Genetics

DNA Copy Number Variants of Known Glaucoma Genes in Relation to Primary Open-Angle Glaucoma

Yutao Liu,[1,2] Melanie E. Garrett,[2] Brian L. Yaspan,[3] Jessica Cooke Bailey,[4] Stephanie J. Loomis,[5] Murray Brilliant,[6] Donald L. Budenz,[7] William G. Christen,[8] John H. Fingert,[9] Douglas Gaasterland,[10] Terry Gaasterland,[11] Jae H. Kang,[12] Richard K. Lee,[13] Paul Lichter,[14] Sayoko E. Moroi,[14] Anthony Realini,[15] Julia E. Richards,[14] Joel S. Schuman,[16] William K. Scott,[17] Kuldev Singh,[18] Arthur J. Sit,[19] Douglas Vollrath,[18] Robert Weinreb,[20] Gadi Wollstein,[16] Donald J. Zack,[21] Kang Zhang,[20] Margaret A. Pericak-Vance,[17] Jonathan L. Haines,[4] Louis R. Pasquale,[5,12] Janey L. Wiggs,[5] R. Rand Allingham,[22] Allison E. Ashley-Koch,[2] and Michael A. Hauser[2,22]

[1]Department of Cellular Biology and Anatomy, Georgia Regents University, Augusta, Georgia, United States
[2]Department of Medicine, Duke University Medical Center, Durham, North Carolina, United States
[3]Genentech, Inc., San Francisco, California, United States
[4]Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, Ohio, United States
[5]Department of Ophthalmology, Massachusetts Eye & Ear, Boston, Massachusetts, United States
[6]Center for Human Genetics, Marshfield Clinic Research Foundation, Marshfield, Wisconsin, United States
[7]Department of Ophthalmology, University of North Carolina, Chapel Hill, North Carolina, United States
[8]Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts, United States
[9]Department of Ophthalmology and Visual Sciences, Carver College of Medicine, University of Iowa, Iowa City, Iowa, United States
[10]Eye Doctors of Washington, Chevy Chase, Maryland, United States
[11]Scripps Genome Center, University of California at San Diego, San Diego, California, United States
[12]Channing Division of Network Medicine, Brigham and Women's Hospital, Boston, Massachusetts, United States
[13]Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, Florida, United States
[14]Department of Ophthalmology and Visual Sciences, University of Michigan, Ann Arbor, Michigan, United States
[15]Department of Ophthalmology, West Virginia University Eye Institute, Morgantown, West Virginia, United States;
[16]Department of Ophthalmology, UPMC Eye Center, University of Pittsburgh, Pittsburgh, Pennsylvania, United States
[17]Institute for Human Genomics, University of Miami Miller School of Medicine, Miami, Florida, United States
[18]Department of Ophthalmology, Stanford University, Palo Alto, California, United States
[19]Department of Ophthalmology, Mayo Clinic, Rochester, Minnesota, United States
[20]Department of Ophthalmology and Hamilton Glaucoma Center, University of California, San Diego, California, United States
[21]Wilmer Eye Institute, Johns Hopkins University Hospital, Baltimore, Maryland, United States
[22]Department of Ophthalmology, Duke University Medical Center, Durham, North Carolina, United States

Correspondence: Yutao Liu, 1460 Laney Walker Boulevard, CB1101, Augusta, GA 30912, USA; yutliu@gru.edu

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Purpose. We examined the role of DNA copy number variants (CNVs) of known glaucoma genes in relation to primary open angle glaucoma (POAG).

Methods. Our study included DNA samples from two studies (NEIGHBOR and GLAUGEN). All the samples were genotyped with the Illumina Human660W_Quad_v1 BeadChip. After removing non–blood-derived and amplified DNA samples, we applied quality control steps based on the mean Log R Ratio and the mean B allele frequency. Subsequently, data from 3057 DNA samples (1599 cases and 1458 controls) were analyzed with PennCNV software. We defined CNVs as those ≥5 kilobases (kb) in size and interrogated by ≥5 consecutive probes. We further limited our investigation to CNVs in known POAG-related genes, including *CDKN2B-AS1*, *TMCO1*, *SIX1/SIX6*, *CAV1/CAV2*, the *LRP12-ZFPM2* region, *GAS7*, *ATOH7*, *FNDC3B*, *CYP1B1*, *MYOC*, *OPTN*, *WDR36*, *SRBD1*, *TBK1*, and *GALC*.

