A Large *GLC1C* Greek Family with a Myocilin T377M Mutation: Inheritance and Phenotypic Variability

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Purpose. POAG is a complex disease; therefore, families in which a glaucoma gene has been mapped may carry additional POAG genes. The goal of this study was to determine whether mutations in the myocilin (*MYOC*) gene on chromosome 1 are present in two POAG families, which have previously been mapped to the *GLC1C* locus on chromosome 3.

Methods. The three exons of *MYOC* were screened by denaturing (d)HPLC. Samples with heteroduplex peaks were sequenced. Clinical findings were compared with genotype status in all available family members over the age of 20 years.

Results. A T377M coding sequence change in *MYOC* was identified in family members of the Greek *GLC1C* family but not in the Oregon *GLC1C* family. Individuals carrying both the *MYOC* T377M variant and the *GLC1C* haplotype were more severely affected at an earlier age than individuals with just one of the POAG genes, suggesting that these two genes interact or that both contribute to the POAG phenotype in a cumulative way. (*Invest Ophthalmol Vis Sci.* 2006;47:620–625) DOI: 10.1167/iovs.05-0631

A leading cause of blindness in the industrial world, glaucoma is a disease predominantly of the elderly.[1] Primary open angle glaucoma (POAG), the most common form of this group of heterogeneous diseases, refers to the open, normal-appearing anterior chamber angle with normal trabecular meshwork in patients. The classic findings in POAG include an increased optic cup-to-disc ratio; characteristic visual field changes; and, in most cases, a high intraocular pressure with no other signs of congenital or secondary glaucoma.

Because the disease is asymptomatic and progresses slowly, diagnosis is often too late, and visual field defects are already severe. Once damage has occurred, the lost peripheral vision cannot be restored. Diagnosis at an early age is paramount, to prevent irreversible glaucomatous optic nerve atrophy by medical or surgical therapy.

Although the etiology of POAG is unknown, at least eight genetic loci are involved.[2–9] The genes for three of these loci—*GLC1A*, *GLC1E*, and *GLC1G*—have recently been identified as *MYOC* (Mendelian inheritance in Man [MIM] 601652;), *OPTN* (MIM 602432), and possibly *WDR36*, respectively.[8,10,11]

POAG is a complex disease that probably results from both genetic and environmental causes. Recent work has suggested that glaucoma may result from interactions between multiple genes within some individuals.[12–16] *MYOC* mutations have been associated with both sporadic cases of POAG and dominant hereditary glaucoma, often with juvenile onset.[10] Within some POAG families, the MYOC mutation is not present in all relatives with glaucoma.[17] This suggests that either additional genes or environmental factors lead to increased susceptibility to POAG in these families. Thus, these pedigrees add to the evidence for complex inheritance in POAG.

We identified the third locus for POAG, *GLC1C*, in a large U.S. family from Oregon.[4] Subsequently, we replicated the *GLC1C* linkage in a family from the Epirus region in Greece.[18] Because of the potential for complex inheritance in these families, we screened both families for mutations in *MYOC*. This is the first report that demonstrates the independent segregation of two genes (one with a known mutation and another mapped by conventional linkage analysis) associated with adult-onset glaucoma in a large family.

Materials and Methods

Two large *GLC1C* families, the first from Oregon in the United States and the second from Epirus, Greece, 56 random Greek patients with POAG, and 121 random U.S. patients with POAG were screened for base-pair variants in the three exons of *MYOC*. The random patients with POAG were recruited from consecutive patients in the Ophthalmology Clinic at the University of Ioannina and the Glaucoma clinic at Kaiser Permanente (Portland, OR). Informed consent was obtained from patients and family members, as approved by the Institutional Review Boards at the Institute of Child Health, Athens; Kaiser Permanente; and the Oregon Health and Science University. The study was conducted in accordance with the Declaration of Helsinki and subsequent revisions.

