# Age-dependent Prevalence of Mutations at the *GLC1A* Locus in Primary Open-angle Glaucoma

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• PURPOSE: To screen a population with primary open-angle glaucoma for mutations in the gene that encodes the trabecular meshwork inducible glucocorticoid response protein (TIGR), also known as myocilin (MYOC).
• METHODS: Ophthalmologic information was collected for study subjects with primary open-angle glaucoma and their relatives. Mutation screening of 74 primary open-angle glaucoma probands was conducted by sequencing TIGR/MYOC coding sequence and splice sites.
• RESULTS: In 23 families we detected 13 nonsynonymous sequence changes, nine of which appear to be mutations likely to cause or contribute to primary open-angle glaucoma. Two mutations, Arg272Gly and Ile499Ser, and one nonsynonymous sequence variant, Asn57Asp, are novel. We found mutations in nine of 25 juvenile glaucoma probands (36%) and two of 49 adult-onset glaucoma probands (4%). Age classification of families rather than individual probands revealed mutations in three of nine families with strictly juvenile primary open-angle glaucoma (33%), and no mutations in 39 families with strictly adult-onset primary open-angle glaucoma (0%). In families with mixed-onset primary open-angle glaucoma containing both juvenile primary open-angle glaucoma and adult-onset primary open-angle glaucoma cases, we found mutations in eight of 26 families (31%).
• CONCLUSIONS: Our data suggest that Gly252Arg, Arg272Gly, Glu323Lys, Gln368STOP, Pro370Leu, Thr377Met, Val426Phe, Ile477Asn, and Ile499Ser are likely to play roles that cause or contribute to the etiology of autosomal dominant primary open-angle glaucoma. Our finding of more TIGR/MYOC mutations in families with mixed-onset primary open-angle glaucoma than in the families with strictly adult-onset primary open-angle glaucoma implies that the presence of relatives with juvenile primary open-angle glaucoma in a family could be used as a basis for identifying a subset of the population with adult-onset primary open-angle glaucoma with higher prevalence of TIGR/MYOC mutations. To address this issue, and to refine estimations of mutation prevalence in these age-defined subpopulations, prospective study of a larger population ascertained entirely through adult-onset primary open-angle glaucoma probands will be needed. (Am J Ophthalmol 2000;130: 165–177. © 2000 by Elsevier Science Inc. All rights reserved.)

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PRIMARY OPEN-ANGLE GLAUCOMA IS ONE OF THE leading causes of blindness in the world. Primary open-angle glaucoma is characterized by a normal-appearing anterior chamber angle and recognizable damage

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to the optic nerve resulting in a characteristic pattern of visual field loss. It is commonly associated with elevation of intraocular pressure. Primary open-angle glaucoma can be subclassified on the basis of the age at which the disease is diagnosed. The most common form of the disease, adult-onset primary open-angle glaucoma, manifests in middle age or later. Juvenile glaucoma is a relatively rare form of primary open-angle glaucoma that occurs in children and young adults. The exact age of the cut off between adult-onset and juvenile-onset disease, which varies from one study to the next, usually falls between 35 and 40 years of age.[1–3]

In 1993, studies of a family with primarily juvenile-onset primary open-angle glaucoma by Johnson and associates[4] and Sheffield and associates[5] led to the mapping of the first primary open-angle glaucoma locus (*GLC1A*) to chromosomal region 1q21–q31. Linkage of juvenile primary open-angle glaucoma to *GLC1A* was confirmed, and the candidate region was narrowed by studies of additional families.[6–12] Further studies showed that the *GLC1A* locus is responsible not only for juvenile primary open-angle glaucoma but also for some cases of adult-onset primary open-angle glaucoma.[11,12] In subsequent studies of *GLC1A*-linked glaucoma, Stone and associates[13–15] found both individuals with juvenile primary open-angle glaucoma and individuals with adult-onset primary open-angle glaucoma with mutations in the gene that encodes the trabecular meshwork inducible glucocorticoid response protein,[16,17] which is also called myocilin.[18] The TIGR/MYOC gene consists of three exons that encode a 504 amino acid protein with an olfactomedin-like domain and a leucine zipper motif hypothesized to mediate protein–protein interactions.[17–19] The majority of sequence changes believed to be likely to cause disease have been localized within the olfactomedin-like domain encoded within the third exon, although apparently benign missense substitutions in both primary open-angle glaucoma and normal populations have been reported throughout the gene.[13–15]

The role of the TIGR/MYOC gene in primary open-angle glaucoma is still under investigation. Stone and associates[13] provided a preliminary estimate of TIGR/MYOC mutation prevalence in patients with familial primary open-angle glaucoma and in unselected patients with primary open-angle glaucoma, at 4.4% and 2.9%, respectively. Other studies have reported mutation frequencies of 3.1%,[3] 3.8%,[14] 4%,[20] and 2.6% to −4.3%.[15] TIGR/MYOC gene mutations have been detected in individuals with adult-onset primary open-angle glaucoma and in families and individuals with juvenile primary open-angle glaucoma.[13–15,20–32] It has been suggested that TIGR/MYOC mutations may be more frequent in familial juvenile primary open-angle glaucoma cases[3,15] and less frequent in sporadic cases,[27] but neither population-based nor family-based studies have indicated whether there might be an identifiable subset of adult-onset primary open-angle glaucoma cases with an increased prevalence of TIGR/MYOC mutations.

We sequenced the TIGR/MYOC gene coding sequences and splice sites to evaluate the prevalence of mutations in 74 unrelated primary open-angle glaucoma cases. We evaluated information on the 74 primary open-angle glaucoma cases and their relatives to determine TIGR/MYOC mutation frequencies in primary open-angle glaucoma population subsets classified according to age at diagnosis of probands or according to information on age at diagnosis of all known affected family members. We present two novel TIGR/MYOC mutations and new information on other previously reported mutations, including information on age-dependent differences in mutation prevalence.

## MATERIALS AND METHODS

INFORMED CONSENT WAS OBTAINED ACCORDING TO A study protocol approved by the University of Michigan Internal Review Board for Human Subjects Studies. Study subjects included 74 unrelated individuals with primary open-angle glaucoma, with age at diagnosis ranging from 12 to 81 years of age (mean, 43 years), and 60 controls ranging in age from 24 to 83 years old (mean, 54 years). The 74 unrelated individuals with primary open-angle glaucoma included 60 Caucasians (81%), 11 African Americans (15%), three Hispanics (4%), and one individual of unknown ancestry (1%). The 60 unrelated normal controls included 43 Caucasians (70%), 11 African Americans (18%), four Hispanics (7%), and one individual of unknown ancestry (1%). Thus, we consider the racial/ethnic composition of the primary open-angle glaucoma and control populations to be comparable but not identical. The age profiles have been selected so that the control population age exceeds the age at diagnosis for the experimental group.