Results. Genomic duplications of *CDKN2B-AS1* and *TMCO1* were each found in a single case. Two cases carried duplications in the *GAS7* region. Genomic deletions of *SIX6* and *ATOH7* were each identified in one case. One case carried a *TBK1* deletion and another case carried a *TBK1* duplication. No controls had duplications or deletions in these six genes. A single control had a duplication in the *MYOC* region. Deletions of *GALC* were observed in five cases and two controls.

Conclusions. The CNV analysis of a large set of cases and controls revealed the presence of rare CNVs in known POAG susceptibility genes. Our data suggest that these rare CNVs may contribute to POAG pathogenesis and merit functional evaluation.

Keywords: DNA copy number variants, POAG, genetics, *SIX6*, *GAS7*

Glaucoma is the second leading cause of irreversible blindness, affecting more than 60 million individuals worldwide, and it is considered to be a heterogeneous group of disorders.[1–4] Primary open-angle glaucoma (POAG) is the most common type and is inherited as a complex trait.[2] Clinically, POAG is characterized by progressive retinal ganglion cell death, optic nerve head excavation, and visual field loss.[5,6] Risk factors for POAG include increased age, elevated IOP, family history, and African ancestry.[2,4,6] Genetic factors have an important role in the pathogenesis of POAG. Family-based linkage studies have identified numerous genomic loci and several genes with varying contribution to POAG, including myocilin (*MYOC*), cytochrome P450 family 1 subfamily B polypeptide 1 (*CYP1B1*), optineurin (*OPTN*), TANK-binding kinase 1 (*TBK1*), and WD repeat domain 36 (*WDR36*).[2,4,6–13] Recently, genome-wide association studies (GWAS) of POAG in Iceland, Australia, Japan, and the United States have successfully identified and confirmed a number of genome-wide significant genetic associations in multiple genes, including caveolin 1/caveolin 2 (*CAV1/CAV2*), *CDKN2B* antisense RNA 1 (*CDKN2B-AS1*), transmembrane and coiled-coil domains 1 (*TMCO1*), SIX homeobox 1/SIX homeobox 6 (*SIX1/SIX6*), fibronectin type III domain containing 3B (*FNDC3B*), S1 RNA binding domain 1 (*SRBD1*), atonal homolog 7 (Drosophila) (*ATOH7*), and the chr8q22 region between zinc finger protein FOG family member 2 (*ZFPM2*), and low density lipoprotein receptor-related protein 12 (*LRP12*).[14–27] The IOP has been associated with variants in several genes, including *TMCO1,* ATP-binding cassette subfamily A (ABC1) member 1 (*ABCA1*) and growth arrest–specific 7 (*GAS7*).[28,29]

In addition, DNA copy number variants (i.e., genomic deletions and duplications) have been shown to have important roles in POAG.[30–32] Genomic deletions of galactosylceramidase (*GALC)* and duplications of *TBK1* have been reported to contribute to POAG pathogenesis.[9,30,31,33,34] A more comprehensive analysis of DNA deletions and duplications of known glaucoma-associated genes is needed to evaluate their possible contribution to the pathogenesis of POAG. To this end, we have studied DNA copy number variants in more than 1599 POAG cases and 1458 eye-examined controls, based on genotype data from the Illumina Human660W_Quad BeadChip (Illumina, San Diego, CA, USA). Here, we report genomic deletions and duplications in a number of known glaucoma-associated genes and their relative contribution to POAG.

Materials and Methods

Study Population

This study adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all study participants. We used POAG cases and controls from two separate glaucoma cohorts: the Glaucoma Genes and Environment (GLAUGEN) study (dbGaP Study Accession: phs000308.v1.p1, available in the public domain at http://www.ncbi.nlm.nih.gov/gap), which is part of the Gene, Environment Association Study consortium, and the National Eye Institute Glaucoma Human Genetics Collaboration (NEIGHBOR) study (dbGAP Study Accession: phs000238.v1.p1, available in the public domain at http://www.ncbi.nlm.nih.gov/gap). Both datasets have been described previously.[20,22,35–42] Briefly, the GLAUGEN study included 976 cases and 1140 controls from two population-based cohorts: the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS), as well as a clinic-based dataset, the Genetic Etiologies of POAG (GEP). The Institutional Review boards of the Massachusetts Eye and Ear Infirmary, Harvard School of Public Health, and Brigham and Women's Hospital approved this study. The NEIGHBOR study consists of 2132 cases and 2290 controls from 12 sites. The institutional review boards of the University of Pittsburgh, Johns Hopkins University, Duke University, University of West Virginia, University of Miami, University of Michigan, Stanford University, Marshfield Clinic, and the University of California, San Diego approved the NEIGHBOR study.

