Glaucoma is the leading cause of preventable irreversible blindness worldwide, with a well-known genetic contribution. Vision damage in glaucoma manifests as visual field loss secondary to optic nerve injury. The disease can be defined as open angle or angle closure depending on the width of the iridocorneal angle. Primary open-angle glaucoma (POAG) is the most common subtype among Caucasians with a 2.37% prevalence in this population, with intraocular pressure (IOP) and age being the most significant risk factors. 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 normal-tension glaucoma (NTG), where untreated IOP is more than two standard deviations above the population mean, and primary open-angle glaucoma (POAG), between 4 and around 40 years for normal-tension glaucoma (NTG), where untreated IOP remains within the normal range (IOP < 22 mm Hg).}

**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 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 TBK1, 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^{-10}).

**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
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%. 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 ITBP2, *602091) in PCG. Additionally, the GLC1C locus has since been linked to the gene IL20RB (interleukin receptor subunit beta) via Sanger sequencing.17

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).8,9 The prevalence of heterozygous MYOC disease-causing variants is around 4% in Caucasian POAG populations overall.8,10 It accounts for a greater proportion in the JOAG subset, with a prevalence of 17% in Australia10 and up to 36% in the United States.11 Disease-causing variants are predominantly located within the third exon and are likely a result of founder effects within European Caucasian populations.12-14 The MYOC glaucoma phenotype is characterized by a young age of onset with high IOP10,11 Optineurin (OPTN) was the second gene to be linked to Mendelian POAG.13 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.16,17 In multiple study cohorts, only the heterozygous p.Glu507fs variant in OPTN has been definitively proven to be disease causing15-18 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.19,20 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.19,22 CYP1B1 (cytochrome P450, family B, polypeptide 1) disease-causing variants have been linked to both PCG and JOAG.25-27 Biallelic CYP1B1 variants transmitted in an autosomal recessive manner were originally linked to PCG and JOAG, 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,26 4.62% in a Taiwanese Chinese JOAG case cohort,28 6.8% in an Australian JOAG case cohort,27 11.1% in an Iranian POAG case cohort.29 Like CYP1B1, variants in ITBP2 (latent transforming growth factor beta binding protein 2) transmitted in an autosomal recessive manner were originally linked to PCG,30,31 with heterozygous variants later suggested in POAG.32 Other genes linked to POAG but with less certainty include ASB10, NTF4, and WDR36.33-35 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.36-39 Heterozygous variants in NTF4 (neutrophin 4) have been suggested to be a rare cause of POAG, ranging from 0.3% in Chinese cohorts40 to 2.26% in German cohorts,44 but were not associated with POAG in US45 and Indian cohorts.42

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.43

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).44-47 Participants with advanced glaucoma and the youngest age at diagnosis with mean age at diagnosis 44.4 ± 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 WES49 (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 disc, deficit on Humphrey visual field testing, and elevation of IOP were required to be related to glaucoma. A larger unexamined disc, deficit on Humphrey visual field testing, and elevation of IOP were required to be related to glaucoma. A larger unexamined disc, deficit on Humphrey visual field testing, and elevation of IOP were required to be related to glaucoma. A larger unexamined disc, deficit on Humphrey visual field testing, and elevation of IOP were required to be related to glaucoma.
Contribution of Known POAG Genes in Early-Onset Disease

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) (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/; in the public domain).

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 ± 8.9 and 18.1 ± 2.9 mm Hg, respectively. The mean age at diagnosis for the POAG cohort was 44.4 ± 10.4 years. Twenty-six HTG participants and 5 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 4.12×10^9 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 ≥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 = 0.01±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 in nine Mendelian POAG genes, predicted to be deleterious by either SIFT or PolyPhen2 HVAR, were recalibrated according to the GATK Best Practices Guidelines.50,51 Variant annotation was performed using ANNOVAR52 software using refGene, SIFT53 (Sorting Intolerant From Tolerant), PolyPhen2 HVAR54 (Polymorphism Phenotyped 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/; in the public domain).

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...
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 study10 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,11 up to 36% of all JOAG was accounted for 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 13.3% 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 OPTN16–18 albeit much less than that in the Japanese discovery cohort (13.5%).15 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.22 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.32,55 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%)26 and New Zealand (6.1%).56 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.27 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

| Table. Numbers and Percentages of POAG Cases or Controls Carrying One or More Qualifying Variants in Nine Mendelian POAG Genes |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| **Gene** | **POAG** | **HTG** | **NTG** | **POAG** | **HTG** | **NTG** | **P Value** |
| ASB10 | 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 (1.62–25.35) | 0.124 |
| IL20RB | 1 (0.46) | 1 (0.67) | 0 | 0 | 1.39 (0.54–3.53) | 1.34 (0.45–4.02) | 1.48 (0.34–6.53) | 0.142 |
| CYP1B1 | 6 (2.75) | 4 (2.67) | 2 (2.94) | 18 (2.00) | 2.17 (0.82–5.71) | 2.1 (0.68–6.47) | 2.32 (0.52–10.43) | 0.069 |
| LTBP2 | 6 (2.75) | 4 (2.67) | 2 (2.94) | 14 (1.29) | 16.62 (7.97–34.67) | 22.72 (10.7–48.22) | 5 (5.34–18.61) | 0.305 |
| MYOC | 29 (13.30) | 26 (17.33) | 3 (4.41) | 10 (0.91) | 1 (0.46) | 1 (0.67) | 3 (4.41) | 7 (0.72) | 2.17 (0.82–5.71) | 2.1 (0.68–6.47) | 2.32 (0.52–10.43) | 0.069 |
| NTF4 | 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 |
| OPTN | 1 (0.46) | 0 | 1 (1.47) | 1 (0.90) | 5.04 (0.31–80.91) | 1.22 (0.34–4.39) | 1.78 (0.49–6.45) | 0.41 (0.1–1.71) | 0.098 |
| TBK1 | 0 | 0 | 0 | 1 (0.90) | 0.52 (0.25–1.09) | 0.57 (0.24–1.32) | 0.41 (0.1–1.71) | 0.098 |
| WDR36 | 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.

**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.
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. Multiple groups have attempted to replicate these results with mixed success. Case-control studies of European Caucasians and Indians found a higher rate of NTF4 mutation carriers in controls compared with POAG subjects. The control cohort used in the European study 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.

In a Chinese WES study, the prevalence of WDR36 variants was high and similar to MYOC; however, no controls were sequenced. 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.

The glaucoma locus GLC1C was originally mapped to 3q21-q24 between markers D3S3637 and D3S1744. It was not until 2014 that the culprit variant for the original linkage signal was identified in IL20RB, p.(Thr104Met), via Sanger sequencing. 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 discasing-causing variants are exceedingly rare, with none identified in the 230 random POAG cases screened in the American study. 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. 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%. 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. The Chinese WES study 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%6 and 8.9% from the Chinese WES study.57 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.10,15 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.44 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 CYP1B1 locus, and none carried disease-causing variants in the latter gene.27 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 contemporaneously on both cases and screened controls, thereby minimizing technical variability. Therefore, it is likely the AOGC control cohort is matched to our cases on ethnicity, with a local subset also having been clinically screened to ensure absence of glaucoma phenotype. Whole-exome 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 contemporaneously on both cases and screened controls, thereby minimizing technical variability.

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