Identification of Mutations in Myocilin and Beta-1,4-galactosyltransferase 3 Genes in a Chinese Family with Primary Open-angle Glaucoma

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Abstract

Background: Glaucoma is a major cause of irreversible blindness worldwide. There is evidence showing that a subset of the disease is genetically determined. In this study, we screened for mutations in chromosome 1q-linked open-angle glaucoma (*GLC1A*) in a Chinese family with primary open-angle glaucoma (POAG).

Methods: A total of 23 members from five generations of a family were enrolled and underwent thorough ophthalmologic examinations. In addition, 200 unrelated healthy Chinese controls were also recruited as normal control. *GLC1A* gene was amplified by polymerase chain reaction, and DNA sequencing was performed to screen for mutations.

Results: Six members were diagnosed as POAG, with severe clinical manifestations, and history of high intraocular pressures. The mean age of disease onset was 26.3 years. However, the others were asymptomatic. In six affected and three asymptomatic members, gene sequencing revealed a mutation c.C1456T in exon 3 of myocilin gene (*MYOC*). Furthermore, we also identified a novel mutation c.G322A in beta-1,4-galactosyltransferase 3 (*B4GALT3*) gene in all six affected and three asymptomatic members, which was not reported previously in POAG patients. The two newly identified variants were absent in other family members as well as controls.

Conclusion: The mutations c.1456C<T (p.L486F) in *MYOC* and c.322G<A (p.V108I) in *B4GALT3* are likely responsible for the pathogenesis of POAG in this family.

Key words: Beta-1,4-galactosyltransferase 3; Genetic Testing; Glaucoma; Myocilin; Primary Open-angle Glaucoma

Introduction

Glaucoma, an anterior optic neuropathy, is the second major cause of irreversible blindness worldwide. It is characterized by optic nerve head changes and corresponding visual field loss.[1] Primary open-angle glaucoma (POAG; OMIM 137760) is the most prevalent subtype of glaucoma, usually displays asymptomatic developments until the late stages of the disease. The prevalence of POAG is predicted to increase to 76.0 million by 2020 and to 111.8 million by 2040 all over the world owing to population aging.[2] POAG was subdivided into two forms according to onset age: juvenile open-angle glaucoma (JOAG) and adult-onset POAG. JOAG develops clinical manifestation between 3 and 35 years of age[3] while adult-onset POAG often shows symptoms after 40 years.[4]

Due to the suboptimal sensitivity of current diagnostic ability, the diagnosis of POAG is usually made after irreversible visual damage has already occurred. Thus, the early diagnosis of POAG is urgently required.[5,6] Genetically, POAG is considered as a complex disease with a substantial fraction showing complicated inheritance patterns, while

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JOAG typically follows an autosomal dominant inheritance. Identification of novel mutations provides opportunities for the understanding of etiology, realizing presymptomatic diagnosis, and improving the prognosis of POAG.

Up to date, several loci have been identified associated with POAG. Among them, four genes, including trabecular meshwork (TM) inducible glucocorticoid response (myocilin [*MYOC*], OMIM 601652), optineurin (OMIM 602432), human dioxin-inducible cytochrome P450 (*CYP1B1*, OMIM: 601771), and WD repeat-containing protein 36 (OMIM 609669), have been identified to be primarily responsible for POAG.[7] As the most frequently mutated gene in POAG families, *MYOC* locates at *GLC1A* (OMIM 601652) locus on chromosome 1q23.[3,8] Therefore, in a newly identified Chinese family with POAG, we screened for mutations at *GLC1A* locus to localize mutations that might be responsible for this pedigree.

Methods

Subjects and clinical examination

This POAG pedigree spans five generations with 23 members recruited. No consanguineous marriage was found in individuals of family. Then, all individuals underwent thorough ophthalmologic examinations including visual acuity, slit-lamp biomicroscopy, intraocular pressure (IOP) measurement, gonioscopy, funduscopy, and perimetry. The 200 controls only with no reported family history of hereditary eye diseases and normal ophthalmic examination findings could be recruited. This study was approved by the Medical Ethics Committee of Anhui Medical University. All participants were informed of the details and all of the procedures, and each individual provided informed consent in accordance with the *Declaration of Helsinki*. In this study, the diagnostic of criteria was defined by a normal anterior chamber angle and two of the following symptoms: (1) IOP >21 mmHg (no IOP lowering therapy); (2) characteristic visual field defects; (3) glaucomatous optic nerve head changes (cup-disc [C/D] ratio >0.6 or notches); JOAG was diagnosed when patients' age at the time of POAG diagnosis was younger than 35 years.