Eligibility for POAG cases and controls has been described previously.[20,35] Briefly, POAG cases had reliable visual field tests showing characteristic visual field defects consistent with glaucomatous optic neuropathy. Elevation of IOP was not a criterion for inclusion. Patients with a diagnosis or history of a secondary glaucoma or ocular trauma were not considered as POAG cases. Trained glaucoma subspecialists reviewed the medical records for all POAG cases and controls to ensure they met inclusion criteria for the study. The examined control individuals were required to have normal optic nerves (cup–disc ratio $\leq$0.6) and normal IOP ($\leq$21 mm Hg).

DNA Genotyping and DNA Copy Number Calling

All DNA samples were genotyped using the Illumina Human660W_Quad_v1 BeadChip at the Broad Institute (GLAUGEN), and the Center for Inherited Disease Research (CIDR) (NEIGHBOR).[20,35] To minimize batch effects, DNA samples were organized for equal representation of POAG cases and controls per 96-well plate from each study site. The details for DNA collection, extraction and plating, genotype calling, single nucleotide polymorpism (SNP) quality control, and the preliminary analyses in GLAUGEN and NEIGHBOR have been described previously.[20,35] After quality control, Illumina's GenomeStudio software was used to generate genotype calls, Log R ratio (LRR), and B allele frequency (BAF) using our established procedure.[31] To have a uniform comparison between cases and controls, we removed DNA samples that were whole genome amplified in vitro or were derived from buccal cells or nonblood samples due to their inconsistent copy number variance (CNV) performance compared to blood DNA sample.[43] Based on our previous experience in CNV analysis,[31] we selected PennCNV software (available in the public domain at www.openbioinformatics.org) to call the CNVs.[44,45] PennCNV software combines multiple sources of information, including LRR and BAF at each SNP marker, the distance between neighboring SNPs, and the allele frequency of SNPs. We applied PennCNV to genotyping data generated from Illumina arrays. During the analysis, we further eliminated DNA samples with a SD of the mean LRR $>$ 0.4 or with SD of the mean BAF $>$ 0.1. We also removed DNA samples for which the total number of CNVs was 2 SDs higher than the mean number of CNVs across the entire dataset. After applying these rigorous quality control measures, 1156 POAG cases and 1079 controls from the NEIGHBOR dataset and 443 POAG cases and 379 controls from the GLAUGEN dataset remained for further analysis. To reduce false positives, we also required the CNVs to be at least 5 kilobases (kb) in size and interrogated by a minimum of five consecutive genotyping probes. We restricted our analysis to genomic regions containing glaucoma-associated genes, including *MYOC*, *OPTN*, *WDR36*, *CYP1B1*, *CDKN2B-AS1*, *TMCO1*, *SIX1/SIX6*, *CAV1/CAV2*, *SRBD1*, and the intergenic chr8q22 *LRP12-ZFPM2* region.

Results

The demographic characteristics of the NEIGHBOR and GLAUGEN participants included in the study are listed in

Investigative Ophthalmology & Visual Science

Table 1. Demographics of POAG Cases and Controls in the NEIGHBOR (1156 Cases and 1079 Controls) and GLAUGEN Datasets (443 Cases and 379 Controls)

	NEIGHBOR, $n = 2235$			GLAUGEN, $n = 822$		
	% Female	Mean Age ± SD	Family History of POAG, %	% Female	Mean Age ± SD	Family History of POAG, %
POAG Case	50.7	58.0 ± 12.7	40.4	56.9	65.3 ± 8.3	52.8
Control	60.4	68.0 ± 11.4	8.0	52.0	64.6 ± 9.5	12.1