The clinical examination protocol included:

1. Applanation tonometry with a recently calibrated Goldmann applanation tonometer (Haag Streit AG, Bern, Switzerland). The anterior segment was examined by clinical slit lamp biomicros-

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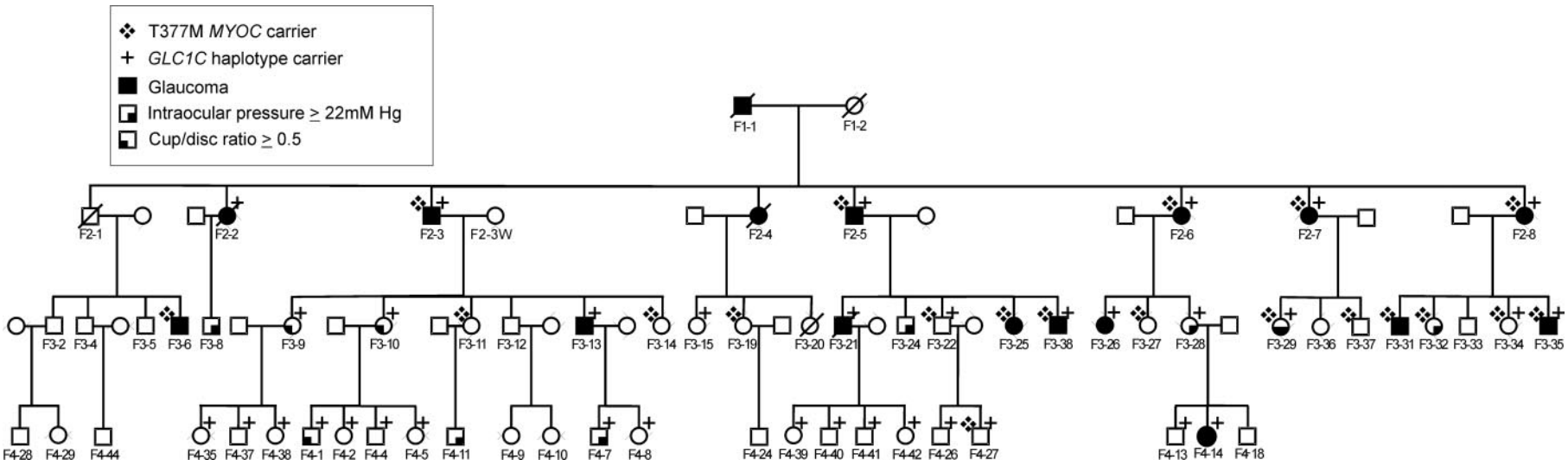

FIGURE 1. Pedigree with the *MYOC* T377M mutation and the *GLC1C* locus. Carrier status for the T377M mutation is shown with a ❖ *above* and to the *left* of the symbol. Carrier status for the *GLC1C* locus is shown with a + *above* and to the *right* of the symbol. Phenotype information is included in the pedigree: *filled symbol*: primary open-angle glaucoma; *right top quadrant half filled*: ocular hypertension; *left bottom quadrant half filled*: CDR ≥0.5; *diagonal line*: deceased. All individuals were examined, with the exception of F1-1, F1-2, F2-1 and F2-4; no DNA samples were available from these four individuals. The pedigree number for each individual who was examined and from whom a blood sample was obtained is listed *below* each symbol.

TABLE 1. Clinical Findings in Family Members with the *GLC1C* Haplotype and the T377M *MYOC* Mutation

ID	Birth Year	Age at Diagnosis	Age at Exam	Maximum IOP	Maximum CDR	Visual Field
F2-3	1909	75	85	28	0.9	SD OU
F2-5	1917	67	84	56	0.95	SD OU
F2-8	1930	54	71	43	0.9	SD OU
F2-6	1924	60	77	23	0.8	SD OU
F2-7	1927	57	74	26	0.4	MD OU
F3-38	1960	36	41	20	0.7*	NA
F3-29	1955		46	27	0.5	NA
F3-34	1964		37	20	0.4	NA
F3-35	1970		35	36	0.3	NA
F4-27	1980		21	12	0.4	NA
F3-22	1948		53	17	0.3	NA

SD, severe defect; MD, moderate defect.
* OS, shunt vessel/vein occlusion.

copy including gonioscopy. Ocular hypertension (OHT) was defined as an intraocular pressure ≥22 mm Hg.

