Whole Exome Sequencing of a large family with Primary Open Angle Glaucoma reveals its vast complexity.

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Abstract

Multiple studies have identified several pathogenic variants, majorly contribute to the pathogenesis of primary open-angle glaucoma (POAG). However, these genetic factors can only explain 5-6% of POAG. To identify pathogenic variants associated with POAG by using Whole Exome Sequencing (WES) data of an Egyptian origin of a large family with POAG settled in South India. We recruited a large five-generation family with a positive family history of POAG from Kayalpatanam, Tamil Nadu, India who basically from Egyptian origin. All participants had a comprehensive ocular evaluation (367 study subjects, including 22 POAG and 20 Suspects). We performed WES for 16 samples (9 POAG and 7 controls). We identified one novel potential pathogenic variants, with low-frequency and several pathogenic variants. The heterozygous pathogenic variant c.G3719A in the RPGR-interacting domain of RPGRIP1 gene is segregated in six POAG cases, which may affect the function of RPGR protein complex. In contrast, the RPGRIP1 variant (G1240E) is relatively common in most populations especially in Africans. Furthermore, we identified a novel c.A1295G variant in Rho guanine nucleotide exchange factors Gene 40 (ARHGEF40) and in Retinitis Pigmentosa GTPase regulator (RPGR) gene, may affect the intraocular pressure regulation by altering the RhoA signaling pathway through RPGR protein complex. Moreover, it is difficult to determine the population frequency for this variant. Even though our study reports rare pathogenic variants in multiple genes and pathways associated in the large family with POAG, epigenetic changes and copy number variations may explored to understand the incredibly complexity of the POAG pathogenesis.

Key words: Whole Exome Sequencing, Large Family with Primary Open Angle Glaucoma, Pathogenic Variants, RPGRIP1 and ARGHEF40 gene.

1. Introduction

Primary open angle glaucoma (POAG) is a leading casuce of irreverisible blindness that affects approximately 60.5 million people worldwide with a worrisome exponential increase. Glaucoma is a well-known second-leading cause of global irreversible blindness after cataract (Resnikoff, et al. 2004). Due to the rapid increase in aging population worldwide, this number is expected to rise to 80 million by 2020 (Quigley and Broman 2006) and 111.8 million people by 2040 compared with 2013 inexplicably affecting people belonging to Asia and Africa (Tham, et al. 2014). Asia alone accounted for approximately 60% of the world's glaucoma cases, and Africa had the second highest number of glaucoma cases with 8.3 million (13%). In India, glaucoma affects 12 million people; this figure is predicted to increase up to 16 million by 2020 (Thylefors, et al. 1995, Vijaya, et al. 2008).

Many risk factors for POAG include advanced age, central corneal thickness, myopia, steroid responsiveness and elevated intraocular pressure (IOP) is associated with this disease (Fingert 2011). However, these risk factors do not capture the full spectrum of the disease. Though, the positive family history is also one of the risk factors for POAG; family-based studies have been useful in discovering candidate genes (MYOC, OPTN, and TBK1) (Fingert 2011, Rezaie, et al. 2002, Stone, et al. 1997) that are capable of causing POAG. These candidate genes were discovered through large pedigrees with a positive family history of glaucoma. In addition, the previous studies have shown that POAG development is associated with various genetic risk factors, including variants of genes with different functions, such as CDKN2B-AS (Nakano, et al. 2012, Osman, et al. 2018, Ramdas, et al. 2011, Wiggs 2012) CAV1/CAV2 (Thorleifsson, et al. 2010), TMCO1 (Koolwijk, et al. 2016) AFAP1 (Gharahkhani, et al. 2014), TXNRD2, FOXC1/GMDS, ATXN2 (Cooke Bailey, et al.

2016), FNDC3B (Hysi, et al. 2014, Liu, et al. 2013), GAS (Koolwijk, et al. 2016), PMM2 (Chen, et al. 2014), TGFBR3 (Li, et al. 2015), and SIX1/SIX6 (Osman, et al. 2018, Ramdas, et al. 2011). Therefore, understanding its genetic causes is thus of prime socio-economic importance.

Earlier, we have reported that known candidate gene variants screening in a single large south Indian family with POAG history failed to detect genetic risk factors (abdul kader, et al. 2016). Therefore, we have performed whole exome sequencing (WES), coding all the exonic regions of the human genome, for sixteen samples including nine POAG and seven unaffected family members a large five generation of South Indian family.

2. Materials and Methods

2.1 POAG subjects and controls

The study was approved by the Institutional Review Board at the Aravind Eye Care System, Madurai, Tamil Nadu, India (IRB2011008BAS). The research followed the tenets of the Declaration of Helsinki. Study subjects were screened as previously described (Abdul kader, et al. 2016). Briefly, the study subjects were recruited from a large five generation of South Indian family at Kayalpatanam region, Tamil Nadu, India. After informed consent, a brief medical history was obtained from each study subject that included information regarding demographics, history of systemic disease, and ocular history after informed consent. The study subjects were clinically examined using slit-lamp, pachymetry, optic disc examination with 90 D lens, and applanation tonometry. Additional examinations including standard automated perimetry with a Humphrey Visual Field Analyzer (Zeiss-Humphrey Systems, Dublin, CA) using SITA 24-2 and 10-2 algorithms were performed for suspected glaucoma subjects at the Aravind Eye Care System and Institute of Ophthalmology at Tirunelveli, Tamil Nadu.