Ophthalmologic examinations included slit-lamp biomicroscopy, optic disk examination, intraocular pressure measurement by applanation, and refraction. Examination of individuals with elevated intraocular pressure and/or glaucomatous optic disk cupping also included gonioscopy. Visual field assessment information was available for all 74 probands. Sixty-one of the 74 individuals were examined by us, whereas diagnosis of the remaining 13 individuals relied on information from medical records from outside ophthalmologists. Information on relatives of the 74 individuals came from a combination of examination, medical records, and family- and self-report. For this study, 24 probands were classified as being affected based on the presence of normal filtration angles, glaucomatous optic disks, glaucomatous visual field changes, and either maximum known intraocular pressure 22 mm Hg or greater or maximum known intraocular pressure 19 mm Hg or greater while on two or more glaucoma medications. The remaining 50 probands were classified as affected with

primary open-angle glaucoma based on clinical findings of a normal filtration angle and an intraocular pressure 30 mm Hg or greater, with the diagnosis confirmed by information indicating glaucomatous optic disk and/or visual field changes for 47 subjects. Five of these cases, and their TIGR/MYOC mutations, are familial cases that we have recently reported.[21,22] Of the remaining 69 cases, eight were sporadic and the rest familial. Because these cases were not sequentially collected, these numbers are not indicative of the proportion of familial or sporadic cases in our clinic population. Information on age at diagnosis for probands and family members came from multiple sources: our examinations, medical records, self-report, and family report.

Two different schemes for classification of glaucoma were used in this study: classification of probands based on their own age at diagnosis and classification of families based on the range of age at diagnosis of all available family members (Table 1). Individuals were classified as having juvenile primary open-angle glaucoma if their own case of primary open-angle glaucoma was diagnosed before 35 years of age, and adult-onset primary open-angle glaucoma if their own case of primary open-angle glaucoma was diagnosed at 35 years of age or later. The 74 families were classified into three groups based on classification of the entire family rather than the classification of the individual proband: juvenile primary open-angle glaucoma if information from all sources, including family-report, indicated that all affected family members were diagnosed before age 35 years; adult-onset primary open-angle glaucoma if information from all sources indicated that all known affected cases were diagnosed at age 35 years or later; and mixed-onset primary open-angle glaucoma if information from all sources indicated that both juvenile primary open-angle glaucoma and adult-onset primary open-angle glaucoma cases were present in the same family. Ten of 26 of the families with mixed-onset primary open-angle glaucoma had probands in whom glaucoma was diagnosed on or after age 35. For individuals with sporadic primary open-angle glaucoma and others with family history but no family age information, diagnosis before age 35 years and diagnosis at or after age 35 resulted in classification as juvenile primary open-angle glaucoma and adult-onset primary open-angle glaucoma, respectively.

One affected individual from each of the 74 families was screened for mutations in all three exons of the TIGR/MYOC gene plus the flanking splice sites. Genomic DNA was extracted from whole blood using a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, Minnesota) according to the manufacturer's protocol. Exons of the TIGR/MYOC gene were amplified by polymerase chain reaction using an AmpliTaq Gold polymerase chain reaction kit (PE Applied Biosystems, Foster City, California) in a 50 μl reaction mixture containing 100 ηg of genomic DNA. Amplifications of exon 1 and exon 2 were carried

**TABLE 1.** Proband and Family Data for Apparently Pathogenic TIGR/MYOC Mutations

| Mutation | Proband Data | | | | | Family Data | | | Population Frequencies | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Proband Type | Family Type | Age at dx (years) | Max IOP (mm Hg) | Racial/Ethnic | Family Number | Age at dx (years) Mean (range) | IOP (mm Hg) Mean (range) | Fraction of POAGs in Family With Mutation | Fraction of POAG With Mutations | Fraction of Normal Controls With Mutations | Other Reports of the Mutation |
| Gly252Arg | J | J | 26 | 62 | Cauc | UM:JG5 | 26 | 62 | 1/1 | 1/74 | 0/43 | 22 |
| Arg272Gly | J | M | 32 | 26 | Cauc | UM:JG10 | 33 (29–45) | 62 (60–65) | 4/4 (9/9*) | 1/74 | 0/60 | |
| Glu323Lys | J | M | 19 | "very high" | Hisp/Ind | UM:GL57 | 19 (9–43) | 43 (23–59) | 11/11 | 1/74 | 0/43 | 22 |
| Gln368Stop | A | M | 37 | 31 | Cauc | UM:GL9 | 36 (28–49) | 37 (26–57) | 2/2 | | | |
| | A | M | 41 | 38 | Cauc | UM:GL92 | 38 (34–41) | 36 (34–38) | 6/6 | 2/74 | 0/60 | 2,3,14,15 |
| Pro370Leu | J | J | 16 | 44 | Cauc | UM:JG1 | 12 (5–27) | 45 (25–66) | 15/15 | 1/74 | 0/43 | 2,3,20,21,24,28 |
| Thr377Met | J | M | 34 | 40 | Cauc | UM:JG7 | 38 (34–44) | 44 (40–51) | 3/3 | 1/74 | 0/60 | 3,14,15 |
| Val426Phe | J | M | 16 | 42 | Cauc | UM:JG3 | 26 (16–46) | 43 (32–52) | 12/12 | 1/74 | 0/60 | 22,27 |
| | J | M | 21 | 35 | Cauc | UM:GL355 | 16 (12–"adult") | 28 | 1/1 | | | |
| Ile477Asn | J | M | 18 | 44 | Cauc | UM:JG2 | 26 (4–80) | 43 (35–52) | 16/16 | 1/74 | 0/43 | 14,15,21,22 |
| Il499Ser | J | J | 31 | 29 | Cauc | UM:GL49 | 28 (25–31) | 16 (16–17) | 2/2 | 1/74 | 0/60 | |

A = adult-onset primary open-angle glaucoma; Cauc = Caucasian; dx = diagnosis; Hisp = Hispanic; Ind = Guaymies Indian; IOP = intraocular pressure; J = juvenile-onset primary open-angle glaucoma; M = mixed-onset primary open-angle glaucoma; POAG = primary open-angle glaucoma.