Table 1. In the NEIGHBOR study, the mean age was 58.0 ± 12.7 years for the cases and 68.0 ± 11.4 for the controls. Similar proportions of female individuals were observed in cases (50.7%) and controls (60.4%). Approximately 40.4% of POAG cases and 8% of controls had a family history of glaucoma in first-degree relatives (i.e., biological parents, siblings, or children). The average number of CNVs (deletions and duplications) in NEIGBHOR was 104 for POAG cases and 103 for the controls. The average size of each CNV was approximately 32 kb for POAG cases and 31 kb for controls. For GLAUGEN samples, the mean age was 65.3 ± 8.3 years for the cases and 64.6 ± 9.5 years for the controls. The proportion of female individuals was similar between cases (56.9%) and controls (52.0%). Of the POAG cases and controls, 52.8% and 12.1%, respectively, had a family history of glaucoma in their first-degree relatives. The average number of CNVs in GLAUGEN was 150 for POAG cases and 163 for the controls. The average size of each CNV in GLAUGEN was approximately 66 kb for POAG cases and 66 kb for the controls.

We examined the genomic deletions and duplications in known POAG-associated genes, including *CAV1/CAV2*, *CDKN2B-AS1*, *TMCO1*, *SIX1/SIX6*, *FNDC3B*, *SRBD1*, chr8q22 region (*LRP12-ZFPM2*), *GAS7*, *CYP1B1*, *OPTN*, *MYOC*, *WDR36*, *TBK1*, *GALC*, and *ATOH7* (Table 2). No deletions or duplications were detected in the *CAV1/CAV2*, *CYP1B1*, or *OPTN* genomic regions in our cohorts. As shown in the Figure, we identified one case with a genomic duplication that spans the *CDKN2B* gene and an intronic region of *CDKN2B-AS1* as well as another case with a duplication that spans a majority of the *TMCO1* genomic region. Both cases with these duplications were diagnosed in their late 40s with elevated IOPs (>21 mm Hg) and a large vertical cup-to-disc ratio (vCDR) of 0.9 in both eyes. Additionally, in both cases, there was a history of glaucoma in first-degree relatives. A genomic deletion spanning the *SIX6* gene and the 5′-end of *C14ORF39* gene was identified in one POAG case. This patient had an elevated IOP (>21 mm Hg) and a large vCDR (0.8 in the affected eye) with a positive family history of glaucoma in the first-degree relatives. Two POAG cases with elevated IOPs (>21 mm Hg) carried duplications that span *MYH13*, *MYH8*, *MYH4*, *MYH1*, and the 5′ end or upstream of the *GAS7* gene, which overlaps with the genomic region associated with IOP.[28,46] One of these patients had a family history of glaucoma, while the other did not. One POAG case carried a 184 kb *TBK1* deletion and another case carried an 886 kb *TBK1* duplication, which are CNVs of novel extent. These cases had normal tension glaucoma without a documented history of elevated IOPs (<21 mm Hg). Deletions in the *GALC* region were found in two controls (one with family history of glaucoma and another without family history) and five POAG cases all of which had elevated IOP (>21 mm Hg) and a positive family history of glaucoma. Deletions of the chr8q22 region (*LRP12-ZFPM2*) were identified in three cases and two controls (one with and another without family history of glaucoma). Duplications and

Table 2. Number of Individuals With DNA Copy Number Variants Within Known POAG-Associated Genes in NEIGHBOR (1156 Cases and 1079 Controls) and GLAUGEN (443 Cases and 379 Controls) Datasets

	NEIGHBOR				GLAUGEN				Total Combined			
	Genomic Deletion		Genomic Duplication		Genomic Deletion		Genomic Duplication		Deletion		Duplication	
	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control
CAV1/CAV2	0	0	0	0	0	0	0	0	0	0	0	0
CDKN2B-AS1	0	0	1	0	0	0	0	0	0	0	1	0
TMCO1	0	0	1	0	0	0	0	0	0	0	1	0
SIX1/SIX6	1	0	0	0	0	0	0	0	1	0	0	0
FNDC3B	3	7	0	0	9	7	0	0	12	14	0	0
SRBD1	6	5	0	0	3	3	14	7	9	8	14	7
LRP12-ZFPM2	2	2	0	0	1	0	0	0	3	2	0	0
GAS7	0	0	1	0	0	0	1	0	0	0	2	0
CYP1B1	0	0	0	0	0	0	0	0	0	0	0	0
OPTN	0	0	0	0	0	0	0	0	0	0	0	0
MYOC	0	0	0	0	0	0	0	1	0	0	0	1
WDR36	0	1	5	3	0	1	3	2	0	2	8	5
TBK1	1	0	1	0	0	0	0	0	1	0	1	0
GALC	4	1	0	0	1	1	0	0	5	2	0	0
ATOH7	0	0	0	0	1	0	0	0	1	0	0	0