Subject with open angles on gonioscopy, glaucomatous optic disc cupping, and consistent visual field defects but not necessarily elevated IOP were characterized as POAG, as previously described (abdul kader, et al. 2016). The study subjects who did not fulfill the above conditions for POAG but still displayed suspicious characteristics like ocular hypertension, suspicious visual fields, or dubious optic discs were characterized as glaucoma suspects. Study subjects were excluded if they were diagnosed with a secondary cause of glaucoma such as ocular surgery, developmental abnormalities, exfoliation syndrome, inflammation, ocular trauma, and pigment dispersion syndrome.

2.2 Sample preparation and Selection

We collected 5 ml of peripheral blood in anticoagulant (EDTA) coated tube from each study subjects. The collected samples were stored at 4°C. Within 12 hours of collection, the genomic DNA was extracted by using a salting-out precipitation method (Miller, et al. 1988). The isolated DNA samples were quantified. Sixteen samples were selected based on their relationship with proband to perform whole exome sequencing.

2.3 Whole exome sequencing

Before proceeding for WES, the DNA concentration and the purity of the samples were analyzed by Nanodrop spectrophotometer. Good quality DNA had an absorbance A260/A280 ratio of 1.7–2.0; also, the samples were run on an agarose gel as a quality control before performing the WES. The ratio of absorption at 260 nm vs. 280 nm was used to determine the purity of DNA. RNA capture baits against approximately 60 Mb of the Human Exome (targeting >99% of regions in CCDS, RefSeq and Gencode databases) were used to enrich regions of interest from fragmented genomic DNA with Agilent's SureSelect Human All Exon V6 kit. Briefly, three µg of each genomic DNA was sheared into 300-350 bp fragments by Covaris. The quality of the fragmentation

was assessed on the Agilent high sensitivity chip by using Bioanalyzer 2100. The fragmented DNA was repaired by using SureSelect XT library kit followed by the adenylation step at the 3' end by an appropriate volume of adenylation mixture, and the paired-end adapters were ligated, and then the adaptor-ligated library was amplified. Each step was followed by purification by AMPure XP beads. Again, the quality of the library was assessed by using bioanalyzer. The prepared libraries were pooled in equal amount to give ~500 ng. The prepared genomic DNA library was then hybridized to a target-specific capture library and then captured using streptavidin-coated beads. The captured library was amplified with the indexing primers contacting 8-bp indexes and cleaned up using AMPure XP beads.

The library preparation and the WES was performed at Centogene, Germany. The generated libraries were sequenced on an Illumina HiSeq 4000 platform to obtain an average coverage depth of ~150x. Typically, ~97% of the targeted bases are covered >10x.

2.4 Data Analysis

We developed an automated pipeline (Supplementary Figure.1) for variant identification from WES data using UNIX script (https://github.com/bharani-lab/WES-pipelines/tree/master/Script). First, raw reads (FASTQ file) were processed to remove the adapter and low-quality sequences using Cutadapt. The processed reads were further aligned against the human genome build GRCh37 using BWA-mem version 0.7.12. GATK version 4.1.0. was used to identify single-nucleotide variants (SNVs) and small Insertion and Deletions (InDels) followed by annotation using ANNOVAR (Wang, et al. 2013). We first filtered rare and low-frequency variants keeping minor allele frequency (MAF) less than or equal to 0.5% in 1000genome, ESP, ExAC and genomeAD. Next, all the protein-coding variants that were either introducing or

removing of stop codon, altering transcripts (frameshift InDels), altering a canonical splice acceptor or splice donor site, and introducing an amino acid change (non-synonymous/missense variant) selected. The non-synonymous variants were further filtered as deleterious variants with two-step process; firstly, variants were selected with the conservation score >2.5 (GERP score) and CADD score greater than 10; secondly, the variants should be predicted to be deleterious with at least three prediction tools among the five (Polyphen2, SIFT, Mutation Taster, FATHMM and LRT). Also, we checked all the variants manually with the help of IGV viewer to avoid mapping errors. All predicted deleterious variants were further filtered based on their presence in at least more than three affected individuals. Finally, the variants were sorted out by their presence in number of affected individuals in the family members and their associated with glaucoma phenotype. We used VarElect software (Stelzer, et al. 2016) to sort the genes based on their direct or indirect association with a glaucoma disease.

We performed pathway and gene ontology analysis using DAVID for all the genes identified final set of variants. A gene network was created using Cytoscape with the enriched pathways and biological processes.

2.5 Confirmation of variant by Sanger sequencing

A novel variant of ARHGEF40 was confirmed by Sanger sequencing and targeted with the following primer set FW-5′-CTGAGCTGACGCCTGAACTT-3′; RV-5′-GGCCGTGGGTACTGAGAAAG- 3′. Polymerase Chain Reaction (PCR) was carried out in a 50 µl reaction mixture containing 100 ng of genomic DNA, 1X buffer (PCR buffer (10 mM TRIS hydrochloride, pH 8.3; 50 mM potassium chloride; 1.5 mM magnesium chloride and 0.001% gelatin)), 0.5 pmol of each primer 200 µM of deoxynucleotide triphosphate and 1 U of Taq DNA polymerase (Sigma Aldrich). We performed the amplification in a DNA Thermal cycler (Applied Biosystems-Invitrogen)

with initial denaturation of 10 minutes at 96°C, followed by 37 cycles at 96°C for 30 seconds, annealing at 58°C for 30 seconds, and final extension at 72°C for 5 mins. The amplified DNA products were purified by QIA quick PCR purification kit method (Bio Basic Inc.,) followed by cyclic PCR. Bi-directional sequencing was performed (3130 Genetic Analyser; Applied Biosystems) and the results were compared with the reference sequence of ARHGEF40 gene using BLAST and Chromas lite (2.1) software.