2. Optic disc appearance was classified as normal, suspicious (vertical cup-to-disc ratio [CDR] ≥0.5), or definitely glaucomatous (CDR ≥0.7). A CDR ≥0.5 has been shown to be a risk factor for POAG in patients with OHT in the Ocular Hypertension Study.[19]
3. Venous blood was obtained for DNA extraction.

The criteria for the diagnosis of glaucoma has been described.[4] In our study, essentially, one of three criteria had to be met: (1) diagnosis of glaucoma before our study with instigation of treatment, (2) definite bilateral nasal steps on Humphrey Glaucoma Hemifield test (Carl Zeiss Meditec, Dublin, CA), or (3) two or more of the following findings: untreated IOP >24 mm Hg, characteristic optic nerve damage, and/or an abnormal Humphrey Glaucoma Hemifield Test result. Characteristic optic nerve damage may include focal neuroretinal rim thinning or a notch extending to the margin, retinal nerve fiber layer defects, disc hemorrhages, or bared circumpapillary vessels. This study has the limitation that some of the Greek family members lived in a rural setting that was fairly remote from the glaucoma clinic in Ioannina. These members were examined in their village as described earlier. Perimetry could not be performed in this location, and therefore Humphrey visual field results are not available for these individuals. Dr. Kitsos diagnosed the illness in all the living Greek family members who had POAG, and the age of diagnosis was therefore determined by his examination.

To gather further information regarding the penetrance of the three different carrier states, we analyzed the proportion of individuals known to carry the mutation who were manifesting a large CDR (≥0.5), OHT or POAG at (1) 30 years of age or older, (2) 40 years of age or older, and (3) 60 years of age. Although a CDR ≥0.5 is not diagnostic of POAG, we used this cutoff for analysis of clinical differences between the four genotype groups, because a larger CDR may represent a susceptibility factor for glaucoma.[19] The Ocular Hypertension Treatment Study (OHTS) has shown a significant association between a CDR >0.38 and the development of glaucoma when combined with baseline clinical and demographic factors.[20]

Mutation and Haplotype Analysis

Mutation analysis of the three exons of MYOC was performed with denaturing high performance liquid chromatography (dHPLC; WAVE system; Transgenomic, Omaha, NE; with WAVEMaker or Navigator software) used to design amplicons that would be optimal for identifying heteroduplex DNA using the system. DNA from 73 members of the Greek *GLC1C* family, 56 random patients with POAG from Greece, 71 members of the Oregon *GLC1C* family and 121 random patients with POAG from the glaucoma clinic at Kaiser Permanente were used for this analysis. PCR reactions contained 1.9 units of polymerase (Optimase; Transgenomic) in a 50-μL volume containing 100 ng of DNA template, 25 picomoles of each forward and reverse primer, 0.2 mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 5 μL of the 10× reaction buffer (Transgenomic) containing 1.5 mM $MgSO_4$. Touchdown PCR was performed (GeneAmp PCR System 9700; Applied Biosystems, Inc., Foster City, CA) with the annealing temperature lowered by 0.5°C for 15 cycles followed by 20 cycles at a constant annealing temperature. The touchdown and annealing temperatures were designed specifically for each PCR product based on the properties of the sequence. The denaturation and extension temperatures were kept constant at 95°C and 72°C, respectively. The final incubation for all the primer sets was at 72°C for 5 minutes. Before the PCR products were applied to the HPLC system (WAVE; Transgenomic), the samples were denatured by raising the temperature to 95°C and then lowering to 25°C by 1.5°C/min.

TABLE 2. Clinical Findings in Family Members with the T377M *MYOC* Mutation

ID	Birth Year	Age at Diagnosis	Age at Exam	Maximum IOP	Maximum CDR	Visual Field
F3-6	1949	36	55	30	0.6	MD OU
F3-25*	1957	34	34	36	0.5	MD OU
F3-31	1958	43	43	26	0.5	MD OU
F3-27	1947		57	14	0.3	NA
F3-11	1940		61	18	0.3	NA
F3-14	1949		55	17	0.2	NA
F3-19	1951		50	20	0.2	NA
F3-37	1958		43	19	0.3	NA
F3-32	1959		42	22	0.2	NA

MD, moderate defect.
* Bilateral trabeculectomy.