CAV1/CAV2, caveolin 1/caveolin 2; *CDKN2B-AS1*, CDKN2B antisense RNA 1; *TMCO1*, transmembrane and coiled-coil domains 1; *SIX1/SIX6*, SIX homeobox 1/SIX homeobox 6; *FNDC3B*, fibronectin type III domain containing 3B; *LRP12-ZFPM2*, low density lipoprotein receptor-related protein 12–zinc finger protein FOG family member 2; *GAS7*, growth arrest–specific 7; *CYP1B1*, cytochrome P450 family 1 subfamily B polypeptide 1; *OPTN*: optineurin; *MYOC*, myocilin; *WDR36*, WD repeat domain 36; *TBK1*, TANK-binding kinase 1; *GALC*, galactosylceramidase; *ATOH7*, atonal homolog 7 (Drosophila).

Investigative Ophthalmology & Visual Science

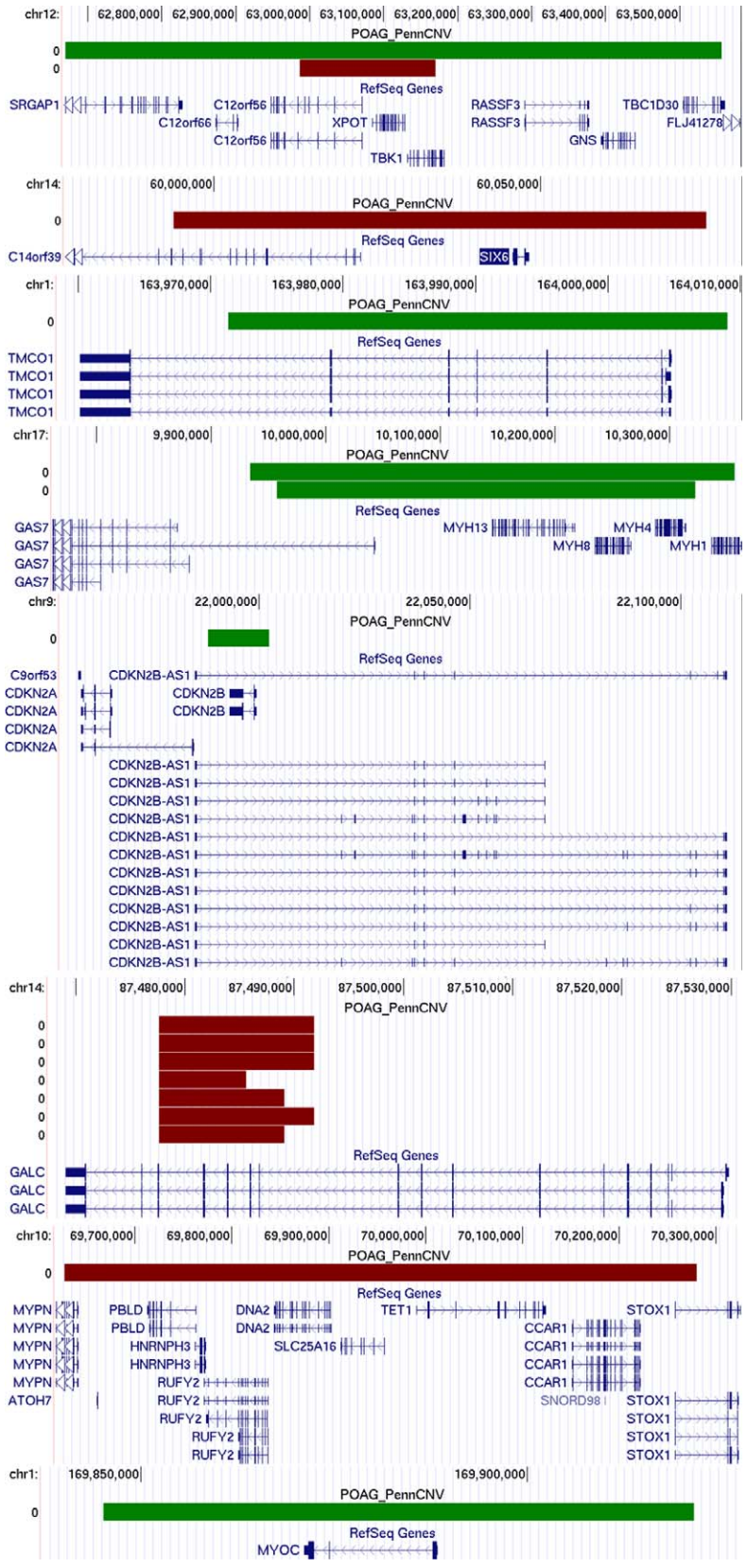

Figure. Representation of rare genetic duplications and deletions in known glaucoma-associated genes in UCSC human genome browser. *Green* represents genomic duplication and *red* represents genomic deletion. The boundary of these deletions and duplications is indicated by the *edge* of these *bars*.

Investigative Ophthalmology & Visual Science

deletions in the regions of *FNDC3B*, *SRBD1*, and *WDR36*, were equally distributed among POAG cases and controls (Table 2). A deletion in the *ATOH7* region was found in one POAG case. This case, with a cup-disc ratio of 0.6 in the left eye and 0.4 in the right eye, had an elevated IOP (>21 mm Hg), reproducible visual field loss, and a positive family history of glaucoma. A duplication in the *MYOC* region was identified in one control without a family history of glaucoma.

Discussion

To our knowledge, our study is the largest case-control analysis of CNVs in POAG, including over 3000 participants, and focusing on known POAG-associated genes. We identified a number of rare CNVs in known glaucoma genes, including *CDKN2B-AS1*, *TMCO1*, *SIX1/SIX6*, *GAS7*, *TBK1*, *GALC*, and *ATOH7*. Many CNVs were observed only in POAG patients. However, we also found that the CNVs in three genomic regions (*WDR36*, *FNDC3B*, and *SRBD1*) were relatively common with a frequency greater than 1% in our study. However, the frequencies were not significantly different between POAG cases and controls. The frequencies of these CNVs have not been reported previously to our knowledge.