DNA extraction

Genomic DNA was extracted from peripheral blood according to the standard procedures using FlexiGene DNA kits (TIANGEN, Beijing, China). DNA samples were stored at −20°C until used. The 1% agarose gel electrophoresis was used to evaluate DNA integrity.

Exome sequencing

Exome sequencing was employed to identify the POAG-associated genes. We performed exome sequencing on DNA samples of the three family members (IV: 1, IV: 9, and V: 4) by Axeq Technology Inc., Seoul, Korea, and prepared each sequenced sample according to the protocols provided by the Illumina Inc., San Diego, USA. In brief, we fragmented one microgram of genomic DNA by nebulization, repaired the fragmented DNA, ligated an "A" to the 3' end, ligated Illumina adapters to the fragments, and the sample was size selected aiming for a 350–400 base pair product. The size-selected product was amplified using polymerase chain reaction (PCR). The Agilent Bioanalyzer (Agilent Technologies Inc., Santa Clara, USA) was used to identify the final product. Then, probes containing the targeted regions of interest were captured by streptavidin beads, and nonspecific binding was washed out. Next, the sequencing libraries were enriched for the desired target by the Illumina Exome Enrichment protocol and the enriched library validation for quality control analysis were performed using the Axeq Technology. For clustering and sequencing, the protein-coding regions of human genome DNA were collected by genomic DNA Illumina TruSeq Exome Capture System (Illumina Inc.). Every captured library was loaded onto the Illumina Hiseq2000 sequencer (Illumina Inc.) and performed by high-throughput sequencing to ensure that each sample met the desired average sequencing depth.

Reads, mapping, and variant detection

The high-quality sequencing reads were aligned to the human reference genome (NCBI build 37.1/ hg19) with SOAP aligner (soap2.21). Based on the SOAP alignment results, we assembled the consensus sequence and called genotypes in target regions using SOAPsnp v1.05. We collected data as lists of sequence variants (single nucleotide polymorphisms [SNPs] and short Indels) and filtered SOAPsnp results for SNP quality control as follows: (1) base quality >20; (2) depth is 4–200; (3) estimate copy number ≤2; (4) the distance between two SNPs longer than 4. BWA (version 0.5.9-r16, Sanger Institute, Cambridge, UK) was used to align all the high-quality reads to the human reference genome. Then, UnifiedGenotyper tool from GATK (version v1.0.4705) was used to perform small Indel detection. SNP and Indel detection were performed only on the targeted exome regions and flanking regions within 200 bp.

Filtering and annotation

The detected variants were annotated and filtered based on four databases including NCBI CCDS (http://www.ncbi.nlm.nih.gov/ RefSeq (http://www.ncbi.nlm.nih.-gov/RefSeq/),CCDS/CcdsBrowse.cgi), Ensembl (http://www.ensembl.org), and Encode(http://genome.ucsc.edu/ENCODE). Prioritized all the high-quality mutations including four major steps: (1) mutations including intronic, intergenic, untranslated regions and synonymous mutations were excluded from downstream analysis; (2) mutations in 1000 genome project (ftp://ftp.1000genomes.ebi.ac.uk/vol1-/ftp), dbSNP137 (http://www.ncbi.nlm.nih.gov/projects/SNP/), YH database (http://yh.genomics.org.cn/), and HapMap Project (ftp://ftp.ncbi.nlm.nih.gov/hapmap) were excluded; (3) SIFT (http://sift.bii.astar.edu.sg/) and Polyphen2 (http://genetics.bwh.harvard.edu/pph 2/) were used to predict possible damaging impacts of each variant on protein structure and function; (4) KEGG pathway annotations (http://www.ebi.ac.uk./clustalw) and gene ontology (http://www.geneontology.org) were used to predict the biological function of each putative gene.

Variants validation

Sanger sequencing was used to determine if any of the potential mutations co-segregated with the disease phenotype in this pedigree. Primers flanking the candidate loci were designed on the basis of genomic sequences of human genome (hg19/build37.1) and synthesized by Invitrogen (USA): *MYOC*-F, 5'-GGGAGACAAACATCCGTAAGC -3'; *MYOC*-R, 5'-GTCTACGCCCTCAGACTACAA -3'; beta-1,4-galactosyltransferase 3 (*B4GALT3*)-F, 5'-AGAGAAGGCTAGGTAAGGATCC-3'; *B4GALT3*-R, 5'-AAAGTGGAAGAGCAGTGC-3'. Genotyping for c.1456C<T in *MYOC* and c.322G<A in *B4GALT3* in the members of family was then confirmed by direct PCR and analyzed on an ABI 3730XL Genetic Analyzer (ABI Inc., Foster City, USA). Sequencing data were compared with the human genome database.

