

# Contribution of Mutations in Known Mendelian Glaucoma Genes to Advanced Early-Onset Primary Open-Angle Glaucoma

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**PURPOSE.** Primary open-angle glaucoma (POAG) and primary congenital glaucoma (PCG) with Mendelian inheritance are caused by mutations in at least nine genes. Utilizing whole-exome sequencing, we examined the disease burden accounted for by these known Mendelian glaucoma genes in a cohort of individuals with advanced early-onset POAG.

**METHODS.** The cases exhibited advanced POAG with young age of diagnosis. Cases and examined local controls were subjected to whole-exome sequencing. Nine hundred ninety-three previously sequenced exomes of Australian controls were called jointly with our dataset. Qualifying variants were selected based on predicted pathogenicity and rarity in public domain gene variant databases. Case-control mutational burdens were calculated for glaucoma-linked genes.

**RESULTS.** Two hundred eighteen unrelated POAG participants and 103 nonglaucomatous controls were included in addition to 993 unexamined controls. Fifty-eight participants (26.6%) harbored rare potentially pathogenic variants in known glaucoma genes. Enrichment of qualifying variants toward glaucoma was present in all genes except *WDR36*, in which controls harbored more variants, and *TBKI*, in which no qualifying variants were detected in cases or controls. After multiple testing correction, only *MYOC* showed statistically significant enrichment of qualifying variants (odds ratio [OR] = 16.62,  $P = 6.31 \times 10^{-16}$ ).

**CONCLUSIONS.** Rare, potentially disease-causing variants in Mendelian POAG genes that showed enrichment in our dataset were found in 22.9% of advanced early-onset POAG cases. *MYOC* variants represented the largest monogenic cause in POAG. The association between *WDR36* and POAG was not supported, and the majority of POAG cases did not harbor a potentially disease-causing variant in the remaining Mendelian genes.

Keywords: exome sequencing, OPTN, CYP1B1, LTBP2, MYOC

Glaucoma is the leading cause of preventable irreversible blindness worldwide, with a well-known genetic contribution.<sup>1</sup> Vision damage in glaucoma manifests as visual field loss secondary to optic nerve injury.<sup>2</sup> The disease can be defined as open angle or angle closure depending on the width of the iridocorneal angle.<sup>3</sup> Primary open-angle glaucoma (POAG) is the most common subtype among Caucasians with a 2.37% prevalence in this population,<sup>1</sup> with intraocular

pressure (IOP) and age being the most significant risk factors.<sup>4,5</sup> Primary open-angle glaucoma can be subclassified into high-tension glaucoma (HTG), where untreated IOP is more than two standard deviations above the population mean, and normal-tension glaucoma (NTG), where untreated IOP remains within the normal range (IOP < 22 mm Hg). Similarly, POAG can be subclassified by age of onset, from less than 4 years for primary congenital glaucoma (PCG), between 4 and around 40



years for early-onset or juvenile-onset POAG (JOAG), to over 40 years for typical POAG. An autosomal dominant mode of inheritance is present in a proportion of individuals with POAG. Estimates place the percentage of Mendelian inherited POAG explained by known causative genes at around 5%.<sup>6</sup> The Online Mendelian Inheritance in Man (OMIM) database (www.omim.org; accessed October 17, 2016; in the public domain) documents seven genes (*MYOC*, \*601652; *OPTN*, \*602432; *CYP1B1*, \*601771; *WDR36*, \*609669; *ASB10*, \*615054; *NTF4*, \*162662; and *TBK1*, \*604834) with potentially disease-causing variants in POAG and two (*CYP1B1*, \*601771 and *LTBP2*, \*602091) in PCG. Additionally, the *GLC1C* locus has since been linked to the gene *IL20RB* (interleukin 20 receptor subunit beta) via Sanger sequencing.<sup>7</sup>