*= Includes genotypes inferred based on model of identity by descent.

out in a 25-μl reaction volume containing 60 ηg of genomic DNA. Polymerase chain reaction conditions were as follows: 10 minutes at 95C followed by 30 to 35 cycles of [45 seconds at 95C, 1 minute at 66C, 1 minute at 72C], and final extension for 10 minutes at 72C. Polymerase chain reaction products were purified with a QIAquick polymerase chain reaction purification kit (Qiagen, Santa Clarita, California). Nucleotide sequences were determined by direct sequencing of both strands of the polymerase chain reaction product with a DNA sequencing kit and an ABI377 DNA sequencer (dRhodamine or Big Dye Cycle Sequencing Ready Reaction; PE Applied Biosystems, Foster City, California) using one of the two polymerase chain reaction primers or one of two internal primers, some of which have been previously published. For exon 1, primers used in polymerase chain reaction amplification and sequencing were 5′-GGC TGG CTC CCC AGT ATA TA-3′, 5′-CTG CTG AAC TCA GAG TCC CC-3′, 5′-AAT TGA CCT TGG ACC AGG-3′, and 5′-CTC CAG AAC TGA CTT GTC TC-3′. For exon 2, primers used in polymerase chain reaction amplification and sequencing were 5′-ACA TAG TCA ATC CTT GGG CC-3′ and 5′-TAA AGA CCA TGT GGG CAC A-3′. For exon 3, primers used in polymerase chain reaction amplification and sequencing were 5′-CTG GCT CTG CCA AGC TTC CGC ATG A-3′, 5′-GGC TGG CTC TCC CCT CAG CCT GCT-3′, 5′-GAG GCC TGC TTC ATC CAC AGC CAA G-3′, 5′-GAG GCC TGC TTC ATC CAC AGC CAA G-3′ and 5′-GAG CTG AAT ACC GAG ACA GTG AAG GC-3′.

Allele-specific oligonucleotide hybridization was used to determine whether additional members of TIGR/MYOC sequence variant families had the sequence variant found in the proband of that family. To evaluate prevalence of the detected sequence variants in the normal population, 60 unrelated normal control individuals were screened by allele-specific oligonucleotide hybridization for the presence of sequence variants Asn57Asp, Arg272Gly, Val329Met, Gln368Stop, Thr377Met, Lys398Arg, Val426Ser, and Ile499Ser. For each of these mutations, a pair of oligonucleotide hybridization probes was constructed with the central nucleotide containing either the altered or the normal base (Table 2). DNA fragments for allele-specific oligonucleotide hybridization were generated by polymerase chain reaction amplification of TIGR/MYOC genomic DNA. Allele-specific oligonucleotide hybridization screening for each sequence variant included polymerase chain reaction fragments from the 60 normal controls, positive controls known to contain the sequence change, and all recruited affected or at-risk members of UM:GL49, UM:GL355, UM:JG10, UM:GL9, UM:GL92, UM:GL80, and UM:GL145, as described above (Figures 1 and 2). For amino acid changes Val426Phe and Gln368Stop, only the members of the UM:GL355, UM:GL92, and UM:GL9 families were screened by allele-specific oligonucleotide hybridization, because normal control and general population data have already been reported elsewhere.[2,3,14,15,26] Allele-specific oligonucleotide hybridization results for screening of Gly252Arg, Glu323Lys, Pro370Leu, and Ile477Asn were previously reported by us,[21,22] and population values for Arg76Lys have been reported previously by Alward and associates[14] and Fingert and associates.[15] No additional affected family members were available to screen for Asn57Asp.

**TABLE 2.** Oligonucleotides Used to Assay for Normal and Variant Alleles of the TIGR/MYOC Gene

| Sequence Variant | Oligonucleotide Sequence |
|---|---|
| Asn57 | 5′-GCC AGT CCC AAT GAA TCC A-3′ |
| Asp57 | 5′-GCC AGT CCC GAT GAA TCC A-3′ |
| Arg272 | 5′-GTG TGG ATG CGA GAC CCC A-3′ |
| Gly272 | 5′-GTG TGG ATG GGA GAC CCC A-3′ |
| Val329 | 5′-GGT GCT GTG GTG TAC TCG G-3′ |
| Met329 | 5′-GGT GCT GTG ATG TAC TCG G-3′ |
| Gln368 | 5′-TAC CAC GGA CAG TTC CCG T-3′ |
| STOP368 | 5′-TAC CAC GGA TAG TTC CCG T-3′ |
| Lys398 | 5′-ATG AGG CCA AAG GTG CCA T-3′ |
| Arg398 | 5′-ATG AGG CCA GAG GTG CCA T-3′ |
| Val426 | 5′-AAG CAG TCA GTC GCC AAT G-3′ |
| Phe426 | 5′-AAG CAG TCA TTC GCC AAT G-3′ |
| Ile499 | 5′-CTT ATG ACA TCA AGC TCT C-3′ |
| Ser499 | 5′-CTT ATG ACA GCA AGC TCT C-3′ |

The Thr377Met mutation in UM:JG7 creates a NlaIII restriction site that can be assayed by polymerase chain reaction followed by cutting with a restriction endonuclease (polymerase chain reaction-RFLP). The 298 base-pair (bp) fragments that contain the mutation site of UM:JG7 were amplified by polymerase chain reaction with primers 5′-GAG CTG AAT ACC GAG ACA GTG AAG GC-3′ and 5′-TCT GCT GAG GTG TAG CTG CTG ACG G-3′. Amplification was carried out using genomic DNA of UM:JG7 family members. Digested polymerase chain reaction products were electrophoresed in 1% agarose gel to distinguish between the 298 bp–fragment length of the normal sequence and the pair of bands at 90 bp and 206 bp generated when the fragment carrying the mutation is cut with NlaIII. Polymerase chain reaction-restriction fragment length polymorphism assays to detect a new AlwNI site generated by the Pro370Leu mutations and a BsrI site destroyed by Glu323Lys were previously reported.[21]

To determine whether a founder effect could account for the presence of the same mutation in families UM:JG3 and UM:GL355, short tandem repeat polymorphisms D1S433, D1S452, D1S210, D1S218, and D1S215 were tested. Relative positions and spacing of these markers and the TIGR/MYOC gene were described previously.[5–7,33,34] DNA was amplified by polymerase chain reaction from genomic DNA obtained from all available affected or at-risk family