Results

Clinical findings

The POAG of this Chinese family in five generations showed an autosomal dominant inheritance [Figure 1]. Six members of this family were diagnosed as JOAG, and the glaucoma relevant characteristics of each affected member are illustrated in Table 1. The mean age at diagnosis was 26.3 years (range from 22.0 to 30.0 years). The proband (IV: 3, a 35-year-old woman) was diagnosed as JOAG 8 years ago (at the age of 27 years), with pretreatment IOPs of 42/36 mmHg (right eye/left eye [OD/OS]), open anterior chamber angle, and enlarged C/D ratio of 0.75/0.70 (OD/OS). Due to the poor efficacy of medical care, she underwent trabeculectomy on both eyes at the age of 27 years, and IOP was controlled (11–15 mmHg OU) until now. No visual field progression was found over the following years. The proband's father (III: 2) recalled vision reduction (approximate 0.1) in his late twenties, had pretherapy IOPs of 40–50 mmHg, and accompanied with symptoms including painful eye bilges and headache. Because of his inactive hospitalized attitude and poor consciousness, POAG was diagnosed a decade later. Although undergoing surgery at the age of 39 years, he still became blind in his later forties. Funduscopy showed glaucomatous optic disc damage with C/D ratio of 1.0/1.0 in both eyes. In addition, he recalled that his grandfather and father (I: 1, II: 1), all became blind between the age of 40 and 50 years. III: 6 were diagnosed as POAG at the age of 39 years with high IOPs and dead 5 years later. Similar to the proband, her uncle (III: 4) recalled that his vision was reduced in his early twenties and performed trabeculectomy at the age of 25 years. The ophthalmologic examinations indicate his visual acuity of 0.04/perception of light, with IOPs of 17/11 mmHg and C/D ratio of 0.90/0.95 in both eyes. Moreover, patient IV: 1 was diagnosed as POAG at the age of 30 years with IOPs of 27/30 mmHg and achieved controlled IOPs after he underwent for trabeculectomy on both eyes. Proband's younger brother and cousin (IV: 7, IV: 9) were diagnosed as POAG at the ages of 27 and 22 years, respectively, and only IV: 9 achieved controlled IOPs after received topical therapy (latanoprost and carteolol hydrochloride eye drops).

Mutations screening of myocilin and beta-1,4-galactosyltransferase 3 in primary open-angle glaucoma

In sequencing analysis, a heterozygous missense mutation, c.1456C<T (p.L486F), was found located at nucleotide 1456