Family-based linkage studies on large affected pedigrees have revealed a number of genes linked to POAG with Mendelian inheritance. Only three genes (*MYOC*, *OPTN*, *CYP1B1*) with disease-causing single nucleotide variants (SNV) and one with copy number variants (*TBK1*) have been unequivocally replicated in discrete glaucoma cohorts. The first and the most prevalent gene discovered in familial POAG is myocilin (*MYOC*).<sup>8,9</sup> The prevalence of heterozygous *MYOC* disease-causing variants is around 4% in Caucasian POAG populations overall.<sup>8,10</sup> It accounts for a greater proportion in the JOAG subset, with a prevalence of 17% in Australia<sup>10</sup> and up to 36% in the United States.<sup>11</sup> Disease-causing variants are predominantly located within the third exon and are likely a result of founder effects within European Caucasian populations.<sup>12-14</sup> The *MYOC* glaucoma phenotype is characterized by a young age of onset with high IOP.<sup>10,11</sup> Optineurin (*OPTN*) was the second gene to be linked to Mendelian POAG.<sup>15</sup> In contrast to *MYOC*, disease-causing *OPTN* variants impart a glaucoma phenotype with normal IOP and are rarer, with percentages ranging from 1.5% to 3.5% of the NTG population.<sup>16,17</sup> In multiple study cohorts, only the heterozygous p.Glu50Lys variant in *OPTN* has been definitively proven to be disease causing<sup>15-18</sup> via a gain-of-function mechanism, thereby explaining the low frequency and lack of variant diversity seen in *OPTN*-related glaucoma. Both *MYOC* and *OPTN* variants are transmitted in an autosomal dominant manner. *TBK1* (TANK-binding kinase 1) has been linked to POAG in the form of autosomal dominant copy number duplication or triplication, with replication in multiple Caucasian POAG cohorts.<sup>19,20</sup> However, the incidence of *TBK1* copy number variant in glaucoma is very rare, accounting for only 0.8% (10 out of 1222 NTG cases) of individuals with NTG in Caucasian and Asian populations.<sup>19-24</sup> *CYP1B1* (cytochrome P450, family 1, subfamily B, polypeptide 1) disease-causing variants have been linked to both PCG and JOAG.<sup>25-27</sup> Biallelic *CYP1B1* variants transmitted in an autosomal recessive manner are involved in PCG, whereas both biallelic and heterozygous variants have been involved in POAG with a lower frequency rate. Deleterious *CYP1B1* variant frequency is highly variable between ethnicities: Prevalence rates of *CYP1B1* variants have been reported as 4.6% in a French POAG case cohort,<sup>26</sup> 4.62% in a Taiwanese Chinese JOAG case cohort,<sup>28</sup> 6.8% in an Australian JOAG case cohort,<sup>27</sup> and 11.1% in an Iranian POAG case cohort.<sup>29</sup> Like *CYP1B1*, variants in *LTBP2* (latent transforming growth factor beta binding protein 2) transmitted in an autosomal recessive manner were originally linked to PCG,<sup>30,31</sup> with heterozygous variants later suggested in POAG.<sup>32</sup> Other genes linked to POAG but with less certainty include *ASB10*, *NTF4*, and *WDR36*.<sup>33-35</sup> *ASB10* (ankyrin repeat and SOCS box containing 10) and *WDR36* (WD repeat domain 36) have shown variability in their replication, with subsequent studies reporting no statistically significant difference in the frequency of potentially pathogenic variants between POAG cases and controls within cohorts of similar

ethnicities.<sup>36-39</sup> Heterozygous variants in *NTF4* (neurotrophin 4) have been suggested to be a rare cause of POAG, ranging from 0.3% in Chinese cohorts<sup>40</sup> to 2.26% in German cohorts,<sup>34</sup> but were not associated with POAG in US<sup>41</sup> and Indian cohorts.<sup>42</sup>

Here we used whole-exome sequencing (WES) to examine the disease burden of these nine known Mendelian POAG genes in a cohort of participants with the most severe glaucoma from a population-based database, the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG).

## METHODS

This study adhered to the principles listed in the revised Declaration of Helsinki and the Australian National Health and Medical Research Council (NHMRC) statement of ethical conduct in research involving humans. Ethical approval was obtained from the Southern Adelaide and Flinders University Clinical Research Ethics Committee. Written informed consent was obtained from all participants following explanation of the research. All peripheral blood samples were collected for genomic DNA extraction as a part of the ANZRAG as previously described.<sup>43</sup>