TABLE 3. Clinical Findings in Family Members with the *GLC1C* Haplotype

ID	Birth Year	Age at Diagnosis	Age at Exam	Maximum IOP	Maximum CDR	Visual Field
F2-2	1907	77	87	34	0.95	SD OU
F3-21	1941	46	60	30	0.7	MD OU
F3-26	1945	43	59	26	0.6	MD OU
F3-13	1947	41	54	23	0.7	MD OU
F4-14	1968	33	33	20	0.8*	NA
F3-9	1936		65	16	0.5	NA
F3-10	1938		63	17	0.5	NA
F4-1	1956		33	21	0.5	NA
F4-39	1967		34	15	0.4	NA
F3-15	1936		68	16	0.3	NA
F3-28	1948		53	22	0.3	NA
F4-35	1955		44	17	0.3	NA
F4-2	1959		45	14	0.2	NA
F4-37	1964		37	17	0.3	NA
F4-38	1964		40	13	0.3	NA
F4-4	1965		39	16	0.3	NA
F4-13	1966		35	18	0.3	NA
F4-40	1968		36	14	0.2	NA
F4-5	1968		33	16	0.2	NA
F4-41	1970		34	12	0.2	NA
F4-42	1971		33	14	0.2	NA
F4-26	1973		28	13	0.3	NA
F4-7	1974		27	23	0.3	NA
F4-8	1977		24	17	0.3	NA

SD, severe defect; MD, moderate defect.
* Disc thin temporally.

dHPLC Analysis. The PCR amplicons were run individually on the system (WAVE; Transgenomic) using 5 μL of each sample. A linear gradient of 5% triethylammonium acetate (TEAA; buffer A) and 25% acetonitrile+5% TEAA (buffer B) was used to elute the sample at a flow rate of 0.9 mL/min. All heteroduplex amplicons were sequenced at the Portland VA Medical Center core facility to identify the specific *MYOC* base-pair change.

Statistical Analysis. We used the Kruskal-Wallis test for the multiple group comparisons and the Mann-Whitney test and the Fisher exact test for the pair-wise comparisons. All computations were performed in R statistical language.[21]

TABLE 4. Clinical Findings in Family Members with Neither the *GLC1C* haplotype Nor the T377M *MYOC* Mutation

ID	Birth Year	Age at Exam	Maximum IOP	Maximum CDR
F3-2	1933	68	13	0.1
F3-4	1939	62	15	0.2
F3-8	1940	61	22	0.2
F3-5	1943	61	16	0.4
F3-12	1945	56	21	0.1
F3-24	1953	48	25	0.3
F3-20	1957	47	15	0.3
F3-36*	1957	44	17	0.3
F3-33	1962	39	14	0.1
F4-11†	1966	35	24	0.1
F4-28	1966	35	21	0.3
F4-9	1974	27	19	0.1
F4-24	1974	27	14	0.2
F4-18	1974	27	20	0.2
F4-10	1977	24	18	0.1
F4-29	1979	22	18	0.3
F4-44	1980	24	15	0.2

Visual field test results are not available for any of these individuals.
* OS nerve fiber layer hemorrhage.
† Extensive nerve fiber mylination.

TABLE 5. Comparison of the Maximum IOP between the Four Genotype Groups

Group	1	2	3	4
1	—	NS	0.009	0.009
2		—	NS	NS
3			—	NS
4				—

Data are probabilities.

RESULTS

We screened for base-pair variations in the three exons of the *MYOC* gene in two large POAG families, previously mapped to the *GLC1C* locus, and a random POAG population consisting of 56 Greek and 112 U.S. patients with POAG.[4,18] The Oregon *GLC1C* family had no disease-causing variants in the MYOC gene. A K398R variant was present in two unrelated spouses and one of their children. All three had normal ocular findings. Two individuals had the Y347Y polymorphism.

An *MYOC* T377M mutation was found in 20 of the 73 family members of the Greek family (Fig. 1). Ten of the 15 affected individuals in the family had the T377M variant, but none of the random patients with POAG possessed this mutation. Additional *MYOC* variants in the Greek family included D380H in one unaffected spouse (F2-3W) and Y347Y in two spouses and several of their children. None of F2-3W's children inherited the D380H variant.