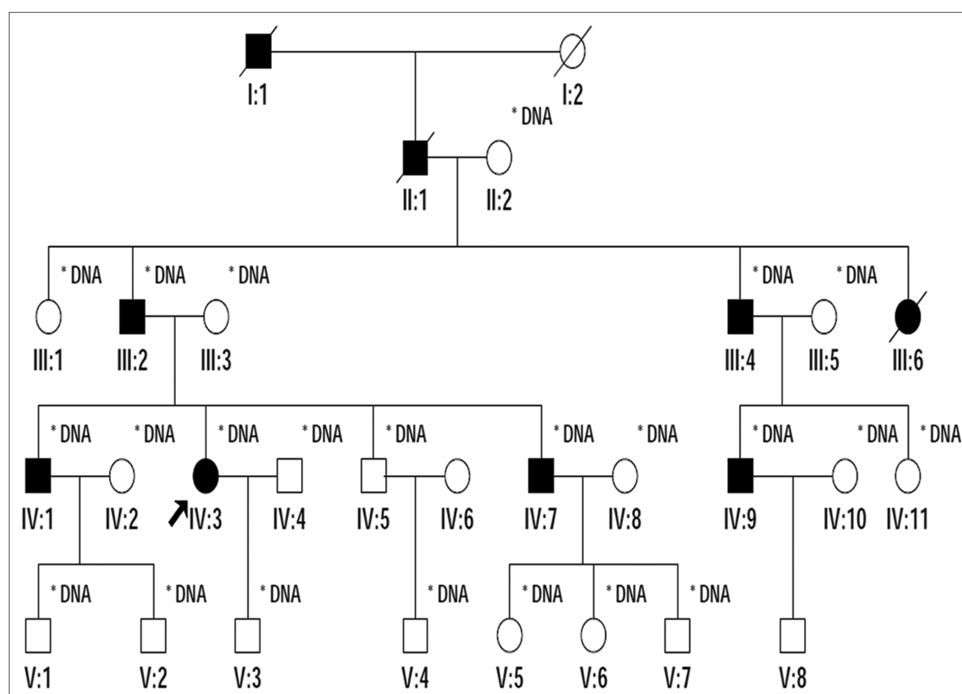

Figure 1: Pedigrees of the Chinese family with primary open-angle glaucoma. Arrow indicates the proband (IV: 3); Filled squares and circles indicate male and female members; solid symbols indicate the carrier; diagonal line indicates deceased subject.

in econ 3 of *MYOC* gene [Figure 2]. This mutation was present in all six affected and three asymptomatic members (IV: 11, V: 3, and V: 5). Meanwhile, sequencing analysis of *B4GALT3* gene revealed a novel heterozygous missense mutation [Figure 3], c.322G<A (p.V108I) of all patients and three unaffected individuals (IV: 11, V: 2, and V: 5). No above-mentioned mutations were found in other asymptomatic individuals and all included controls. We did not find these two variants in public databases including dbSNP137, 1000 genome project, YH database, and HapMap project.

Bioinformatic analysis

The mutation, c.1456C<T, in the exon 3 of *MYOC* gene would lead to the replacement of leucine by phenylalanine. DNA sequence alignment in eight different species suggests that leucine 486 is a highly conserved residue in *MYOC* protein [Figure 4]. SIFT analysis predicts the p.L486F mutation of *MYOC* as "affecting protein function" with a score of 0.00 (score <0.05 means the protein function may be affected by the amino acid change). Similarly, valine 108 was highly conserved in *B4GALT3* protein among different species. SIFT also predicts that the p.V108I mutation of *B4GALT3* may affect its function by replacing valine to isoleucine at position 108 (a score of 0.00).

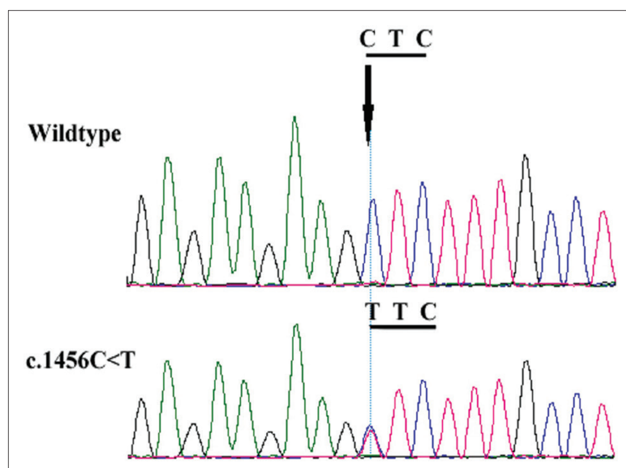

Figure 2: Sequencing results of the *MYOC* gene. A heterozygous missense mutation, c.1456C<T (p.L486F, black arrow), was found located at nucleotide 1456 in exon 3 of *MYOC* gene of all six affected and three unaffected members (IV: 11, V: 3, and V: 5). *MYOC*: Myocilin.

Discussion

MYOC gene locates at *GLC1A* locus on chromosome 1q23, includes three exons and encodes for 57kD *MYOC* protein, which is the first gene identified to be responsible for POAG.[8,9] *MYOC* protein is expressed in various ocular tissues, including cornea, sclera, iris, ciliary body, retina, and optic nerve head, but most abundant in TM cells.[10] Most glaucoma-causing *MYOC* mutants are misfolded, aggregation-prone, detergent-insoluble, and accumulated in the endoplasmic reticulum, which result in cytotoxicity in TM cells. It has been suggested that *MYOC* mutation is associated with the blockage of aqueous outflow through TM, and thus leads to elevated IOPs.[11] There are more glaucoma risk mutations found in *MYOC* than any other glaucoma-associated genes.[12] Over eighty different mutations have been identified in *MYOC*, and majority of them are clustered in exon 3, which constituted an important olfactomedin domain.[13] Mutations of *MYOC* have been reported associated with POAG pedigrees