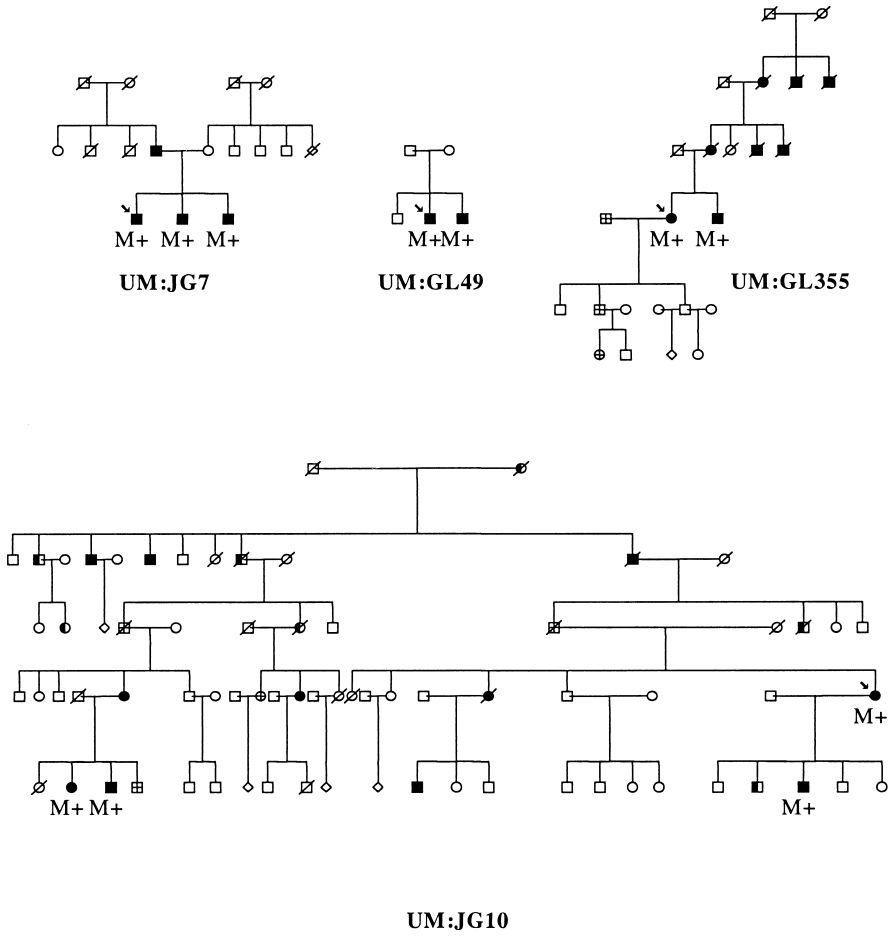


**FIGURE 1. Pedigrees of families with missense mutations. M+ marks the presence of both the normal sequence and the variant sequence found for that family as listed in Table 1. Genotyping is not presented for individuals who were unaffected or had other phenotypes, such as ocular hypertension. The youngest generation in UM:JG10, in which there are no known affected individuals, is not shown here. Phenotypic assignment of individuals who were genotyped is based on examination and medical records. Some information on individuals not screened may also come from self-report and family-report. Genotyping and pedigrees for some families not shown in this figure were previously reported by Rozsa and associates[21] and by Richards and associates[22] and are not presented here. Families with missense sequence variants deemed unlikely to cause disease are not shown here.**

members in UM:GL355 and UM:JG3 using 32P-end-labeled primers, as previously described.[8,22] Polymerase chain reaction products were analyzed by size fractionation on 6% denaturing acrylamide gels, followed by autoradiography of the dried gel.

To determine if these mutations cause any changes in predicted structure of trabecular meshwork inducible glucocorticoid response/myocilin protein, the protein sequences for normal trabecular meshwork inducible glucocorticoid response/myocilin and its sequence variants were analyzed with the Genetyx-Mac 9.0 program (Software Development, Ltd, Tokyo, Japan) using the predictive algorithms of Chou and Fasman[35] and Garnier and associates.[36,37] Protein motifs in trabecular meshwork inducible glucocorticoid response/myocilin were identified from the Blocks,[38] Prints,[39] and Prosite[40] databases, as described previously.[21] Predicted protein structure change caused by the mutations and protein motifs identified near the mutation sites are listed in Table 3.

Eleven of the TIGR/MYOC nonsynonymous sequence variants identified in this study were evaluated by a previously reported Triton X-100 solubility assay that can distinguish known pathogenic mutations, which produce insoluble trabecular meshwork inducible glucocorticoid response/myocilin protein, from apparently benign sequence variants, which produce soluble trabecular meshwork inducible glucocorticoid response/myocilin protein (Table 3, Figure 3).[41] Site-directed mutagenesis was employed to create TIGR/MYOC cDNA sequence variant constructs tagged with the FLAG epitope.[42] Constructs were introduced into human embryonic kidney 293T cells[43] by transient transfection, and Triton solubility of the expressed variant proteins was assessed by detergent extraction and immunoblot, as previously described.[41]

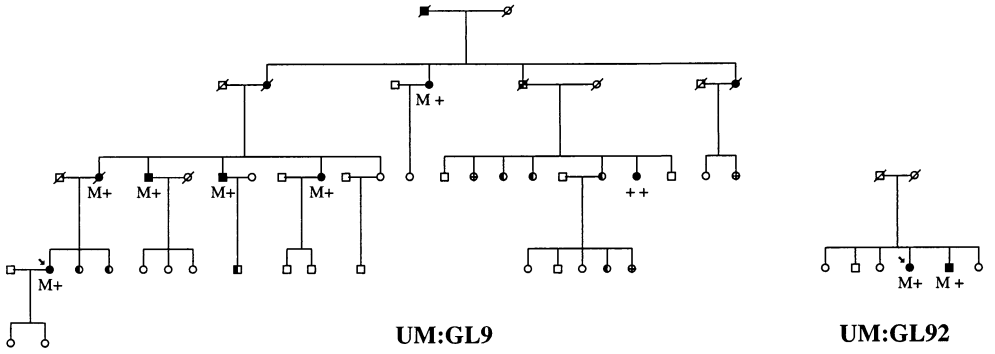


**FIGURE 2. Pedigrees of families with the Gln368STOP mutation. M+ marks the Gln368STOP sequence in one copy of the TIGR/MYOC gene. No one was found to carry the Gln368 STOP mutation on both copies of the gene. Genotyping is not presented for individuals who were unaffected or had other phenotypes, such as ocular hypertension. Phenotypic assignment of individuals who were genotyped is based on examination and medical records. Some information on individuals not screened may also come from self-report and family report.**

## RESULTS

SEQUENCING OF THE COMPLETE TIGR/MYOC GENE REvealed a total of 13 nonsynonymous sequence variants in 25 of the 74 families with primary open-angle glaucoma screened (Table 1). To the best of our knowledge, three of the nonsynonymous sequence variants presented here (Asn57Asp, Arg272Gly, and Ile499Ser) and two of the five nonsynonymous sequence variants presented in our previous reports[21,22] (Gly252Arg and Glu323Lys) are novel. The remaining eight nonsynonymous sequence variants have also been observed by other research groups[2,3,14,15,23–27]

Five previously reported synonymous DNA sequence changes that do not alter the amino acid sequence of the trabecular meshwork inducible glucocorticoid response/myocilin protein were detected in eight of the 74 families with primary open-angle glaucoma (Pro13Pro in UM:GL7 and UM:GL 270, Leu159Leu in UM:GL7 and UM:GL270, Thr204Thr in UM:GL136, Thr325Thr in UM:GL7 and UM:GL69, Leu159Leu in UM:GL7, Tyr347Tyr in UM:GL43 and UM:GL188, and Thr204Thr in UM:GL136).[13–15,21] It is interesting to note that two of 11 African-American families in this study each have the three synonymous polymorphisms, Pro13Pro, Leu159Leu, and Thr325Thr, and that no one else in the study has any of those three polymorphisms. Pro13Pro is homozygous in the proband of one family. Allele-specific oligonucleotide hybridization and polymerase chain reaction-RFLP screening of relatives with glaucoma-related phenotypes identified relatives with their family's sequence variant, as shown in Figures 1 and 2. Although screening results are not shown for unaffected individuals, some of them also have their family's sequence variant.