Genomic duplications of *TBK1* have been specifically associated with POAG in normal tension glaucoma patients.[9,33,34] We also identified a genomic duplication of novel extent that spans *TBK1* in a POAG case with normal pressure. Our finding provided further support for the genetic contribution of *TBK1* duplication in normal tension glaucoma (NTG) pathogenesis. Additionally, for the first time we have identified a genomic deletion of *TBK1* in an NTG patient. These results suggested that duplication and deletion of *TBK1* contribute to glaucoma. The *TBK1* gene encodes a kinase that phosphorylates optineurin, which also is known to cause NTG.[10,47,48] Both TBK1 and OPTN participate in autophagy and NF-kB signaling,[4,8,49] suggesting that *TBK1* CNVs may cause disease by dysregulating these pathways. However, further work is necessary to determine the responsible molecular events.

Variants in *TMCO1* and *GAS7* genes recently have been associated with IOP and POAG.[28,29,50,51] However, it remains unclear how the protein products from these two genes regulate IOP and affect POAG development. Mutations in *TMCO1* have been associated with craniofacial dysmorphism, skeletal anomalies, mental retardation (OMIM *614123) and cerebro-facio-thoracic dysplasia (OMIM #614132).[52–55] Ocular phenotypes, including IOP elevation or glaucoma, have not been reported previously in patients carrying *TMCO1* mutations.[52–54] However, most of these patients, aged from 3 to 39 years, have macrocephaly, fusion of cervical or thoracic spine, and prominent cerebrospinal fluid (CSF) space,[54] which might affect CSF pressure. Decreased CSF pressure has been proposed as a risk factor for glaucoma by increasing the translaminar pressure difference.[56–58] The *GAS7* gene, as part of the growth arrest–specific gene family, is involved in neurite outgrowth and motor neuron function associated with muscle strength maintenance.[59,60] The *GAS7* regulates the expression of RUNX2 and its dependent transcriptional expression. Mutations in *GAS7* increase body fat levels and decrease bone density in mouse models.[61] Although variants in the *GAS7* genomic region have been associated with IOP level and POAG risk,[28,62] the mechanism remains unknown. In addition, ocular phenotypes, including altered IOP, have not been described in the *GAS7*-deficient mouse.[59] The presence of duplications of *GAS7* overlapping with IOP-associated regions in two glaucoma patients, but not controls, supports the potential involvement of GAS7 in POAG. However, this does not exclude the possible role of other genes contained within the duplicated region.

