normalizing band intensities to the initial time point (C) were significantly different (p < 0.001) and correspond to half lives of 255.8 min. for mutated (red squares and red line) and 636.9 min. for normal ADAMTS10 (blue diamonds and blue line) (C). Molecular weight markers are shown in the left-most lanes of the blots (A and B) with mw in kDa indicated.

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glaucoma in Marfan syndrome most often presents as POAG, with elevated intraocular pressure and open iridocorneal angles [33]. As type I fibrillin is involved in microfibril formation and function, presentation of POAG in patients with Marfan syndrome caused by type I fibrillin mutations suggests that microfibril defects may be involved in POAG pathogenesis. This notion is supported by another common ocular manifestation in WMS and Marfan syndrome, ectopia lentis (dislocated or malpositioned lens). Consistent with a defect in microfibril structure or function, ectopia lentis is caused by defects in the zonule fibers that hold the lens in place and are composed of fibrillin-containing microfibrils [34]. Recently, mutations were found in other members of the ADAMTS superfamily, ADAMTS17 in autosomal recessive WMS [29] and ADAMTS14 in isolated ectopia lentis [35], supporting a role for ADAMTS family members in microfibril structure and function.

Ultrastructural studies of human trabecular meshwork have shown changes with age that are more pronounced in POAG patients, including a thickening of sheaths that surround elastin fibers and are composed of extracellular matrix, including fibrillin and fine fibrils, as well as an accumulation of sheath-derived plaques in the aqueous humor outflow pathway [36]. We have previously described similar changes in the trabecular meshwork of POAG-affected Beagles [37], which could be explained by microfibril defects caused by the Gly661Arg variant in the ADAMTS10 gene. Additionally, microfibrils play an important regulatory role in the homeostasis of extracellular matrix by controlling the activation and localization of TGFβ [32], which is elevated in the aqueous humor of glaucomatous eyes [38,39]. Involvement of microfibril defects in glaucoma is further suggested by recent findings in primary congenital glaucoma of a null mutation in LTBP2, which shares homology with fibrillins and is a structural and functional component of microfibrils [40]. Identification of the Gly661Arg variant of ADAMTS10 in the POAG Beagles in this study provides genetic evidence that microfibril abnormalities may be involved in increased resistance to outflow of aqueous humor through the trabecular meshwork in POAG.

The precise mechanisms of increased resistance to outflow of aqueous humor have remained a long-standing puzzle in glaucoma research. Current treatments for POAG patients involve reduction of intraocular pressure by inhibiting production of aqueous humor, or bypassing the diseased outflow pathway, but do not address the root of the problem. The robust expression of ADAMTS10 in the trabecular meshwork suggests that any defect in ADAMTS10 function caused by the Gly661Arg variant could have particularly pronounced effects on the functioning of the trabecular meshwork, specifically affecting aqueous humor outflow. Identification of ADAMTS10 as a candidate gene in the POAG Beagles suggests that altered processing of extracellular matrix and/or defects in microfibril structure or function may be involved in raising intraocular pressure, offering specific biochemical targets for future research and treatment strategies.

Materials and Methods

SNP genotyping

Blood samples from dogs were obtained by licensed veterinarians or veterinary technicians by standard venipuncture, in accordance with the Institutional Animal Care and Use Committees of Vanderbilt University and the University of Florida. A total of 48 canine DNA samples, including 30 dogs from the POAG Beagle colony, 7 unrelated normal Beagles and 11 unrelated mixed-breed dogs, were genotyped using version 2 of the Affymetrix genome-wide SNP genotyping array (http://www.broadinstitute.org/mammals/dog/caninearrayfaq.html). For combined genotype data of all dogs, 40,600 informative SNPs had call rates >90%, and were heterozygous for <30% of dogs. For duplicate samples, 99.6% of SNPs received identical calls. The disease status of dogs was determined by clinical eye exams by veterinary ophthalmologists. The minimal age of the dogs at final diagnosis was 2.2 years.