## Participants

The individuals composing the study cohort were Caucasian participants from the ANZRAG database, collected in a prospective unselected manner and previously analyzed for common disease-associated variants in genome-wide association studies (GWAS).<sup>44-47</sup> Participants with advanced glaucoma and the youngest age at diagnosis with mean age at diagnosis  $44.4 \pm 10.4$  years were included in this study. Advanced glaucoma was defined as a glaucomatous visual field loss involving at least two of the four central fixation squares with a pattern standard deviation of less than 0.5% on a reliable Humphrey 24-2 field (Carl Zeiss, Dublin, CA, USA), or a mean deviation of worse than  $-15$  dB in the worse affected eye. For participants without formal visual field testing, their best-corrected visual acuity had to be worse than 20/200 owing to glaucomatous damage. The less affected eye was also required to have glaucomatous optic neuropathy as measured by a reliable Humphrey 24-2 field, with corresponding neuroretinal rim thinning. Participants with HTG or NTG were included. High-tension glaucoma was defined as having a maximum recorded untreated IOP of greater than 21 mm Hg. Individuals with secondary glaucoma were excluded from this study. Individuals with disease-causing *MYOC* variants detected on capillary sequencing were not analyzed by WES<sup>10</sup> (Supplementary Table S1), but were included for analysis to enable relative proportions of POAG cases potentially explained by each individual gene to be assessed.

All local control participants were interviewed to exclude a family history of glaucoma and further examined to exclude glaucoma or phenotypic traits including cupping of the optic disc, deficit on Humphrey visual field testing, and elevation of IOP that could be related to glaucoma. A larger unexamined control cohort from the Australian Osteoporosis Genetics Consortium (AOGC)<sup>48</sup> was included for in silico analysis. All AOGC participants ( $n = 993$ ) were females with either high or low bone mass and were otherwise self-identified as healthy.

## Whole-Exome Sequencing

DNA was extracted from peripheral blood samples using the QIAamp DNA blood kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Samples were prepared with

SureSelect Human All Exon V4 enrichment kit (Agilent, Santa Clara, CA, USA) per manufacturer's protocol for whole-exome capture and enrichment. Enriched DNA was sequenced on a HiSeq2000 (Illumina, San Diego, CA, USA) with 100 bp paired end reads (Macrogen Next Generation Sequencing Services, Marcogen, Inc.). Local controls served as both technical and phenotypic controls. Raw sequencing data were used to generate binary alignment map (BAM) files and joint-called with previously sequenced AOGC exomes that were captured with Nimblegen Human Exome Capture V2 (Roche, Basel, Switzerland) and sequenced on the HiSeq2000 (Illumina) at the University of Queensland Centre for Clinical Genomics, Brisbane, Australia. FASTQ files were aligned to human genome build hg19 with Novoalign (version 3.02.08; Novocraft Technologies Sdn Bhd, Petaling Jaya, Selangor, Malaysia). Duplicate reads were marked with Picard MarkDuplicates (version 1.124; <http://broadinstitute.github.io/picard/>; in the public domain). Local indel realignment and base quality recalibration were performed with GATK<sup>49</sup> (version 3.2.2). Variant calling of SNV and small indels was performed with the UnifiedGenotyper module in GATK, and variant quality scores were recalibrated according to the GATK Best Practices Guidelines.<sup>50,51</sup> Variant annotation was performed using ANNOVAR<sup>52</sup> software using refGene, SIFT<sup>53</sup> (Sorting Intolerant From Tolerant), PolyPhen2 HVAR<sup>54</sup> (Polymorphism Phenotyping v2), NHLBI GO Exome Sequencing Project (ESP) (<http://evs.gs.washington.edu/EVS/>; in the public domain), 1000 Genomes Project (<http://www.1000genomes.org/>; in the public domain), and Exome Aggregation Consortium (ExAC) databases ([exac.broadinstitute.org/](http://exac.broadinstitute.org/); in the public domain).

### Bioinformatic Analysis

Whole exomes from all glaucoma cases not carrying pathogenic *MYOC* variants ( $n = 189$ ) and controls ( $n = 1096$ ) were analyzed concurrently. Only protein coding exonic and splicing site variants were selected for analysis following filtering with in-house UNIX codes. The quality control threshold was set at a Genotype Quality (GQ) score of 20. In order to include only potentially disease-causing variants, all variants with a minor allele frequency (MAF) of greater than 0.01 in dbSNP, NHLBI GO Exome Sequencing Project (ESP), 1000 Genomes Project, and ExAC were excluded. Pathogenicity filtering further removed all synonymous variants, and missense variants considered "tolerated" or "possibly damaging" by both SIFT and PolyPhen2 HVAR, respectively. The qualifying variants for analysis consisted of all protein-truncating variants in the canonical gene transcript, including nonsense, frameshift, and essential splice site variants, as well as missense variants predicted to be deleterious by either SIFT or PolyPhen2 HVAR. Variants in nine Mendelian POAG genes, *ASB10*, *CYP1B1*, *IL20RB*, *LTBP2*, *MYOC*, *NTF4*, *OPTN*, *TBK1*, and *WDR36*, were selected. Of these genes, *LTBP2* has been linked to PCG, *CYP1B1* to both PCG and POAG, and the rest to POAG only. Variant loads per gene were calculated for the glaucoma case and control cohorts by summing the minor allele counts of all qualifying variants in the same gene and dividing by the average number of captured alleles for those variants to adjust for capture rate. Odds ratios were generated by comparing the glaucoma variant load with the control using Fisher's exact test to calculate  $P$  values.