3. Results

3.1 Clinical Evaluation of patients

We enrolled 84 members of the family after screening 240 family members based on their relation to proband (as seen in the Supplementary Figure 2). Among 84, 14 were diagnosed with POAG. POAG in the family has a relatively early age of onset with a mean of 50 ± 14 years and a range of 23-68 years. Maximum recorded IOP in family members ranged from 14-36 mmHg with a mean of 22.5 ± 6.5mmHg. CCT had a mean value of 529 ± 37.8 microns and cup-disc ratio ranges from 0.6 to 0.9 with a mean of 0.74 ± 0.14. Moderate to severe visual field losses were detected.

3.2 Exome Sequencing and Variant Filtering

We selected nine POAG cases and seven unaffected family members for Whole Exome Sequencing study. The human exonic regions of about 60 Mb (targeting >99% of regions in CCDS, RefSeq and Gencode databases) was enriched from fragmented genomic DNA with Agilent's SureSelect Human All Exon V6 kit. The enriched libraries were sequenced on an Illumina HiSeq 4000 platform to obtain an average coverage depth of ~150x. The raw data were initially pre-processed and analyzed to produce a VCF file containing all the variants with annotations. Approximately 60,000 single-nucleotide variants (SNVs) and small insertion and deletion (InDels) changes were identified in each patient's exome by comparison with the human reference build

GRCh37. Further, we followed stringent variant filtering and prioritization strategy (as mentioned in the methods section, Figure 1) to provide pathogenic variants in the POAG samples.

3.3 Pathogenic variants

We identified six pathogenic variants (5 non-synonymous, one frameshift variants) based on their co- segregated in the family (Table.1). We found a heterozygous variant c.G3719A altering amino acid (p.G1240E) with a deleterious effect might affect the Retinitis Pigmentosa GTPase regulator-interacting protein1 (RPGRIP1) gene, which showed direct association with glaucoma disease (Varlect score of 8.35). Among the WES screening, the variant was segregated in the family with the phenotype (Supplementary Figure 3). Also, Ferna´ndez-Martı´nez et al showed that mutations in the RPGRIP1gene might cause or increase the susceptibility to various forms of glaucoma including POAG (Fernández-Martínez, et al. 2011). Followed by RPGRIP1 gene, we found a novel variant c.A1295G (p.Q432R) in the ARHGEF40 gene, which also segregated with phenotype in the family. Further, we validated the c.A1295G (p.Q432R) variant in other family members by Sanger sequencing, and we detected in eight POAG and two unaffected family members (Supplementary Figure 2). The reaming variants were detected in OR11G2 (c.847delC p.H282fs), OR4K14 (c.A355G p.M119V), RNASE13 (c.C338T p.S113F) and OR11H12 (c.T719G p.V240G) genes. Interestingly, all the pathogenic variants were found in the genetic loci of chromosomal location between14q19 and 14q21.

We also identified 54 pathogenic variants in 51 genes. Of that were not co-segregated basesd on exome data, 52 were missense and 2 were InDel variants with frameshifting the coding region as shown in table 2. From the top list based on the glaucoma phenotype, RPGR gene variants may affect its protein partner RPGRIP1 in the RPGR

proteasome complex (Roepman, et al. 2000). The next on the list, the PLK4 gene has been reported to be involved eye abnormalities (Martin, et al. 2014) Mutations in PLK4, encoding a master regulator of centriole biogenesis, cause microcephaly, growth failure and retinopathy. Interestingly, six variants were identified as novel variants. The top variants (c.A1841T p.D614V) in neural cell adhesion molecule 1 (NCAM1) gene, was further confirmed in six POAG and two unaffected family members by Sanger sequencing. The NCAM1 has been reported to be altered in the optic nerve, which is associated with elevated intraocular pressure (Ricard, et al. 2000).

3.4 Functional network analysis

To investigate the pathways and the biological processes involved in the glaucoma pathogenesis, we constructed a functional network of all genes identified with pathogenic variants. Initially, DAVID database was used to integrate all genes with KEGG pathways and Gene Ontology (GO) process. In total, 60 genes submitted were significantly enriched into three pathways, and 17 GO biological processes ($P < 0.01$). These pathways are Focal adhesion, ECM-receptor interaction and PI3K-Akt signaling pathway. Further in the Gene-functional network (Figure. 2), NCAM1, LAMB4 and PDGFRA genes connected all the three pathways to other GO process. Of these genes, NCAM1 was shown to be connected to the top gene list RPGRIP1 and ARHGEF40 with pathogenic variants through RPGR protein interaction, and GO processes of positive regulation of GTPase activity and visual perception.

4. Discussion

Due to the late onset of disease, recruiting families with POAG was a challenging assignment. With the support of study subjects, we have recruited a large South Indian family with 240 participants. We clinically characterized 22 members as POAG and 20 members as POAG suspect; the rest of the members are unaffected and considered as

controls. Earlier, we have reported the clinical characterization and mutational screening of reported POAG gene of these five generational south Indian family (abdul kader, et al. 2016). We have shown that the reported POAG gene screening failed to detect pathogenic variants in the family through conventional Sanger Sequencing (abdul kader, et al. 2016) method. Therefore, in this study, we have performed whole exome sequencing (WES) of family members with POAG and unaffected.

To perform WES, we have selected nine POAG cases and seven unaffected family members based on their relation to the proband (III-2). More than sixty thousands of SNVs and InDels were detected in each family members. Following low population frequency analysis and pathogenic predictions using several bioinformatics tools (Figure 1), several potential variants were detected. Further, we have prioritized the variants based on theor segregation and their associated with phenotype. This filtering led us to identify six pathogenic variants including five non-synonymous in ARHGEF40, RPGRIP1, OR4K14, RNASE13 and OR11H12, and one frameshift InDel in OR11G2 gene. All these pathogenic variants were present in the chromosome 14q, which has previously been reported to have potential POAG loci (Fan, et al. 2011, Osman, et al. 2018, Wiggs, et al. 2000). In addition, we have shown that fifty-four variants including fifty-one non-synonymous and two frameshift InDel.