Although 73 family members of the Greek *GLC1C* POAG family were examined, only the 61 individuals 20 years and older are reported, because all the younger family members had normal ocular findings. One family member had juvenile glaucoma (F3-25), all other affected family members had adult-onset POAG. Analysis of the pedigree reveals that 11 individuals have both the T377M mutation and the *GLC1C* haplotype (referred to below as group 1), 9 family members carried only the T377M mutation (group 2), 24 have just the *GLC1C* haplotype (group 3), and 17 individuals had neither the *MYOC* mutation nor the *GLC1C* haplotype (group 4).

Information on the age at diagnosis and examination, maximum recorded IOP and CDR for the family members is shown grouped by genotype in Tables 1, 2, 3 and 4. No significant difference in age at diagnosis or examination was found between the four groups by the Kruskal-Wallis test. Maximum IOPs were significantly higher in group 1 than in groups 3 and 4, by Mann-Whitney test ($P = 0.009$ for both), but not significantly different from group 2 (Table 5). The maximum IOPs were not significantly different between groups 2, 3, and 4. The CDR was also significantly greater in group 1 than in the three other groups ($P = 0.025$, 0.020, and 0.000 in groups 2, 3, and 4, respectively), as shown in Table 6. The maximum CDR in groups 2 and 3 were significantly higher than in group

TABLE 6. Comparison of the Maximum CDR between the Four Genotype Groups

Group	1	2	3	4
1	—	0.025	0.020	0.000
2		—	NS	0.029
3			—	0.001
4				—

Data are probabilities.

TABLE 7. Penetrance of Clinical Characteristics of POAG

Age (y)	Interaction (T377M + GLC1C)		T377M		GLC1C		Normal (wild type)
	n	*P*	*n*	*P*	*n*	*P*	*n*
≥30	7/9	0.07	4/9	0.64	9/21	0.46	3/11
≥40	7/8	0.04	4/9	0.62	8/13	0.18	2/8
≥60	5/5	0.04	0/1	1.00	3/4	0.49	1/4

n, number of individuals with CDR ≥ 0.5 and/or OHT/total number of persons in age group; *P* (two-sided) by the Fisher exact test for each group against the normal group.

4 ($P = 0.020$ and 0.001, respectively) No significant difference was found between groups 2 and 3. One person, F3-25, who had the T377M *MYOC* mutation but not the *GLC1C* haplotype, required trabeculectomy. None of the other affected family members had had surgical intervention.

Variable expressivity was observed in all three groups of mutation carriers. In those family members with both the *GLC1C* haplotype and T377M mutation, the age of the youngest affected member with ocular hypertension was 21 and the oldest unaffected individual was 53. In the family members with just the T377M mutation, the youngest affected member was 43 at diagnosis, and the oldest unaffected member was 61. In the *GLC1C* group, the earliest age of diagnosis was 33, and the oldest unaffected member was 68 years of age. As shown in Table 7, the penetrance of OHT and/or a large CDR was higher at age 40 and older in individuals carrying both mutations compared with individuals with just the *MYOC* or the *GLC1C* mutation.

To determine the incidence of *MYOC* mutations in the general Epirus POAG population, 56 random patients with POAG from the University of Ioannina Ophthalmology Clinic were screened. Two polymorphisms were found, Y347Y in four patients and Y647Y in one patient for an incidence of 7.1% and 1.8%, respectively. The T377M mutation was not found in this sample.

DISCUSSION

We originally mapped the locus for *GLC1C* on chromosome 3 in two large POAG families independently.[4,18] We have extended both families and re-examined individuals who may have developed POAG in the years since the original publications, to facilitate identification of the *GLC1C* gene. In the Oregon *GLC1C* family, two additional family members received the diagnosis of POAG. Both carried the *GLC1C* disease haplotype, and one had a recombination that allowed us to refine the region from 11 to 4 cM.[22] In the Greek *GLC1C* family, six additional family members have received definite diagnoses of POAG since the original report. These include three individuals who carried the *GLC1C* haplotype (F3-21, F3-35, and F4-14), and three others who did not (F3-6, F3-31, and F3-25, who had juvenile-onset POAG).