### RESULTS

This study included 218 participants with advanced POAG from the ANZRAG and 1096 controls (103 without clinical features of glaucoma and 993 unexamined Australian controls).

There were 150 HTG and 68 NTG participants. The mean maximum recorded IOPs of each group were  $32.9 \pm 8.9$  and  $18.1 \pm 2.9$  mm Hg, respectively. The mean age at diagnosis for the POAG cohort was  $44.4 \pm 10.4$  years. Twenty-six HTG participants and 3 NTG participants were found to carry *MYOC* disease-causing variants upon capillary sequencing and were excluded from WES. Whole-exome sequencing was performed on the remaining 189 cases and 103 examined controls. The mean percentage of mappable reads was 99.4%, with an average total of  $4.12 \times 10^9$  on-target reads per sample, and an average depth of 73 reads per target base. Coverage of at least 10-fold was achieved at an average of 97.9% of all targeted exonic regions. Australian Osteoporosis Genetics Consortium controls had an average depth of 24 reads per target base and  $\geq 10$ -fold coverage of 75.1%.

Qualifying variants in known primary glaucoma genes (all nine genes) were identified in 58 cases (58/218, 26.6%) and 128 controls (128/1096, 11.7%) generating an odds ratio of 2.74 (1.93–3.90,  $P = 1.01 \times 10^{-7}$ ) (Supplementary Table S2 shows all variants found in glaucoma cases). Only one participant with POAG carried qualifying variants in more than one gene, with qualifying variants in both *CYP1B1* and *WDR36*. Variants (heterozygous) for *MYOC* accounted for most carriers of qualifying variants in all the nine genes (26 or 17.3% of HTG, 3 or 4.4% of NTG, 29 or 13.3% of all POAG), and demonstrated significant enrichment within the POAG cohort (odds ratio [OR] = 16.62, Fisher's  $P = 6.31 \times 10^{-16}$ ) (Table). All other genes except *TBK1* and *WDR36* exhibited nominal enrichment in POAG; however, the difference was not statistically significant (Table). No *TBK1* SNV or indels were detected in the POAG cohort, and only one SNV was detected in 1096 controls.

Primary congenital glaucoma caused by mutations in both *CYP1B1* and *LTBP2* genes has an autosomal recessive pattern of disease inheritance, although both heterozygous and compound heterozygous variants have previously been reported in POAG cohorts.<sup>27,32</sup> Four cases carried two qualifying variants in these genes. One participant was compound heterozygous and two were homozygous for *CYP1B1* qualifying variants. Another participant was compound heterozygous for *LTBP2* qualifying variants. Another three and five participants were heterozygotes for qualifying variants in *CYP1B1* and *LTBP2*, respectively. No control was homozygous or potentially compound heterozygous for qualifying variants in *CYP1B1* (Fisher's  $P = 0.0047$ ) or *LTBP2* (Fisher's  $P = 0.17$ ). Carrier rates for *CYP1B1* and *LTBP2* in the control cohort are shown in the Table.

*WDR36* was the only gene to harbor more qualifying variants in the control cohort than in cases (OR = 0.52, 0.25–1.09). For the remaining eight Mendelian POAG genes, a total of 50 POAG cases (22.9%) carried qualifying variants. The carrier rate for qualifying variants in each gene excluding *WDR36* and *TBK1* in cases versus controls is shown in Figure 1. With *WDR36* excluded, no cases carried qualifying variants in multiple Mendelian POAG genes. The proportion of cases carrying qualifying variants in each Mendelian POAG gene is presented in Figure 2.