In this study, we used phenotype sorting tool to sort the variants that directly or indirectly associated with the glaucoma phenotype. The pathogenic variant in retinitis pigmentosa GTPase regulator-interacting protein 1 (RPGRIP1) gene is observed with highest phenotype score and existed in six POAG cases, suggesting that it may play an important role in POAG. Ferna´ndez-Martı´nez et al., has shown that the heterozygous non-synonymous variants in C2 domain of RPGRIP1gene might cause the various forms of glaucoma including POAG (Fernández-Martínez, et al. 2011). Further, they

have shown that RPGRIP1 interaction with NPHP4 protein plays an important role in the pathogenesis of glaucoma (Fernández-Martínez, et al. 2011). In this study, the heterozygous non-synonymous variant is detected in the RPGR-interacting domain of RPGRIP1. This is in contrast to the previous study (Fernández-Martínez, et al. 2011) and also all homozygous or compound heterozygous variants detected in RPGRIP1 that are associated to photoreceptor dystrophies (Booij, et al. 2005, Dryja, et al. 2001). Interestingly, we observed a pathogenic variant in RPGR gene, which is existed in four POAG cases. RPGRIP1 and its interacting partner RPGR, have been shown to express in human retina and also outside of the retina (Castagnet, et al. 2003, Ferreira 2005, Mavlyutov, et al. 2002, Roepman, et al. 2000) may regulate cilia genesis, maintenance, and function mainly through signalling pathways (Patnaik, et al. 2015). Luo et al., 2014 has reported that the primary cilia of trabecular meshwork (TM) mediates intraocular pressure sensation through signaling pathway in the eye, and further highlighted that defect in the signaling pathway leads to Lowe syndrome that developed congenital glaucoma at birth (Luo, et al. 2014). RPGR and its protein partners play an important role in actin cytoskeleton remodeling of cilia through these signaling pathways by activating the small GTPase, RhoA (Gakovic, et al. 2011).

In the current study, we have identified one novel pathogenic variant in Rho guanine nucleotide exchange factor 40 (ARHGEF40) gene using WES data. Further, the variant was confirmed in the family members (Supplementary Figure 3). Study show that Rho guanine nucleotide exchange factors Gene Family protein (ARHGEF12) has been implicated as a risk factor of glaucoma by increasing intraocular pressure through RhoA/RhoA kinase pathway (Abiko, et al. 2015). Furthermore, the activation of the Rho/ROCK pathway results in trabecular meshwork (TM) contraction, and the inhibition of this pathway would aggravate relaxation of TM with a consequent increase

in outflow facility and, thereby, decrease intraocular pressure (Wang and Li 2010). In the present study, we speculate that ARGHEF40 variant may affect the RhoA signaling through RPGRIP1 and its interacting partner RPGR in actin cytoskeleton remodeling of trabecular meshwork (TM) cilia, which may subsequently increase the intraocular pressure.

The pathogenic variants detected in other genes have not been reported to be directly associated with POAG. Therefore, we constructed a network of genes using GO and pathway enrichment. We have shown three pathways Focal adhesion, ECM-receptor interaction and PI3K-Akt signaling pathway to be associated with the pathogenesis of POAG. Furthermore, the highlighted genes ARHGEF40, RPGRIP1 and RPGR were enriched through visual perception and positive regulation of GTPase activity. Intriguingly, the genes NCAM1, HSP1 and PDGFRA including ARHGEF40 and RPGR in the biological process of positive regulation of GTPase activity is prioritized as top pathogenic variants based on the phenotype score. A study has shown that neural cell adhesion molecule (NCAM) participate in the optic nerve changes associated with elevated intraocular pressure (Ricard, et al. 2000).

5. **Conclusion**

Altogether, this study provides an panel of pathogenic variants in multiple genes, and the interaction of these genes may directly or indirectly be associated with pathogenesis of POAG in the five generational South Indian family. Although this study needs a larger sample size to confirm the results, this study supports the idea of genetic heterogeneity in POAG.

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Ethics approval and consent to participate

The study adhered to the tenets of the Declaration of Helsinki, and ethics committee approval was obtained from the Institutional Review Board of the Aravind Eye Care System (IRB2011008BAS). All study participants read and signed informed consent after explaining the nature and possible significances of the study.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Author's contributions

Mohd Hussain Shah done the sample preparation, sanger sequencing and wrote the manuscript. Manojkumar Kumaran analysed the whole exome sequencing data and wrote the manuscript. Periasamy Sundaresan and Bharanidharan Devarajan designed the work and co-wrote the manuscript. Mohideen Abdul Kader, R Ramakrishnan and Subbiah R Krishnadas assisted in clinical diagnosis and sample collection. All authors read and approved the final manuscript.

Availability of data and material

The pipeline lines used for analysis in this study and a detailed tutorial is openly available for the public at (https://github.com/bharani-lab/Wole-Exome-Analysis-Pipeline).

We have submitted the data that support the finding of this study to SRA project ID PRJNA555016. Data can be accessed upon request

Supportive/Supplementary Materials

Figure Legends

Figure 1. Work Flow for variant prioritization

Figure 2. Functional network enriched with pathways and Gene Ontology (GO) on genes identified with pathogenic variants. Hexagon represents the gene; rectangle represent pathways and Diamond represents GO.