Linkage analysis

Initial power calculations were performed using the SIMILINK V 4.12 program (http://esg.sph.umich.edu/boehnke/simlink.php). Two-point and multipoint linkage analyses of the genome-wide SNP data were performed using SuperLink Online [41] assuming an autosomal recessive model with complete penetrance. Dogs 1 through 31, except dog 6, were included in the analysis (Figure 1). SNPs uninformative for Beagles were removed from analysis. Mendelian error checking was performed and inconsistent SNPs removed for all individuals. Minor allele frequencies were calculated using SNP data from 8 unaffected Beagles whose unrelatedness was confirmed using the Graphical Representation of Relationship (GRR) software package [42].

Microarray-based DNA capture

Enrichment for genomic sequence within the 4 Mb locus was carried out using capture microarrays designed and manufactured by Roche NimbleGen, using build 2 of the canine genome. The capture arrays consisted of 385,000 capture probes >60 bp in length, designed to capture all non-repetitive sequence from base position 55,800,000 to 59,850,000 on canine chromosome 20. Sequence capture was carried out on 3 dogs from the POAG Beagle colony (dogs 3, 9, and 11, Figure 1), essentially as described in Albert et al. [43] and Okou et al. [44], with modifications to optimize for the Illumina Genome Analyzer II sequencing platform.

Illumina sequencing

Hybridization of the captured DNA fragments to the flow cell and amplification to form clusters was performed using the Illumina cluster station, following the standard Illumina protocol. The captured DNA fragments were used at a final concentration of 5 pM during hybridization/cluster generation to achieve cluster density of ~160,000 clusters/tile. Paired end, 38 base pair read sequencing was carried out with the Illumina Genome Analyzer II. Illumina pipeline software. Paired end alignments to the canine genome build 2 were carried out using Bowtie [45]. For the 3 samples, 53.7% of the reads aligned to the 4.05 Mb target region, representing 0.17% of the genome, yielding a 316-fold enrichment of the target sequence. The percentage of the capture region covered >8-fold ranged from 91.3% to 92.1%. The average coverage of genes, represented by the human protein alignment track in the UCSC genome browser (http://genome.ucsc.edu/) ranged from 93.1% to 94.4%. For the 29 SNPs in the capture region represented on the SNP genotyping array, complete concordance in genotype calls was found between the Illumina sequencing and SNP array data. Bases different from the reference canine sequence (variant SNPs) were identified using SAMtools [46] (http://samtools.sourceforge.net). SNPs segregating with disease were defined as being a homozygous variant in the affected dog and heterozygous for the carrier dog, with the additional criterion that the normal dog could not be homozygous for the variant found in the affected dog. Segregation of the 56097365 G->A variant with disease status was confirmed by Sanger sequencing of affected, carrier and normal dogs from the POAG Beagle colony. To determine the disease allele frequency in
Western blotting of eye tissue protein extracts

Postmortem eyes from dogs were obtained by veterinary technicians in accordance with the Institutional Animal Care and Use Committee of Vanderbilt University. Eyes were removed from normal dogs free of eye disease within 30 min after sacrifice. Cornea, trabecular meshwork, iris, ciliary body and optic nerve were isolated by dissection under a stereo microscope. Protein was extracted by homogenization in 150 mM LiCl, 50 mM Tris/ pH 7.5, 1 mM diethioctanoic acid, protease inhibitors and 1% lithium dodecyl sulfate. Protein concentration was determined using a fluorescence-based protein assay (Nano-Orange Protein Assay, Invitrogen). Lysates of HEK293 cells transiently transfected with either empty vector or vector containing an epitope-tagged, full-length human ADAMTS10 construct (Origene) were used as controls. For SDS-PAGE under reducing conditions, 10 μg of total protein from eye tissues or 5 μg from cell lysates were loaded into wells of 10% pre-cast polyacrylamide gels (Criterion, Bio-Rad). After SDS-PAGE, proteins were transferred to PVDF membrane (Bio-Rad). Standard Western blotting was performed using 1 μg/ml goat anti-human ADAMTS10 antibody (Santa Cruz) or 3.3 μg/ml mouse anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (clone 6C5, Millipore). Blots were imaged and molecular weights determined using an Odyssey infrared imaging system (Li-Cor Biosciences). A single immunoreactive band for ADAMTS10 ran at an apparent molecular weight of 130 kDa, the same as previously reported for the intact ADAMTS10 zymogen [15].