### DISCUSSION

This study explored the genetic contribution of the nine known primary glaucoma genes in 218 Caucasian POAG participants from the ANZRAG with advanced disease and young age at diagnosis. We examined odds ratios associated with each gene to determine their relative contributions to POAG cases and controls. Our results highlight the overwhelming influence of *MYOC* among the known Mendelian POAG genes, but emphasize that the majority of POAG cases

TABLE. Numbers and Percentages of POAG Cases or Controls Carrying One or More Qualifying Variants in Nine Mendelian POAG Genes

Gene	Number of Individuals (%)				OR (95% CI)			P Value POAG
	POAG	HTG	NTG	Ctrl	POAG	HTG	NTG	
<i>ASB10</i>	4 (1.83)	1 (0.67)	3 (4.41)	7 (0.72)	2.59 (0.75-8.93)	0.93 (0.11-7.62)	6.4 (1.62-25.33)	0.124
<i>IL20RB</i>	1 (0.46)	1 (0.67)	0	0	NA	NA	NA	0.120
<i>CYP1B1</i>	6 (2.75)	4 (2.67)	2 (2.94)	18 (2.00)	1.39 (0.54-3.53)	1.34 (0.45-4.02)	1.48 (0.34-6.53)	0.142
<i>LTBP2</i>	6 (2.75)	4 (2.67)	2 (2.94)	14 (1.29)	2.17 (0.82-5.71)	2.1 (0.68-6.47)	2.32 (0.52-10.43)	0.069
<i>MYOC</i>	29 (13.30)	26 (17.33)	3 (4.41)	10 (0.91)	16.62 (7.97-34.67)	22.72 (10.7-48.22)	5 (1.34-18.61)	<b>6.31</b> × 10 <sup>-16</sup>
<i>NTF4</i>	3 (1.38)	3 (2.00)	0	11 (1.13)	1.22 (0.34-4.39)	1.78 (0.49-6.45)	NA	0.731
<i>OPTN</i>	1 (0.46)	0	1 (1.47)	1 (0.09)	5.04 (0.31-80.91)	NA	16.33 (1.01-263.94)	0.305
<i>TBK1</i>	0	0	0	1 (0.09)	NA	NA	NA	1
<i>WDR36</i>	8 (3.67)	6 (4.00)	2 (2.94)	75 (6.86)	0.52 (0.25-1.09)	0.57 (0.24-1.32)	0.41 (0.1-1.71)	0.098

The data from 218 cases and 1096 controls are presented. Odds ratios were calculated using POAG cases versus all controls with Fisher's exact test for P value. Bold font indicates statistical significance. Ctrl, control; CI, confidence interval.

are not accounted for by mutations in the known Mendelian glaucoma genes. These unexplained cases are likely to be accounted for by a combination of common GWAS alleles, rare variants in glaucoma risk genes, noncoding variants not found in WES, copy number variations, and large structural variations that are difficult to detect.

The proportions of our POAG cases explained by individual genes were largely in accord with previous publications, despite differences in cohorts, sequencing methods, and qualifying variant definitions. The prevalence of *MYOC* disease-causing variants in advanced JOAG patients was reported to be 17% in our previous study<sup>10</sup> with an age at diagnosis cutoff of 40 years. The current study includes all *MYOC* JOAG participants (age at diagnosis less than 40 years) from our previous study as well as more recently identified *MYOC* participants up to 55 years old at diagnosis. In another study in which the inclusion criterion for age at diagnosis was even younger at 35 years,<sup>11</sup> up to 36% of all JOAG was accounted for by disease-causing variants in *MYOC*. Although there was no strict limit to age at diagnosis in this study, the mean age of our POAG cohort was older than the thresholds for both previous studies but still relatively young for POAG at 44.4 ± 10.4 years. Therefore, our finding of 13.30% prevalence for *MYOC* glaucoma is in keeping with previous data.

In our POAG cohort, there was one NTG participant out of 68 (1.5%) who carried the *OPTN* p.Glu50Lys disease-causing variant. This rate is comparable with that in other replication

studies on *OPTN*,<sup>16-18</sup> albeit much less than that in the Japanese discovery cohort (13.5%).<sup>15</sup> Our data also demonstrate the highly conserved nature of the *OPTN* gene, with only one person in the control cohort (0.1%) carrying a qualifying variant. This generated a highly skewed odds ratio toward NTG cases for this gene (OR = 16.33 [1.01-263.94]). However, this was not statistically significant due to the rarity of qualifying variants in cases. It should also be noted that the p.Glu50Lys variant is suspected to be a gain-of-function variant.<sup>22</sup> Like copy number gains in *TBK1*, this may represent a pathogenic mechanism that our filtering criteria are not well positioned to detect.