Table Legends

Table.1. List of the Pathogenic variants co- segregated with phenotype. Varlect score with symbol † represents the direct association with glaucoma phenotypes and ‡ represent the indirect association. * represent the Novel variant.

Table 2. List of pathogenic variants. Varlect score with symbol † represents the direct association with glaucoma phenotypes, and ‡ represent the indirect association. * represent the Novel variant.

Supplementary Figure Legends

Supplementary Figure 1. Modular Pipeline

Supplementary Figure 2. Pedigree from south India Family. Family members diagnosed with POAG are shaded with black.

Supplementary Figure 3. Pedigree of selected family members from large south India family as shown in supplementary figure 1 (A). Sanger sequencing results of novel variant c.A1295G in ARGEF40 gene (marked with down arrow). The variant is detected in the family members II-2, III-1, III-2 and III-3 (B).

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Chromosome Position	Accession number	Nucleotide changes	Gene Name	Amino acid change	dbSNP	Varlect	Number of cases (sample ID)
14:21816432	NM_020366.3	c.G3719A	RPGRIP1	p.G1240E	rs34725281	8.35†	6 (III-3;III-2;II-2;III-32;IV-26;IV-27)
14:21550588	NM_001278529.2	c.A1295G	ARHGEF40*	p.Q432R	.	*1.59*‡	*6 (III-3;III-2;II-2;III-32;IV-26;IV-27)*
14:20666340	NM_001005503.1	c.847delC	OR11G2	p.H282fs	rs528205284	*0.99*‡	*6 (III-3;III-2;II-2;III-32;IV-26;IV-27)*
14:20482998	NM_001004712.1	c.A355G	OR4K14	p.M119V	rs7157076	*0.95*‡	*6 (III-3;III-2;II-2;III-32;IV-26;IV-27)*
14:21502110	NM_001012264.4	c.C338T	RNASE13	p.S113F	rs114504351	*0.71*‡	*6 (III-3;III-2;II-2;III-32;IV-26;IV-27)*
14:19378312	NM_001013354.1	c.T719G	OR11H12	p.V240G	rs61969158	*0.22*‡	*6 (III-3;III-2;II-2;III-32;IV-26;IV-27)*

Table.1. List of the Pathogenic variants co- segregated with phenotype. Varlect score with symbol † represents the direct association with glaucoma phenotypes and ‡ represent the indirect association. * represent the Novel variant.

Chromosome Position	Accession number	Nucleotide changes	Gene Name	Amino acid change	dbSNP	Varlect	Number of cases (sample ID)	Number of controls (sample ID)
X:38144822	NM_001034853.2	c.G3430A	RPGR	p.V1144I	rs12688514	9.02†	4 (III-3;III-2;II-2;II-15)	1 (III-41)
4:128816154	NM_001190799.2	c.C2513T	PLK4	p.T838I	rs557954721	6.01†	6 (III-3;III-2;III-32;IV-26;IV-27;II-15)	1 (III-4)
14:45605338	NM_001308133.2	c.C104T	FANCM*	p.P35L	.	*4.84*‡	6 (III-3;III-2;III-32;IV-26;IV-27;II-15)	1 (III-4)
6:56394545	NM_015548.5	c.A9427G	DST	p.N3143D	rs530170321	*4.2*‡	6 (III-3;III-2;II-5;II-15;III-32;IV-27)	2 (III-4;III-16)
13:101759922	NM_001350750.2	c.A2408G	NALCN	p.Y803C	rs549182297	4.11†	8 (III-3;III-2;II-2;II-5;III-19;III-32;IV-27;II-15)	3 (III-41;III-4IV-10)
1:145534254	NM_001303041.1	c.A1330G	ITGA10	p.T444A	rs782732004	*3.98*‡	3 (III-3;III-2;II-2)	1 (III-4)
1:145541806	NM_001303041.1	c.T2900C	ITGA10*	p.L967P	.	*3.98*‡	3 (III-3;III-2;II-2)	1 (III-4)
2:179528378	NM_001267550.2	c.G36508A	TTN	p.E12170K	rs2163008	*3.78*‡	4 (III-3;III-2;II-2;III-32)	1 (IV-28)
19:55350963	NM_001281971.2	c.509_510insCCCGGAGCTCCTATGACATGTA	KIR2DS4	p.S151fs	rs551456772	3.56†	5 (III-3;II-5;III-32;IV-26;IV-27)	2 (IV-11;III-4)
4:55147769	NM_001347827.2	c.C2345T	PDGFRA	p.T782M	rs2291591	3.56†	4 (III-32;IV-26;IV-27;II-15)	1 (IV-28)