Homology modeling

The homology model of ADAMTS10 was calculated using the program I-TASSER [47] and is based on the structure of ADAMTS13 (PDB entry 3GHM; [20]). Superposition of the calculated model with ADAMTS13 in the program O [48] resulted in a RMS deviation of 1.0 Å for 347 Cα atoms. Figure 8 was made using MOLSCRIPT [49] and RASTER3D [50]. The domain nomenclature and color coding follow those of Akiyama et al. [20].

Protein half-life measurement

Canine aqueous humor was obtained from laboratory-quality dogs using protocols approved by the Institutional Animal Care and Use Committee of Vanderbilt University and placed immediately in sealed sterile tubes and stored at −80°C. An expression vector with a T7 promoter upstream of a cDNA insert encoding full-length human ADAMTS10 corresponding to NCBI accession number NM_030957 with a c-terminal Myc-DDK tag was obtained from Origene. The ADAMTS10 insert was verified by Sanger DNA sequencing on both strands. A PCR-based mutagenesis kit (Quick Change II, Stratagene) was used to introduce the G to A mutation found in the POAG-affected Beagles, resulting in a glycine to arginine substitution at amino acid 661 into the expression construct. Mutagenesis was confirmed by Sanger sequencing of the entire construct. A rabbit reticulocyte-based in vitro coupled transcription/translation kit (TNT Quick, Promega) was used to express normal and mutated ADAMTS10 protein from the expression vector constructs following the manufacturer’s protocol. Modified lysine-specific tRNA was included in the reaction (Transcend tRNA, Promega) to produce ADAMTS10 protein with biotinylated lysines. To measure protein half-life, samples were made with 4 μl of in vitro reaction mixed with 26 μl aqueous humor in sterile O-ring-sealed tubes and placed in a 37°C water bath. At various times, samples were removed from the water bath and placed in −80°C. The aqueous humor used in the experiments was pooled from 3 individual dogs and included 50 μg/ml cycloheximide (Sigma) to prevent protein synthesis during incubation. Samples were separated by SDS-PAGE using 7.5% pre-cast polyacrylamide gels (Criterion, Bio-Rad). After SDS-PAGE, proteins were transferred to PVDF membrane (Bio-Rad). The membrane was blocked 1 h in PBS/1% casein and then probed with streptavidin conjugated to IRDye 680 (Li-Cor Biosciences). Membranes were imaged and molecular weights and background subtracted band intensities determined using an Odyssey infrared imaging system (Li-Cor Biosciences). A single band at the expected molecular weight of ~130 kDa was detected, similar to that reported previously [15] and found in eye tissue in this study. Protein decay was assumed to follow the equation:

$$A(t) = A(t=0) \times 2^{-t/h}$$

where $A(t)$ is the amount of protein at time $t$, $A(t=0)$ is the amount of protein at time $t = 0$ and $h$ is the half-life. The decay equation can be rearranged to:

$$\log_2[A(t)] = \log_2[A(t=0)] - (1/h) \times t$$

By plotting the Log2 of the band intensity versus time of incubation, the half-life of the protein was determined as the negative inverse of the slope of the linear fit to the data. Four independent experiments were performed.

Supporting Information

Figure S1 SNPs satisfying the zygosity criterion. The chromosome 20 base pair location and genotypes for all affected, all carrier as well as a normal unrelated Beagle introduced into the colony are shown. Found at: doi:10.1371/journal.pgen.1001306.s001 (0.37 MB TIF)

Figure S2 Haplotypes for chromosome 15 regions of interest. The base pair locations and genotypes are shown for haplotypes representing region R1 (A) and R2 (B), with symbols used to represent the haplotypes shown below. Found at: doi:10.1371/journal.pgen.1001306.s002 (0.43 MB TIF)

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Author Contributions

Conceived and designed the experiments: JK RWK. Performed the experiments: JK TR EOM KNG RWK. Analyzed the data: JK LMO. Contributed reagents/materials/analysis tools: TJLH RWK. Wrote the paper: JK TMI RWK.

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