Although *CYP1B1* and *LTBP2* were initially linked to autosomal recessive PCG, there is evidence that heterozygous variants in these two genes may contribute to POAG.<sup>32,55</sup> In *CYP1B1*, the proportion of POAG cases with qualifying variants was 2.75% and was lower than in previous reports in Caucasian populations in France (4.6%)<sup>26</sup> and New Zealand (6.1%).<sup>56</sup> This difference may be in part due to the younger age at diagnosis in the French JOAG study (median = 40, range, 13-52), as our previous study examining the prevalence of *CYP1B1* variants in JOAG found a 6.8% rate.<sup>27</sup> Additionally, the three cases in the French study harbored a known polymorphic variant, p.(Ala443Val), which brings their true prevalence to 3.4%. The case cohort in the New Zealand study included pseudoexfoliative glaucoma and ocular hypertension as well as POAG suspects, which makes any direct comparison

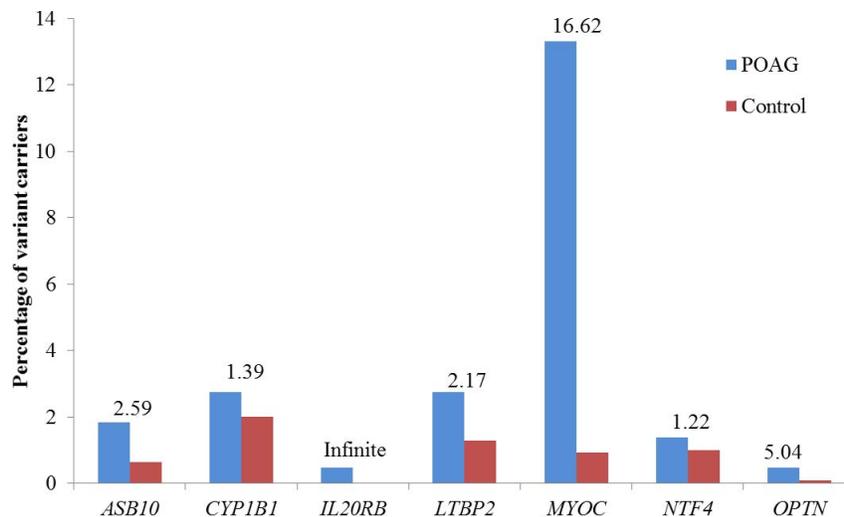


FIGURE 1. Carrier percentages of one or more qualifying variants in known glaucoma genes. The odds ratios of POAG cases versus controls are shown above each column pair.

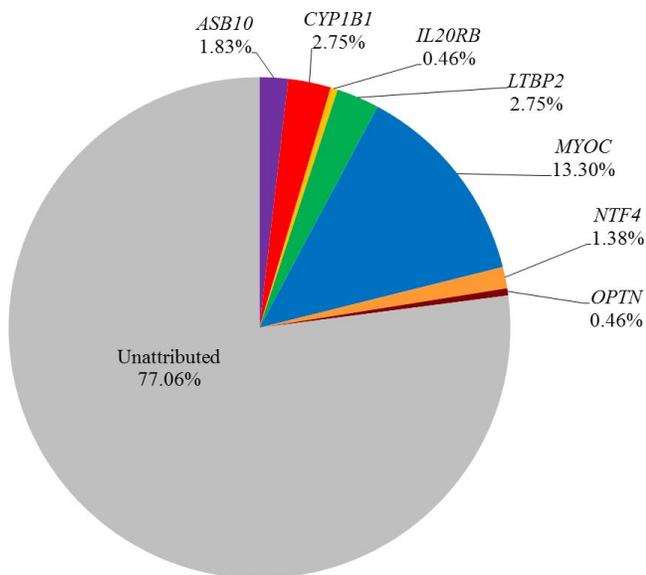


FIGURE 2. Comparative percentages of advanced POAG cases with one or more qualifying variants in Mendelian glaucoma genes.

less meaningful. The biallelic rate (compound heterozygous or homozygous) in this study was 1.4%, which is similar to the 0.85% in the French study<sup>26</sup> and 1.8% in the New Zealand study.<sup>56</sup>