We also identified a rare duplication of *CDKN2B/CDKN2B-AS1* in a POAG case. Variants in this genomic region have been associated with POAG, especially with normal-tension glaucoma and advanced glaucoma.[16,17,20,50,63] Elevated levels of *CDKN2B-AS1*[64] suppress the expression of cell cycle regulators *CDKN2A* and *CDKN2B*. Thus, duplication of *CDKN2B-AS1* intronic region might increase risk of POAG by elevating gene expression to suppress the expression of *CDKN2A* and *CDKN2B*. Of course the actual mechanism remains unknown at this time. Recently, two groups reported that *SIX6* is the gene associated with POAG in the *SIX1/SIX6* genomic region.[38,65] We identified a heterozygous deletion in the *SIX6* region. The homozygous loss of SIX6 function in human patients, including homozygous deletion of a large genomic region containing *SIX6* gene, has been reported to be associated with variable degrees of retinal hypoplasia, absence of the optic chiasm and optic nerve, and bilateral anophthalmia.[66–68] However, the individual we have identified with a heterozygous deletion in *SIX6* has a much less severe phenotype. It has been proposed that the level of SIX6 expression is associated with POAG although the exact mechanism remains unknown.[38,65] Deletions of the *GALC* genomic region also have been associated with POAG risk.[31] In this study we identified more *GALC* deletions in cases compared to controls. Although the distribution of deletions between cases and controls was not significantly different, it provides further support on the potential contribution of this gene to POAG.[4] Variants in *ATOH7* have been associated with optic disc size and vertical cup-to-disc ratio[14,69,70] as well as POAG.[16,25,71] The *ATOH7* knockout mouse lacks retinal ganglion cells and optic nerves.[72] Deletion of a remote enhancer of *ATOH7* disrupts retinal neurogenesis and causes nonsyndromic congenital retinal nonattachment.[73] Homozygous *ATOH7* mutations were reported in patients with global eye developmental defects, including microphthalmia.[74] The POAG case with a heterozygous deletion of *ATOH7* represents an intermediate phenotype, suggesting the dosage effect of *ATOH7*. This is the first report of *ATOH7* deletion in a POAG patient.

We acknowledge that this study had several limitations. First, the detection of CNVs in our samples was based on an Illumina SNP-genotyping array and computational algorithm. Even though this array was designed with the ability to detect CNVs, there are nonetheless limitations to the number of SNP/CNV probes and their ability to detect CNVs. Second, our study focused on genes and loci that have a known or suspected association with POAG and, as such, does not provide information on novel associations, which would have been unlikely due to the uncommon occurrence of CNVs in general. Due to the small number of CNVs in cases and controls, the statistically significant association could not be demonstrated. The frequency of these CNVs in these known glaucoma genes have not yet been reported. This study was designed to lend support to known genes and examine the potential role of gene dosage mechanisms in POAG. Recently, three new studies identified several new genes associated with POAG.[26,27,29] However, these results were not available in time to be included in our analysis. Third, these CNVs have not been confirmed by other technologies, such as comparative genomic hybridization array or probe-based real time PCR. Fourth, in this set of unrelated cases and controls, we are not able to demonstrate that interesting CNVs associated with POAG exhibited familial segregation.

In summary, we reported the analysis of DNA copy number variants of known glaucoma-associated genes in two large POAG case-control sets—NEIGHBOR and GLAUGEN. We identified DNA duplications or deletions in the *CDKN2B-AS1*, *TMCO1*, *SIX1/SIX6*, *GAS7*, *ATOH7*, *TBK1*, and *GALC* genomic

Investigative Ophthalmology & Visual Science

regions in POAG cases. These data suggested that rare CNVs in known glaucoma-associated genes may contribute to POAG pathogenesis potentially through gene dosage effects, and merit further analysis and functional evaluation.

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