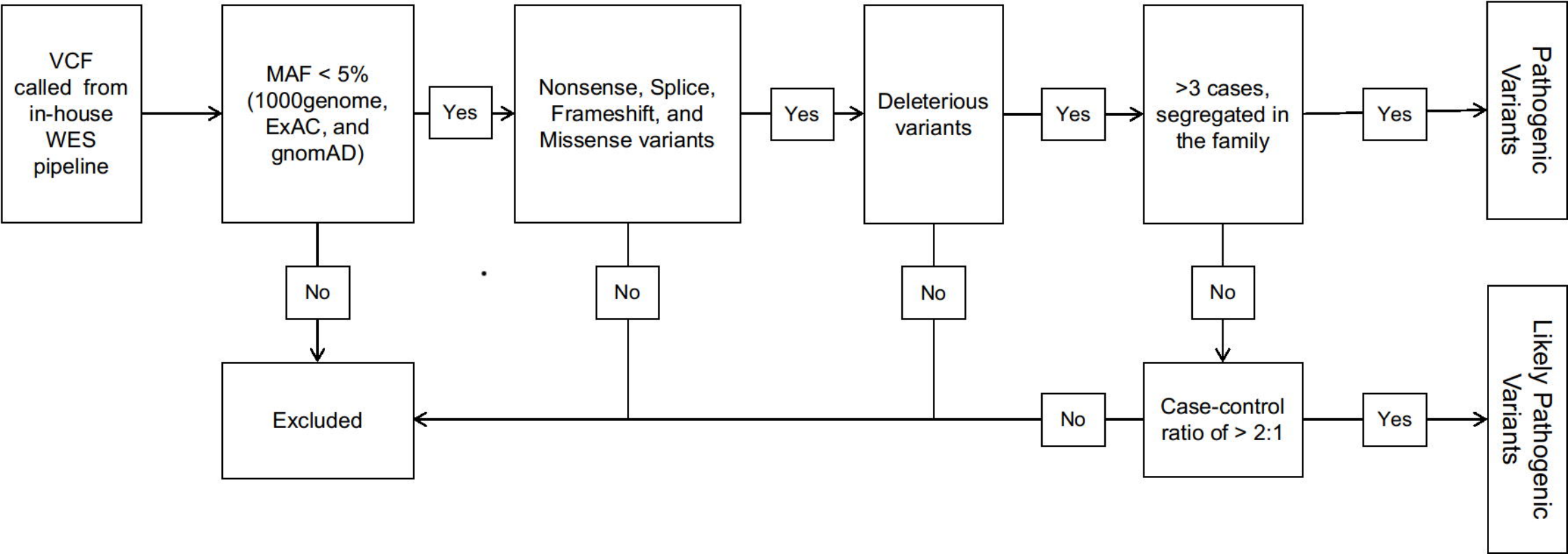
4:141483476	NM_021833.5	c.C680T	UCP1	p.T227I	rs148598275	*3.51*‡	6 (III-3;III-2;III-32;IV-26;IV-27;II-15)	1 (III-4)
1:175046835	NM_022093.2	c.C281T	TNN	p.T94M	rs41266080	*3.23*‡	4 (III-3;III-2;II-5;II-15)	2 (III-41;III-34)
3:141526640	NM_139209.2	c.G1204A	GRK7	p.D402N	rs150840377	*3.21*‡	8 (III-3;III-2;II-5;III-32;IV-26;IV-27;III-19;II-15)	4 (III-34;IV-11;IV-28;III-41)
5:33951693	NM_001012509.4	c.G1122C	SLC45A2	p.L374F	rs16891982	*3.17*‡	5 (III-2;III-32;IV-26;IV-27;II-15)	1 (III-4)
11:113126641	NM_000615.7	c.A1841T	NCAM1*	p.D614V	.	3.16†	6 (III-3;III-2;III-32;IV-26;IV-27;II-15)	2 (IV-28;III-4)
19:45853924	NM_177417.3	c.C1298T	KLC3*	p.S433F	.	*3.05*‡	6 (III-3;III-2;II-5;IV-27;III-32;IV-26)	3 (IV-28;III-4;III-16)
19:50752298	NM_024729.3	c.T1360G	MYH14	p.W454G	rs572234218	*2.94*‡	6 (III-3;III-2;II-5;III-32;IV-26;IV-27)	3 (IV-28;III-16;III-4)
1:209791929	NM_001318046.2	c.C2777A	LAMB3	p.A926D	rs2076222	*2.6*‡	7 (III-3;III-2;II-5;III-32;IV-26;IV-27;III-19)	3 (IV-11;III-4;IV-10)
10:100189242	NM_001322492.1	c.C808T	HPS1	p.P270S	rs34533614	*2.55*‡	3 (III-3;III-2;II-2)	1 (III-4)
10:100202987	NM_000195.5	c.T11C	HPS1	p.V4A	rs58548334	2.55†	6 (III-3;III-2;III-32;IV-26;V-27;II-15)	3 (III-41;III-34;IV-28)
10:97192237	NM_001034957.1	c.C173T	SORBS1	p.P58L	rs200179325	*2.49*‡	6 (II-5;III-32;IV-26;IV-27;II-15;III-19)	1 (IV-10)