*LTBP2* was originally discovered as the gene responsible for PCG linked to the *GLC3D* locus.<sup>30</sup> Since then, it has also been implicated in POAG in a study of 42 Iranian individuals. The prevalence of *LTBP2* deleterious coding variants was found to be 11.9% (5 out of 42) in the Iranian study.<sup>32</sup> However, a WES study failed to detect any deleterious *LTBP2* variants in Chinese POAG, JOAG, and PCG patients.<sup>57</sup> The prevalence of *LTBP2*-predicted pathogenic variants in our Australian cohort (2.8%) was lower than the rate reported in the Iranian population. Taken with the evidence from the Chinese study, the prevalence rate of *LTBP2* in POAG appears to be heavily dependent on ethnicity, just like the prevalence rate of *CYP11B1*.<sup>32,57</sup>

The relevance of *ASB10* in POAG pathogenesis has been debated. Rare nonsynonymous variants in *ASB10* in POAG have been reported in 6.0% of Caucasian POAG cases compared with 2.8% of controls in a mixed cohort from the United States and Germany.<sup>58</sup> A later study of a smaller US cohort ( $n = 158$ )<sup>36</sup> found a 7.0% rare nonsynonymous variant rate in POAG cases, which was not significantly higher than the 3.7% in controls ( $n = 82$ ). Within the Japanese population, one study reported significant association of microsatellite polymorphisms in the *GLC1F/ASB10* locus with NTG.<sup>59</sup> A more recent Pakistani study of 238 cases found a significant association of *ASB10* rare nonsynonymous variants in POAG with 9.7% prevalence in cases compared to 1.3% in controls.<sup>60</sup> However, the variants did not segregate in the familial cases, and one variant identified in cases but not in the controls (p.(Arg453Cys)) has an allelic frequency of 2.2% in the ExAC population database, emphasizing the importance of large control cohorts to avoid selection bias. The *ASB10* variant p.(Arg304Cys) found in 0.23% of our cases was previously reported in 0.4% of cases in the Pakistani study.<sup>60</sup> The other three variants found in our study have not been previously reported. Our variant rates in cases and controls were lower than in both previous studies due to the additional qualifying condition of predicted pathogenicity via software prediction prior to analysis. In our cohort, there was skewing of *ASB10*

variants toward NTG cases, which is supportive of the findings from the Pakistani study and the study of microsatellite polymorphisms in the Japanese NTG cohort. Given the rarity of predicted pathogenic variants in *ASB10*, larger case cohorts will be required to definitively examine its role in POAG pathogenesis.

The prevalence of *NTF4* variants in our cases was comparable to, albeit slightly lower than, the 1.7% reported in the discovery cohort of European Caucasians.<sup>34</sup> Multiple groups have attempted to replicate these results with mixed success. Case-control studies of European Caucasians<sup>41</sup> and Indians<sup>42</sup> found a higher rate of *NTF4* mutation carriers in controls compared with POAG subjects. The control cohort used in the European study<sup>41</sup> was significantly younger than the original discovery cohort and may include cases that develop glaucoma later in life. The mean age of our cases was even younger than in the discovery cohort used in the European study. Our slightly lower prevalence rate may be attributable to the stringent filtering with pathogenicity prediction software and a possible “winner’s curse” in the original discovery cohort. Meanwhile, two separate studies in Chinese populations have identified low rates of nonsynonymous variation in *NTF4* from 0.3% to 0.6% in POAG cases and none in controls, suggesting that it may be a rare cause of POAG in Chinese people.<sup>40,61</sup>

In a Chinese WES study, the prevalence of *WDR36* variants was high and similar to *MYOC*; however, no controls were sequenced.<sup>57</sup> Our study found that *WDR36* variants were the second most frequent after *MYOC*, although by a large margin. However, it is highly unlikely that *WDR36* variants are causative for POAG in our cohort of advanced glaucoma participants given the higher prevalence of *WDR36* variants in our age-matched local controls and AOGC controls than in POAG cases. Our findings are similar to those in several previous studies that also reported no significant association between *WDR36* and glaucoma pathogenesis owing to the high prevalence of coding variants in controls.<sup>37–39</sup>

The glaucoma locus *GLC1C* was originally mapped to 3q21-q24 between markers D3S3637 and D3S1744.<sup>62</sup> It was not until 2014 that the culprit variant for the original linkage signal was identified in *IL20RB*, p.(Thr104Met), via Sanger sequencing.<sup>7</sup> The variant was reported to reduce receptor function in primary dermal fibroblasts from patients. Human trabecular meshwork cells expressed the *IL20RB* protein and demonstrated its upregulation in response to cytokine treatment, indicating its role in aqueous outflow resistance in POAG. However, *IL20RB* disease-causing variants are exceedingly rare, with none identified in the 230 random POAG cases screened in the American study.<sup>7</sup> In this study, we report the presence of one novel nonsynonymous variant, p.(Arg140Ter), in 1 HTG case and no rare nonsynonymous variants in 1096 controls. Our results support the involvement of *IL20RB* in IOP regulation.