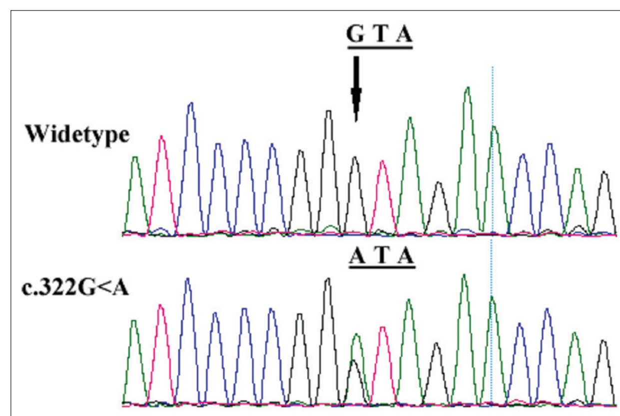

Figure 3: Sequencing results of the *B4GALT3* gene. A novel heterozygous missense mutation, c.322G<A (p.V108I, black arrow), of all patients and three unaffected individuals (IV: 11, V: 2, and V: 5). *B4GALT3*: Beta-1,4-galactosyltransferase 3.

Table 1: Clinical data of patients in this Chinese family with POAG

Member ID	Gender	Age at study (years)	Onset age (years)	OD/OS				Therapy
				Visual acuity	IOP (mmHg)	C/D ratio	Visual field damage	
III: 2	Male	58	28	NPL/NPL	NR/NR	1.0/1.0	S/S	Trabe
III: 4	Male	47	23	0.04/PL	17/11	0.90/0.95	S/S	Trabe
III: 6	Female	P	39	NR/NR	NR/NR	NR/NR	NR/NR	Trabe
IV: 1	Male	38	30	0.5/0.6	16/14	0.80/0.65	S/M	Trabe
IV: 3	Female	35	27	0.5/0.8	11/12	0.75/0.70	S/S	Trabe
IV: 7	Male	30	28	0.6/0.6	25/27	0.85/0.85	S/S	Med
IV: 9	Male	24	22	1.0/0.8	10/11	0.50/0.65	M/M	Med

OD: Right eye; OS: Left eye; C/D: Cup-disc; PL: Perception of light; NPL: No perception of light; NR: No recordable; Trabe: Trabeculectomy; P: Pass away; S: Severe defect; M: Moderate defect; Med: Medical treatment; POAG: Primary open-angle glaucoma; IOP: Intraocular pressure.

p.Leu486Phe	
Human	LTIPFKNRYKYSSMIDYNPLEKKLFAWDNLNMVTYDIKLSKM
Chimpanzee	LTIPFKNRYKYSSMIDYNPLEKKLFAWDNLNMVTYDIKLSKM
Rhesus Monkey	LTIPFKNRYKYSSMIDYNPLEKKLFAWDNLNMVTYDIKLSKM
Dog	LSIPFKNRYKYSSMIDYNPLEKKLFAWDNFNMVTYDIRLSKM
Cattle	LTVPFKNRYKYSSMIDYNPLERKLFAWDNFNMVSYDIKLSRL
House Mouse	LTIPFTNRYKYSSMIDYNPLERKLFAWDNFNMVTYDIKLLEM
Marway Rat	LTIPFKNRYKYSSMVDYNPLERKLFAWDNFNMVTYDIKLSEM
Chicken	LSIPFENRFRYLSMVDYNPAERRLFAWDSYNMVAYPVRLAHA
Zebrafish	ISVPFKNRYRYNSMVDYNSAKRKLYAWDNYYMVSYSVRLGKQ

Figure 4: The c.1456C<T (p.L486F) of *MYOC* involved a highly conserved residue. DNA sequence alignment in eight different species suggests that leucine 486 is a highly conserved residue in *MYOC* protein. *MYOC*: Myocilin.

in many different ethnic groups.[14-21] As a result, in this study, we first screened for the mutations in *MYOC gene* in a five-generation Chinese POAG pedigree. A heterozygous missense mutation, c.1456C<T (p.L486F), was found to locate at nucleotide 1456 in exon 3 of *MYOC* gene of all six affected and three unaffected members (IV: 11, V: 3, and V: 5). Huang *et al*. evaluated mutations in several glaucoma-associated genes in 683 Chinese patients with primary glaucoma and also found c.1456C<T (p.L486F) mutation of *MYOC* in a sporadic POAG patient, which indicates that the c.1456C<T variants in *MYOC* are likely to be responsible for the pathogenesis of POAG in this family.[22] The majority of patients in this pedigree were JOAG as the onset of clinical manifestations occurs before the age of 30 years, and the average onset age was 26.3 years. This may explain why IV: 11, V: 3, and V: 5 (age of 19, 8, and 11 years, respectively) harbor the same mutation, yet without clinical manifestations. A careful follow-up for these members may help us further clarify the onset age of disease and the pathological changes at the early stage.