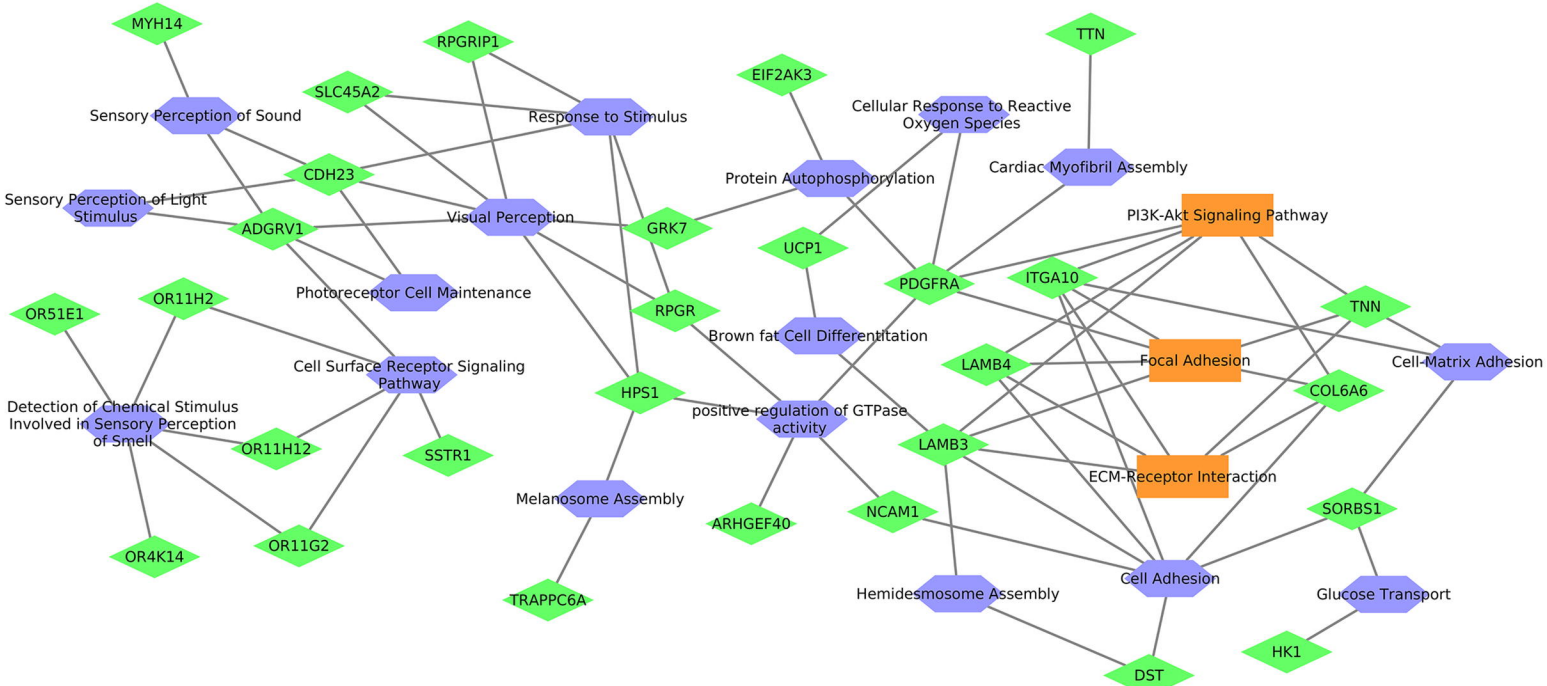
7:107746432	NM_001350531.2	c.C700T	LAMB4	p.H234Y	rs2074749	*2.47*‡	8 (III-3;III-2;II-5;III-32;IV-26;IV-27;II-15;III-19)	2 (III-41;III-16)
10:90530612	NM_001289967.1	c.G683C	LIPN	p.G228A	rs201135817	*2.33*‡	9 (III-3;III-2;II-2;II-5;II-15;III-32;IV-26)	4 (III-16;IV-28;IV-27;III-41;III-19;III-4)
3:130361856	NM_001102608.2	c.G5216A	COL6A6	p.R1739Q	rs16830494	*2.17*‡	6 (III-3;III-2;II-5;III-32;IV-26;IV-27)	3 (III-4;III-16;;IV-28)
19:44571252	NM_013361.6	c.1506dup	ZNF223	p.R424fs	rs562593501	*2.05*‡	7 (III-3;III-2;II-5;III-32;IV-26;II-15;III-19)	3 (IV-11;III-4;III-16)
5:90016871	NM_032119.4	c.G9743A	ADGRV1	p.G3248D	rs16869032	2.05†	8 (III-3;III-2;II-5;III-32;IV-26;II-15;III-19)	4 (III-16;IV-28;IV-27;III-41;III-34)
10:73434888	NM_001171930.2	c.G1469C	CDH23	p.G490A	rs1227049	2.05†	6 (III-3;III-2;II-5;III-32;IV-27;III-19)	2 (III-34;III-16;)
10:71160787	NM_001322367.1	c.C2554G	HK1*	p.P852A	.	2.05†	6 (III-3;III-32;IV-26;II-15;III-2;II-5)	2 (III-16;III-4)
5:96117554	NM_001040458.3	c.C2290T	ERAP1*	p.P764S	.	*1.95*‡	7 (III-3;III-2;II-2;II-5;IV-26;IV-27;III-19)	0 ()
3:182788862	NM_001293273.1	c.A335G	MCCC1	p.E112G	rs142629318	*1.83*‡	6 (III-3;III-32;IV-26;IV-27;II-15;II-5)	2 (III-16;III-4)
3:179408072	NM_003940.3	c.A338G	USP13	p.N113S	rs771971543	1.78‡	7 (III-3;III-2;II-5;III-32;IV-26;IV-27;II-15)	3 (III-4;III-16;IV-28)