No qualifying variants were found in *TBK1* in our POAG cohort and only 0.1% of the control population carried any qualifying variant in the gene. Published work on *TBK1* implicated copy number gain in glaucoma pathogenesis.<sup>19,20</sup> Our data complement this evidence by showing an absence of potential disease-causing *TBK1* SNVs in our POAG cohort. However, one could not exclude altogether the possibility that *TBK1* SNVs or indels may be responsible for POAG given the rarity of disease-causing copy number variation in *TBK1*.

The estimate of monogenic burden in POAG is traditionally reported at around 5%.<sup>6</sup> Another WES study similar to this study also found *MYOC* to be the gene with the largest genetic contribution to POAG in a Chinese cohort.<sup>57</sup> The Chinese WES study<sup>57</sup> of predominantly POAG cases (125 WES) stated that 8.9% of its cohort harbored a known monogenic cause, of

which 5.6% carried *WDR36* variants. The percentage of our POAG cohort carrying variants in known glaucoma genes (26.6%) was significantly higher than previous estimates of 5%<sup>6</sup> and 8.9% from the Chinese WES study.<sup>57</sup> This finding may be largely due to our extreme disease phenotype selection, skewed toward a younger age of disease onset, as is seen in comparison of JOAG versus POAG cohorts.<sup>10,15</sup> By selecting for only the most severe POAG disease phenotype, we have enriched our case cohort for genetic causes of POAG with higher penetrance. This strategy maximizes our ability to identify disease-causing variants, and has been valuable for discovering common disease alleles with modest cohort sizes.<sup>44</sup> However, given the rarity of disease-causing variants in most POAG-causing genes, our sample size was insufficiently powered to detect statistically significant association in genes other than *MYOC*.

The strength of our study lies in our study cohort and experimental design. All our glaucoma cases had detailed clinical examinations to ascertain their phenotypes. Our large control cohort is matched to our cases on ethnicity, with a local subset also having been clinically examined to ensure absence of glaucoma phenotype. Whole-exome sequencing was performed contemporaneously on both cases and screened controls, thereby minimizing technical variability. One drawback of our study design is the potential underreporting of non-*MYOC* variants in cases carrying *MYOC* variants. As *MYOC*-positive cases were excluded from exome sequencing, the co-occurrence of variants in the other POAG genes cannot be excluded. This scenario is likely to be rare, as the frequency of cases potentially explained by genes other than *MYOC* in this advanced POAG cohort is 9.2%, which equates to an underestimation of non-*MYOC* variants by two to three participants (9.6% of 29, assuming no excessive enrichment of these variants in the *MYOC* cohort). Moreover, JOAG participants in ANZRAG with *MYOC* disease-causing variants have been previously sequenced at the *CYP11B1* locus, and none carried disease-causing variants in the latter gene.<sup>27</sup> Other limitations of this study relate to WES. The ANZRAG cases and controls were captured with a different enrichment protocol from that for the AOGC controls. Although sequencing was performed on the same platform, batch effects on top of capture differences could confound our data. We attempted to minimize this systematic error by employing joint-calling of both datasets using the same bioinformatic pipeline. Additionally, although our local 103 controls were clinically screened to exclude glaucoma or its related endophenotypes, no ophthalmic clinical examination was performed on the 993 AOGC controls. Therefore, it is likely the AOGC control cohort harbors the background rate of POAG in the Caucasian population at around 2.37%. This reduces the power of this study to detect significant differences between our cases and the AOGC controls.

In conclusion, the majority of genetic causes for POAG remain unidentified. In our selective cohort of participants with early-onset advanced disease; *MYOC* had the single greatest influence on POAG, accounting for up to 13.3% of all cases. A role for *WDR36* variants in POAG was not supported in our cohort. The other seven Mendelian POAG genes did not show a significant enrichment of qualifying variants in POAG cases, although they may additively account for up to 9.6% of the POAG burden, providing an overall prevalence of up to 22.9% for known glaucoma genes in advanced early-onset POAG.

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