Normal controls were screened for the presence of the nonsynonymous sequence variants. Gly252Arg, Glu323Lys, Pro370Leu, and Ile477Asn were not present among 43 normal controls previously described,[21,22] and Asn57Asp, Arg272Gly, Val329Met, Gln368Stop, Thr377Met, Val426Phe, and Ile499Ser were not present in a panel of 60 normal controls ranging in age from 24 to 83 years old (mean, 54 years) screened as part of the present project. Lys398Arg was present once in the panel of 60 normal controls. Controls were not screened for Arg76Lys, because Alward and associates[14] and Fingert and associates[15] have previously provided evidence of Arg76Lys presence in more than 6% of their controls. In other reports, Pro370Leu, Thr377Met, Val426Phe, and Ile477Asn were also found to be missing from control populations.[13–15,24–26] Fingert and associates[15] found Lys398Arg in 20 of 1703 primary open-angle glaucoma cases (1.2%) and seven of 793 controls (0.9%), and they found Val329Met in two of 312 African-American primary open-angle glaucoma cases (0.6%), one of 50 African-American general population controls (2%), and none of their Caucasian or Japanese primary open-angle glaucoma cases or controls.

Data on cosegregation and population frequencies suggest that seven nonsynonymous sequence variants are mutant alleles that are likely to cause or contribute to primary open-angle glaucoma. Our data on Arg272Gly, Glu323Lys, Pro370Leu, Val426Phe, and Ile477Asn show significant evidence of cosegregation with primary open-angle glaucoma, indicating that they are mutant alleles (Figure 1, Table 1). Combining our moderate evidence for cosegregation of primary open-angle glaucoma with Gln368STOP and Thr377Met with cosegregation data from other studies[1,3,14] results in highly significant evidence that these are also mutant alleles (Figure 1, Table 1).

Our finding of Lys398Arg in one of 74 primary open-angle glaucoma cases (1.4%) and one of 60 controls (1.7%) is consistent with a previous report that Lys398Arg is unlikely to cause glaucoma (Table 4).[14,15] Of the four other sequence variants found in families in which few

**TABLE 3.** Predicted and Assayed Protein Alterations for 13 TIGR/MYOC Nonsynonymous Sequence Variants

| Protein Change | DNA Change | Charge Change | Triton Solubility Assay | Hypothetical Motifs and Structural Changes |
|---|---|---|---|---|
| Asn57Asp | AAT→GAT | U→− | Not done | Eliminates the Asn to which N-linked oligosaccharide attachment is predicted based on presence of the motif Asn-Glu-Ser. |
| Arg76Gly | AGA→AAA | +→U | Not done | Interjected turn (CF) and no motifs altered. |
| Gly252Arg | GGA→AGA | U→+ | Insoluble | *B*-strand→*a*-helix (CF) across conserved PCK motif also hit by Thr256Met. GOR predicts interjection of an additional turn at this PKC motif. |
| Arg272Gly | CGA→GGA | +→U | Insoluble | No significant structural change (CF, GOR) and no motifs altered. |
| Glu323Lys | GAA→AAA | −→+ | Insoluble | No predicted structural change and no motifs altered. Impact on translocational pausing reported by Zimmerman and associates.[45] |
| Val329Met | GTG→ATG | None | Soluble | B-strand→a-helix (GOR), no motifs altered. |
| Gln368STOP | CAG→TAG | | Insoluble | Premature truncation. |
| Pro370Leu | CCG→CTG | None | Insoluble | Interjected turn (GOR) near conserved CK2 motif that is also hit by Thr377Met and Asp380Gly. |
| Thr377Met | ACG→ATG | U→N | Insoluble | No predicted structural change (CF, GOR), but it alters the target residue of a conserved CK2 motif that is also hit by Asp380Gly. |
| Lys398Arg | AAA→AGA | None | Soluble | B-strand→a-helix (GOR) near a CK2 motif that is conserved across species but not across gene family. |
| Val426Phe | GTC→TTC | None | Insoluble | No predicted structural change (CF, GOR). Altered residue adjoins cAMPK motif also hit by Arg422Cys, Arg422His, Lys423Glu, and Ser425Pro. Motif is conserved across species but not across gene family. Target serine residue is predicted to be phosphorylated by CK2 in some members of gene family lacking cAMPK motif. |
| Ile477Asn | ATT→AAT | N→U | Insoluble | Interjected turn (GOR) with a CK2 motif that is also hit by Tyr437Cys and Asn480Lys. |
| Ile499Ser | ATC→AGC | N→U | Insoluble | B-strand becomes random coil (GOR) or a turn is interjected (CF) next to a C-terminal trafficking signal, in a region also hit by Ile499Phe and neighboring Lys500Arg. |

cAMPK = cyclic AMP–dependent kinase; CF = Chou Fasman algorithm[35]; CK2 = casein kinase 2; GOR = Garnier, Ogusthorpe, Robson algorithm[36,37]; − = negatively charged; none = no charge; N = nonpolar; + = positively charged; U = uncharged.

affected individuals were available for testing, Arg76Lys and Val329Met have not been classified as causative mutations because of previous reports of their frequencies in control and primary open-angle glaucoma populations.[14,15] Gly252Arg and Ile499Ser cannot be assigned to benign or pathogenic mutation categories based on currently available family or population data but were classified based on functional analyses presented below. All individuals with TIGR/MYOC mutant alleles were heterozygotes, and no compound heterozygotes were found.