We recently screened the Greek POAG family and 56 random Greek patients with POAG for *MYOC* base-pair variants and identified a T377M mutation in the family. The 15 living affected family members have all been examined by GK. Ten of the 15 POAG individuals carry the T377M variant. It appears that the *GLC1C* gene on chromosome 3 and the MYOC mutation on chromosome 1 segregate independently in this family.

Relatives with both the *GLC1C* haplotype and T377M mutation were more severely affected as a group than those individuals with one mutant POAG gene. Maximum-recorded IOPs were significantly higher in individuals with both mutant genes compared to family members with just the *GLC1C* haplotype but not compared with those with the *MYOC* T377M mutation. Thus, *MYOC* may be more fundamentally involved in regulating IOP than is the *GLC1C* gene. However, CDRs were significantly higher in the group with both the *GLC1C* gene and the T377M variant compared with those with just the T377M mutation or the *GLC1C* gene. This suggests that the *GLC1C* gene and *MYOC* may interact synergistically.

Screening of the Oregon *GLC1C* family revealed no disease-causing *MYOC* mutations, although two polymorphisms, K398R and Y347Y, were identified in four individuals, all of whom had IOPs <22 mm Hg and normal-appearing optic nerves. This clearly suggests that, although the *GLC1C* gene and *MYOC* may interact to cause POAG in some cases, *GLC1C* also appears to act on its own to cause glaucoma.

Segregation of *MYOC* variants is not always concordant with disease within families, suggesting complex inheritance of the POAG phenotype.[23–25] Consistent with this, in the Greek *GLC1C* family, the *MYOC* T377M variant does not segregate cleanly with glaucoma; five of the 15 affected individuals do not carry this variant. This explains why the *GLC1A* locus had been excluded in this pedigree.[26] *MYOC* T377M mutations have also been reported in four Australian families, two U.S. families, one Indian family, a Finnish family, and in a Moroccan individual.[13,23,24,27–30] Consistent with our findings, the T377M variant did not show complete segregation with POAG in many of these families, although some of them are too small to evaluate. This suggests that the T377M variant is a susceptibility factor for POAG, rather than a causal gene.

Penetrance

Existence of variation in age-dependent penetrance for the *MYOC* T377M mutation is shown in the literature. *MYOC* T377M mutations have been reported in four Australian families, originating from Greece, the former Yugoslav Republic of Macedonia, and Great Britain.[24] Not all affected family members carried the T377M variant, which is consistent with our findings. The penetrance of OHT/POAG in the Australian families was 90% at 40 years or more, which is similar to the penetrance of 88% in the carriers of both the *GLC1C* and T377M variant in this report. However, in our study, those individuals with only the T377M mutation had a much lower penetrance of 44% at 40 years or older. This is similar to the Finnish study in which the penetrance of the T377M mutation was 40% at ages 36 to 50 years and 45% at >50 years. The difference in age-related penetrance in these families suggests that there may be an additional POAG susceptibility or modifier gene(s) segregating through these families.

General Greek POAG Population

The finding of four families originating from a common geographical area sharing the T377M mutation raised the question of whether this *MYOC* variant is common in this region. Screening of 56 random patients with POAG from the Oph-

thalmology Clinic at the University of Ioannina in Epirus revealed no *MYOC* T377M in any of the individuals. Therefore, the T377M mutation found in the *GLC1C* family may be a rare mutation in the general Greek POAG population. We are currently screening additional Greek patients with POAG to determine the incidence of the T377M variant in a larger population.

POAG is a complex disease commonly arising from the interaction of two or more genes and/or the environment.[31] Segregation of more than one gene through a pedigree has been shown for the *CYP1B1* and *MYOC* genes in families with early-onset glaucoma.[15,16] In this study, the *MYOC* and *GLC1C* genes appeared to act synergistically, as family members with both genes were more severely affected and the disease was diagnosed at an earlier age. The finding of two POAG genes, previously mapped independently as Mendelian traits, occurring in one large glaucoma family adds further support to the idea that the variable POAG phenotype, even within the same family, results from the interaction of multiple genetic and environmental factors.

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