4:141483476	NM_021833.5	c.C680T	TMEM63B	p.R82P	rs371238478	1.69‡	6 (III-3;II-5;III-32;IV-26;IV-27;II-15)	3 (III-4;IV-10;III-16)
19:40363916	NM_003890.2	c.G14726A	FCGBP	p.T1975M	rs372872173	1.66‡	6 (III-3;III-2;II-5;IV-27;III-32;IV-26)	3 (IV-28;III-4;III-16)
19:40366240	NM_003890.2	c.C13994T	FCGBP	p.R4909H	rs77005739	1.66‡	3 (III-3;II-2;IV-27)	1 (IV-28)
3:141526640	NM_139209.2	c.G1204A	FCGBP	p.P4665L	rs62106922	1.66‡	3 (III-3;II-2;IV-27)	1 (IV-28)
5:96117554	NM_001040458.3	c.C2290T	EIF2AK3	p.S136C	rs867529	1.62†	7 (III-3;III-2;II-5;III-32;IV-27;II-15;III-19;)	2 (III-4;III-16)
2:179528378	NM_001267550.2	c.G36508A	TIGD4	p.C204F	rs576908904	1.5‡	6 (III-3;III-2;II-5;III-32;IV-26;IV-27;IV-10)	3 (III-16;IV-28)
15:82934639	NM_001322400	c.G941A	GOLGA6L10	p.R314H	rs200928526	1.564‡	7 (III-3;III-2;II-5;III-32;IV-26;IV-27;II-15)	3 (III-4;III-16;IV-28)
X:38144822	NM_001034853.2	c.G3430A	PEX5L	p.F173C	rs141827659	1.54‡	7 (III-3;III-2;II-5;III-32;IV-26;IV-27;II-15)	3 (III-4;III-16;IV-28)
17:39622068	NM_001017402.1	c.C665A	KRT32	p.S222Y	rs2071561	1.1†	6 (III-3;III-2;III-32;IV-26;IV-27;II-15)	1 (III-4)
11:103006630	NM_001080463.2	c.G2527A	DYNC2H1	p.A843T	rs548461924	0.97‡	6 (III-3;III-2;III-32;IV-26;IV-27;II-15)	2 (III-4;IV-28)
4:128816154	NM_001190799.2	c.C2513T	PDZD3	p.Q226K	rs147147532	0.85‡	6 (III-3;III-2;III-32;IV-26;IV-27;II-15)	0
3:179529649	NM_001349404.2	c.T518G	OR51E1	p.A156T	rs202113356	0.84‡	6 (III-3;III-2;III-32;II-15;IV-26;IV-27)	3 (III-4;III-16;IV-28)

11:119058667	NM_001168468.2	c.C676A	OR11H2	p.P269S	rs2815979	0.82‡	6 (III-3;III-2;III-32;IV-26;IV-27;II-15)	0 (III-4;IV-28)
21:33735605	NM_014825.3	c.T1369A	URB1	p.S457T	rs148292685	0.81‡	7 (III-3;III-2;II-5;III-32;IV-26;IV-27;II-15)	3 (III-4;III-16;IV-28)
11:30974115	NM_001350255.1	c.G1449T	DCDC1	p.R483S	rs158633	0.78‡	6 (III-3;III-32;IV-26;IV-27;II-15;III-19)	2 (III-41;III-34)
11:31123752	NM_001350255.1	c.A812G	DCDC1	p.D271G	rs183555899	0.78‡	6 (III-3;III-32;IV-26;IV-27;II-15;III-19)	2 (III-41;III-34)
19:49969382	NM_001145396.2	c.G1627C	ALDH16A1	p.A543P	rs555667637	0.42‡	6 (III-3|III-2|II-5|IV-27|III-32|IV-26|)	3 (III-4_C|III-16_C|IV-28_C|)
2:88913273	NM_004836.7	c.C407G	EFHC1	p.R353W	rs527295360	0.42‡	6 (III-3;III-32;IV-26;IV-27;II-15;II-5)	2 (III-4;III-16)
1:209791929	NM_001318046.2	c.C2777A	KLHL24	p.D197A	rs116961268	0.42‡	7 (III-3;III-2;II-5;III-32;IV-26;IV-27;II-15)	3 (III-4;IV-28;III-16)
19:50752298	NM_024729.3	c.T1360G	MAGIX	p.P221L	rs781930221	0.28‡	7 (III-2;II-2;II-5;III-32;IV-26;IV-27;III-19)	3 (III-4;III-34;III-16)
5:33951693	NM_001012509.4	c.G1122C	PIEZO1	p.I2265V	rs1803382	0.24‡	6 (III-3;III-2;III-32;IV-26;IV-27;II-15)	1 (III-4)
10:90530612	NM_001289967.1	c.G683C	LARP1B*	p.I178F	.	0.21‡	7 (III-3;III-2;II-5;III-32;IV-26;IV-27;II-15)	3 (III-4;III-16;IV-28)
6:44103070	NM_001318792.1	c.G245C	SSTR1	p.T390delinsTLX	rs775405351	0.18†	6 (III-3;III-2;II-5;IV-27;II-15;III-32)	2 (IV-28;III-16)

Table 2. List of pathogenic variants. Varlect score with symbol † represents the direct association with glaucoma phenotypes, and ‡ represent the indirect association. * represent the Novel variant.


VCF called from in-house WES pipeline
MAF < 5% (1000genome, ExAC, and gnomAD)
Yes
Nonsense, Splice, Frameshift, and Missense variants
Yes
Deleterious variants
Yes
>3 cases, segregated in the family
Yes
Pathogenic Variants
No
No
No
No
Excluded
No
Case-control ratio of > 2:1
Yes
Likely Pathogenic Variants


MYH14
RPGRIP1
SLC45A2
Sensory Perception of Sound
Response to Stimulus
CDH23
Sensory Perception of Light Stimulus
ADGRV1
Visual Perception
GRK7
Photoreceptor Cell Maintenance
RPGR
OR51E1
OR11H2
Cell Surface Receptor Signaling Pathway
Detection of Chemical Stimulus Involved in Sensory Perception of Smell
OR11H12
SSTR1
HPS1
OR4K14
OR11G2
Melanosome Assembly
TRAPPC6A
EIF2AK3
Cellular Response to Reactive Oxygen Species
TTN
Protein Autophosphorylation
Cardiac Myofibril Assembly
PI3K-Akt Signaling Pathway
UCP1
PDGFRA
ITGA10
TNN
Brown fat Cell Differentitation
LAMB4
Focal Adhesion
Cell-Matrix Adhesion
COL6A6
positive regulation of GTPase activity
LAMB3
ECM-Receptor Interaction
ARHGEF40
NCAM1
SORBS1
Cell Adhesion
Hemidesmosome Assembly
Glucose Transport
HK1
DST