One mutation found in two different families appears to originate from a shared common ancestor. The Val426Phe missense mutation in family UM:GL355 was identical to the Val426Phe mutation that cosegregated with primary open-angle glaucoma in UM:JG3 (Table 1). Screening of five polymorphic markers (D1S433, D1S452, D1S210, D1S218, and D1S215) spanning a 10.7-cM interval containing the TIGR/MYOC gene identified the same Val426Phe-containing haplotype: [148, 221,121, 272, 199] in both families. It was not possible to evaluate whether these families share ancestry with the one other reported Val426Phe family,[26] but information on ethnic origins of the families would be consistent with such a possibility. Founder effects have also been reported for other TIGR/MYOC mutations, such as Gln480Lys in French families,[44] and a more complex mutation in which Gly367 and Gln368 are replaced by a valine residue in Italian families.[30]

Functional testing of the 11 nonsynonymous sequence variants in exon 3 resulted in assignment of nine of them into the pathogenic category (Table 3). In a Triton solubility assay,[41] Gly252Arg, Arg272Gly, Glu323Lys, Gln368STOP, Pro370Leu, Thr377Met, Val426Phe, Ile477Asn, and Ile499Ser produced insoluble protein typical of pathogenic TIGR/MYOC mutations (Figure 3, Table 3). In the same assay, Lys398Arg and Val329Met

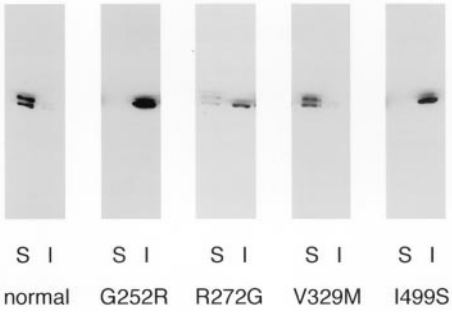


**FIGURE 3. Triton solubility assay of trabecular meshwork inducible glucocorticoid response/myocilin protein produced by sequence variants identified in this study. DNA constructs encoding FLAG epitope-tagged versions of normal or mutant trabecular meshwork inducible glucocorticoid response/myocilin protein (0.2 micrograms each) were separately transfected into human embryonic kidney cells by lipofection, and a Triton extraction procedure was carried out, as described previously.[41] Triton soluble (S) and insoluble protein (I) were detected by immunoblot with an anti-FLAG monoclonal antibody. Scoring of Triton solubility results for all exon 3 nonsynonymous sequence variants in this article appear in Table 3.**

produced the soluble protein that has previously been shown to be indicative of benign sequence variants (Figure 3, Table 3). Results on exon 1 variants are not present here, because it is unclear whether this test is relevant for mutations outside of exon 3.[41] In a different assay system, the functional impact of Glu323Lys on translocational processing of the trabecular meshwork inducible glucocorticoid response/myocilin protein reported by Zimmerman and associates[45] also offers functional support for assignment of Glu323Lys into the pathogenic category.

All of the mutations presented here are predicted to alter the protein's charge and/or secondary structure (Table 3) and change residues that are conserved across species, except residues Gln368 (histidine in mouse),[46–48] Lys398 (arginine in rat),[49] and Ile477 (valine in rat).[50] Only Val329Met alters a residue that is conserved both across species and across gene family.[21,46–48] Three of the four predicted charge change mutations (Gly252Arg, Arg272Lys, and Glu323Lys) are located in an area in which most known mutations effect a positive charge change. Note that the Lys398Arg sequence variant reported here and elsewhere[14,15] changes the residue at position 398 to the arginine residue naturally present at that position in the rat gene. Asn57Asp is of potential interest, because it eliminates the only residue on the protein that is predicted to be N-glycosylated.

Frequencies of TIGR/MYOC mutant alleles, that is, those alleles that appear to be associated with disease based on genetic, population, or functional data, differed according to age at diagnosis in both individual and family categories (Table 5). We found mutations in three of nine families with juvenile primary open-angle glaucoma (33%), eight of 26 families with mixed-onset primary open-angle glaucoma (31%), and zero of 39 families with adult-onset primary open-angle glaucoma (0%). If we classify by proband age at diagnosis instead of the family age information, we find almost an order of magnitude difference in mutation frequency between the two age categories, with mutations in nine of 25 (36%) of the juvenile primary open-angle glaucoma probands and two of 49 (4%) of the adult-onset primary open-angle glaucoma probands. Although our overall frequency is 11 of 74 (15%), this frequency is unlikely to be meaningful, because the screened population does not represent an unbiased random and/or sequential sample of the population with primary open-angle glaucoma.

Substantial representation of adult-onset primary open-angle glaucoma cases in the families with mixed-onset primary open-angle glaucoma is demonstrated by the presence of 37 adult-onset primary open-angle glaucoma cases of 112 affected family members (33%) in nine families with mixed-onset primary open-angle glaucoma with mutations and 42 adult-onset primary open-angle glaucoma cases of 63 affected family members (67%) in 17 families with mixed-onset primary open-angle glaucoma without mutations (Table 5). These adult-onset primary open-angle glaucoma cases within families with mixed-onset primary open-angle glaucoma are not just juvenile primary open-angle glaucoma cases that slipped across our age boundary because of a slight delay in diagnosis, because many of these individuals were diagnosed in their sixties and later (Figure 4).

A small bias in age classifications for Caucasians and African Americans suggested that representation in different family-based age groups differed by race in our sample set. When we examined classification of primary open-angle glaucoma probands based on their own age at diagnosis, the differences were not substantial. We found that four of 11 African Americans (36%) and 19 of 59 Caucasians (32%) were classified as having juvenile primary open-angle glaucoma, and seven of 11 African Americans (64%) and 40 of 59 Caucasians (68%) were classified as having adult-onset primary open-angle glaucoma. However, when family classifications of primary open-angle glaucoma probands were examined, four of 11 African Americans (36%) and 34 of 59 Caucasians (58%) were in families with adult-onset primary open-angle glaucoma, three of 11 African Americans (27%) and six of 59 Caucasians (10%) were in families with juvenile primary open-angle glaucoma, and four of 11 African Americans (36%) and 19 of 59 Caucasians (32%) were in families with mixed-onset primary open-angle glaucoma. Thus, African Americans were represented more than Caucasians in the families with juvenile primary open-angle glaucoma and less than Caucasians in the families with adult-onset primary open-angle glaucoma. Because the total number of African Americans being assigned to

**TABLE 4.** Proband and Family Data for Nonsynonymous TIGR/MYOC Sequence Variants Not Assigned to Mutation Category

| Sequence Variant | Proband Type | Family Type | Age at Diagnosis Mean (range) | Max IOP (mm Hg) Mean (range) | Racial/Ethnic | Fraction of POAG Families With Variant | Fraction of Unrelated Controls With Variant | Fraction of Unrelated Controls With Variant* (Fingert and associates[15]) | Other Reports of the Sequence Variant |
|---|---|---|---|---|---|---|---|---|---|
| Asn57Asp | J-POAG | J-POAG | 19 | 44 | Caucasian | 1/75 | 0/60 | Not reported | |
| Arg76Lys† | A-POAG | A-POAG | 56 (35–80) | 30 (27–35) | Caucasian (all) | 11/75 | not done | 18/288 | 14,15 |
| Val329Met | A-POAG | A-POAG | 54 (30–70) | 35 (32–38) | African American | 1/75 | 0/60 | 1/793 (1/50‡) | 14,15 |
| Lys398Arg | A-POAG | A-POAG | 37 (35–39) | 24 (19–28) | Caucasian | 1/75 | 1/60 | 7/793 | 14,15 |

Abbreviations as in Table 1.
*Model-dependent interpretation of population data presumes autosomal dominant inheritance.
†Includes pooled data from all 11 Arg76Lys families.
‡African-American subset of their data.

different categories is small, care must be taken to avoid overinterpreting these data.