Furthermore, sequencing analysis of *B4GALT3* gene revealed a novel heterozygous missense mutation, c.322G<A (p.V108I) of all patients and three unaffected individuals (IV: 11, V: 2, and V: 5). *B4GALT3* locates on chromosome 1q23.3 and is one of seven *B4GALT* genes. The coding region of *B4GALT3* has a single initiation codon, immediately before a sequence encoding a hydrophobic transmembrane segment. *B4GALT3*'s coding sequence encodes Type II transmembrane glycoprotein which includes an N-terminal cytoplasmic domain of four residues, a stem region, a transmembrane segment, and a catalytic domain with four N-linked glycosylation sites.[23] All seven B4GALT proteins have distinct functions in the synthesis of glycoconjugates and saccharide structures. The *B4GALT3* gene encodes an enzyme that may be mainly involved in the synthesis of the first *N*-acetyllactosamine unit of poly-*N*-acetyllactosamine chains. *In vitro* assay predicted that *B4GALT3* and B4GALT4 proteins exhibit similar kinetic properties and functions.[24] Recently, it has been reported that *B4GALT3* protein plays an important role in invasion, proliferation, and metastasis in extravillous trophoblast, colorectal cancer, and neuroblastoma.[25-27] The mutation of *B4GALT3* gene was first identified in a POAG pedigree. POAG is a complex neurodegenerative disease which results in the stress in retinal ganglion cells and optic nerve axons. The outgrowth of an axon is critical for the regeneration of nerve fibers during wound healing. It has been suggested that B4GALT proteins play a role in maintaining the normal function of nerve cells, increasing neurite outgrowth, and promoting the regeneration of injured neurons.[28-31] Although the association between the mutations in *B4GALT* gene/protein and the risk of POAG has not been reported, the p.V108I mutation of *B4GALT3* protein may affect its function by replacing valine to isoleucine at position 108 and may also contribute to the pathogenesis and process of POAG in our reported kindred. Similar to the mutations of *MYOC*, the c.G322A mutation of *B4GALT3* was also found in three asymptomatic members (IV: 11, V: 2, and V: 5).

Among these two reported mutations, we propose that c.C1456T in exon 3 of *MYOC* plays a major role in the pathogenesis of POAG. The reasons are as follows: on one hand, the same mutation has been found and reported in a Chinese sporadic POAG patient; on the other hand, although the exact intraocular physiological functions of B4GALT protein are largely unknown, abnormal B4GALT protein may affect its function to maintain the normal activity of nerve cells, increase neurite outgrowth, and promote the regeneration of injured neurons. Considering its known functions, it may cooperate with the mutated *MYOC* to accelerate the progression of POAG after retinal ganglion cells and optic nerve axons being damaged, rather than being a main cause to initiate the onset of POAG. It is possible that c.1456C<T (p.L486F) mutation of *MYOC* leads to the pathogenesis of POAG in this family, in which c.G322A (p.V108I) variant of *B4GALT3* accelerates its progression. The role of c.G322A (p.V108I) variant in this pedigree will be further clarified by the follow-up studies of V: 2 and V: 3 who only possess c.1456C<T (p.L486F) mutation of *MYOC* and c.G322A (p.V108I) variant of *B4GALT3*, respectively.

In this pedigree, the majority of patients were JOAG. The onset symptoms occur before the age of 30 years. In addition, severe clinical manifestations, high IOPs, unstable to topical therapy, and most of the patients requiring surgery to avoid the progress of disease characterize this pedigree's phenotype, which is different from the typical phenotypes of POAG. Thus, early diagnosis and proper interventions are very critical for protecting patients from the loss of visual acuity, and especially, preventing young members who harbor the same mutations in this family develop blindness at youth.

In conclusion, the mutations c.1456C<T (p.L486F) in *MYOC* and c.322G<A (p.V108I) in *B4GALT3* are likely responsible for the pathogenesis of POAG in this family. The new variants in these two genes provide a significant

opportunity to further clarify the etiology of POAG, as well as to improve the presymptomatic diagnosis and prognosis of POAG. Furthermore, the mechanisms of POAG could further be investigated in animal model using these two genetic mutations in the future.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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