## DISCUSSION

SINCE THE FIRST IDENTIFICATION OF TIGR/MYOC mutations in cases of primary open-angle glaucoma in 1997, research groups around the world have identified more than 50 missense or nonsense variants in TIGR/MYOC.[2,3,13–15,20–32,44,51] Of 74 unrelated subjects with primary open-angle glaucoma screened, we have identified nine apparent mutations in 11 families among a total of 13 nonsynonymous sequence variants seen in 25 families. Five of the nonsynonymous sequence variants identified appear to be novel (Asn57Asp, Gly252Arg, Arg272Gly, Glu323Lys, and Ile499Ser). A combination of genetic data and functional information were used to assign nine of the 13 nonsynonymous sequence variants into the pathogenic mutation category (Table 1). We have provided genetic and/or functional information indicating that Gly252Arg, Arg272Gly, Glu323Lys, Gln368STOP, Pro370Leu, Thr377Met, Val426Phe, Ile477Asn, and Ile499Asn are all mutant alleles that are likely to cause or contribute to primary open-angle glaucoma.

Four other nonsynonymous sequence variants were not classified as causative mutations (Table 4). We have family and population results that are consistent with previous reports that Lys398Arg and Val329Met are unlikely to cause disease,[15] and our Triton solubility assay results produced the soluble protein indicative of benign variants. For the purposes of this article, we are accepting the statement of prior reports that Arg76Lys is unlikely to cause disease.[14,15] We cannot currently tell whether Asn57Asp is pathogenic. It is not present in our normal control population, but it has not been evaluated for cosegregation with glaucoma or presence in large populations, although its absence from both primary open-angle glaucoma and normal populations in the report by Fingert and associates[15] suggests that it might be a truly rare variant that cannot be classified based on simple population values. However, our conclusions regarding age-dependent prevalence of mutations would not be altered if this one sequence variant were actually found to be pathogenic rather than benign, because it was found in one of the subjects with juvenile-onset primary open-angle glaucoma.

Four sequence variants were found only in African Americans in our study. Val329Met may be a population-specific polymorphism, because it has been found only in African Americans, both in our study and in Fingert and associates'.[15] The co-occurrence of the three synonymous polymorphisms Pro13Pro, Leu159Leu, and Thr325Thr in 18% of our African-American families and none of our Caucasian families is consistent with Fingert and associates'[15] observation that frequencies of each of these three

**TABLE 5.** Frequencies of Families With Different Categories of TIGR/MYOC Sequence Variants

| Classification | Age Groups | All Sequence Variants | Nonsynonymous Variants | Mutations Implicated in Disease |
|---|---|---|---|---|
| J-POAG probands | J-POAG individuals with onset prior to age 35 years | 11/25 (44%) | 9/25 (36%) | 9/25 (36%) |
| A-POAG probands | A-POAG individuals with onset at or after age 35 years | 17/49 (35%) | 14/49 (29%) | 2/49 (4%) |
| J-POAG families | Families with only J-POAG individuals | 5/9 (55%) | 3/9 (33%) | 3/9 (33%) |
| M-POAG families | Families with both J-POAG and A-POAG individuals | 10/26 (39%) | 9/26 (35%) | 8/26 (31%) |
| A-POAG families | Families with only A-POAG individuals | 13/39 (33%) | 11/39 (28%) | 0/39 (0%) |
| J-POAG and M-POAG families combined | Families with any J-POAG individuals | 15/35 (46%) | 12/35 (37%) | 11/35 (31%) |
| M-POAG and A-POAG families combined | Families with any A-POAG individuals | 23/65 (35%) | 20/65 (31%) | 8/65 (12%) |
| All families | Complete data set | 28/74 (38%) | 23/74 (31%) | 11/74 (15%) |

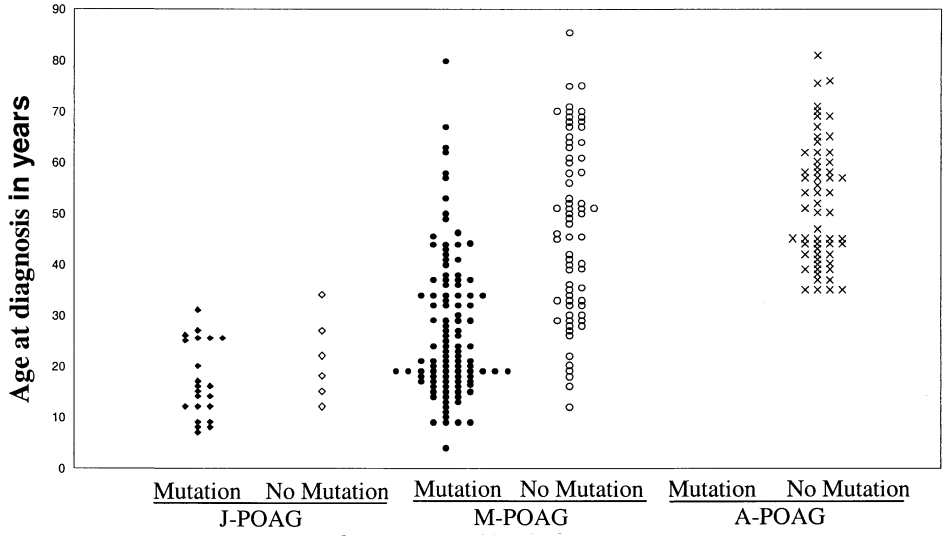


**FIGURE 4. Scatter graph showing age at diagnosis of individuals within families classified according to presence of mutations and according to age-at-diagnosis information for the overall family. Nonmutation categories contain data points from individuals in families without sequence variants, with synonymous sequence variants, or with missense changes deemed unlikely to cause or contribute to disease, as discussed in text and listed in Table 4. Mutation categories contain data points from affected individuals in families with missense or nonsense changes deemed likely to cause disease, as discussed in the text and presented in Table 1. Note that there are no data points in the category called adult-onset primary open-angle glaucoma with mutation.**

polymorphisms are elevated in the African-American population compared with the Caucasian population. The co-occurrence of these three polymorphisms in the same individuals in our study raises questions about whether they might be in linkage disequilibrium, but we do not have haplotype data that would allow us to draw a firm conclusion regarding physical clustering of these three variants. Because Fingert and associates'[15] study does not identify individuals in which more than one sequence variant occurs, it is not possible to confirm this hypothesis from their data.

Our data suggest that information on age at diagnosis may be a helpful indicator of the probability that someone has a TIGR/MYOC mutation. Because TIGR/MYOC mutations occur in fewer than 5% of unselected primary open-angle glaucoma cases[13–15] and negative test results offer little information, identification of a population with primary open-angle glaucoma enriched for TIGR/MYOC mutations would greatly enhance the usefulness of TIGR/MYOC genotyping.

We found TIGR/MYOC mutations in 36% of juvenile primary open-angle glaucoma probands and 4% of adult-onset primary open-angle glaucoma probands. An increased mutation frequency in juvenile primary open-angle glaucoma cases is not a surprising result, because the *GLC1A* locus was originally mapped and otherwise characterized relative to juvenile primary open-angle glaucoma families.[4–13] These findings support the previous suggestion

by Adam and associates[25] and Wiggs and associates[3] that individuals with juvenile primary open-angle glaucoma are more likely to have TIGR/MYOC mutations than are adult-onset primary open-angle glaucoma cases, and the suggestion of Brezin and associates[44] that *GLC1A*-linked glaucoma generally has an earlier age –at diagnosis. However, this information is of limited use in making decisions about screening of the overall population with primary open-angle glaucoma, most of whom are adult-onset cases with an apparently low probability of having a TIGR/MYOC mutation.

Something more surprising and potentially more useful resulted from analysis of the three family age categories assigned on the basis of information from the whole family instead of the proband. We found an increased frequency of TIGR/MYOC mutations (31%) in families with mixed-onset primary open-angle glaucoma, a value comparable to the 33% found for families with juvenile primary open-angle glaucoma, whereas we found no mutations in the families with strictly adult-onset primary open-angle glaucoma (0%; Figure 4, Table 5). Thus, it is worthwhile to consider whether the presence of a juvenile primary open-angle glaucoma relative might predict an increased probability of a positive TIGR/MYOC mutation test for an individual with adult-onset primary open-angle glaucoma. We believe it is also worthwhile to consider whether information on age at diagnosis of relatives might have predictive value for unaffected at-risk individuals and at-risk individuals with ocular hypertension making decisions about undergoing genetic testing for TIGR/MYOC mutations. However, these findings should not be overinterpreted, because our initial study design and basis for ascertainment were not originally designed to ask whether age at diagnosis in relatives has predictive value.

Although Gln368STOP mutations have previously been reported as being associated with later onset of disease,[2,3,14] both Gln368STOP mutations that we found were in families with mixed-onset primary open-angle glaucoma (Table 1). Although both families were identified through adult-onset primary open-angle glaucoma probands, mean age –at diagnosis was 37 years (range, 27 to 49 years), which is markedly younger than values reported by Allingham and associates[2] and Wiggs and associates[3] (mean, 62 years; range, 41 to −75 years) or Alward and associates[14] (mean, 59 years; range, 36 to 77 years). Exclusion of families with mixed-onset primary open-angle glaucoma from the screened population can explain the older age at diagnosis in the Allingham and associates[2] and Wiggs and associates[3] studies but may not explain the older age findings in the Alward study.[14] The Gln368STOP mutation is present in all members of UM:GL9 with primary open-angle glaucoma. Individuals with normal adult exams and ocular hypertension are found in both mutation and nonmutation categories, and we identified no characteristics that distinguish the ocular hypertension cases with mutations from the cases without mutations (Figure 2). Our findings are consistent with the reports by Allingham and associates[2] and Wiggs and associates[3]that Gln368STOP was found in multiple normal individuals suspected of having glaucoma who have ocular hypertension. Although the combination of population and family data suggests that individuals with Gln368STOP should be monitored because of increased risk of glaucoma and ocular hypertension, further studies will be needed before it will be possible to offer clinically useful predictions regarding disease risk or prognosis for this mutation. The appearance of a Gln368STOP mutation in a case of normal-tension glaucoma reported elsewhere[51] and a case of pseudo-exfoliation with ocular hypertension in UM:GL9 raises questions about just how variable the phenotype can be. The presence of the Gln368STOP mutation in many older ocular hypertension cases in our families and others raises questions about whether a Gln368STOP mutation is predictive of glaucoma, or whether it is simply predictive of the initial intraocular pressure elevation without implied progression to glaucoma. The cause of such great variation in Gln368STOP expressivity remains to be determined. Caution must be exercised in evaluating the implications of any TIGR/MYOC mutant, because nonpenetrance, reduced penetrance, phenocopies, and variation in age at diagnosis all appear to complicate any prediction that can be made regarding the future development of glaucoma in any one individual.

The type and location of the sequence substitutions found in the trabecular meshwork inducible glucocorticoid response/myocilin polypeptide offer insights into potential regions of functional importance. Three predicted charge change mutations (Gly252Arg, Arg272Gly, and Glu323Lys) are located in an area in which almost all known mutations effect a positive charge change, suggesting that there might be a charge-sensitive domain within this 107-residue region (246–352). A prediction that modification of the protein may be involved in normal function and disease processes arises from the observation that five of the mutations presented in this article (Gly252Arg, Pro370Leu, Thr377Met, Val426Phe, and Ile477Asn) sit in clusters of mutations predicted to impact conserved phosphorylation motifs (Table 3). However, much further work will be needed to evaluate this model, because trabecular meshwork inducible glucocorticoid response/myocilin has been reported to be phosphorylated,[16] but it has not yet been determined whether the predicted phosphorylation points near the mutations are the residues that are actually modified in vivo.

In summary, we have found nine mutations (two of them novel) among 13 nonsynonymous TIGR/MYOC sequence variants in a group of 74 unrelated primary open-angle glaucoma cases, predicted the existence of a charge-sensitive domain and three functionally important phosphorylation sites in TIGR/MYOC exon 3, and detailed ways in which age –at diagnosis may help identify

individuals at increased risk of harboring a TIGR/MYOC mutation. Efficacy of TIGR/MYOC mutation screening will improve as we learn more about the pathogenic role of specific TIGR/MYOC sequence changes and identify subsets of the population with primary open-angle glaucoma who could most benefit from such testing. A larger study with a different ascertainment basis will be needed to provide more precise estimation of mutant allele frequencies in different age groups and to validate our hypothesis that presence of juvenile primary open-angle glaucoma relatives predicts an increased probability of a TIGR/MYOC mutation.

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