Objective: To search for the genetic cause of juvenile-onset open-angle glaucoma (JOAG) in a Chinese family.

Methods: In a 3-generation glaucoma family affected with JOAG or ocular hypertension, we screened myocilin (MYOC) and optineurin (OPTN) for mutations and investigated apolipoprotein E (APOE) polymorphisms in 6 family members, 2 of them patients with JOAG, 2 patients with ocular hypertension, and 2 patients who were asymptomatic. Normal controls included 200 unrelated Chinese subjects. The COS-7 cell line was transfected with expression vectors encoding wild-type or mutated MYOC complementary DNA. Cellular and secreted MYOC proteins were characterized by Western blotting.

Results: One missense MYOC mutation, 734G>A: Cys245Tyr, was identified. It occurred in all 4 family members afflicted with JOAG or ocular hypertension but not in asymptomatic family members. No OPTN variations were observed. APOE polymorphism frequencies were similar to those for controls. The Cys245Tyr MYOC mutation cosegregated with the disorder within the family. It was absent in the 200 control subjects. The Cys245Tyr mutant MYOC protein formed homomultimeric complexes that migrated at molecular weights larger than their wild-type counterparts. These mutant complexes remained sequestered intracellularly in COS-7 cells.

Conclusions: The Cys245Tyr MYOC mutation was the genetic cause of JOAG in this Chinese family. This mutation may alter covalent bonds that formed between MYOC cysteines.

Clinical Relevance: Genetic tests of MYOC mutations may be beneficial to predict new cases of the disease in families with JOAG.

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even normal-tension glaucoma.\textsuperscript{22,23} Among 73 reported MYOC mutations, 63 (86.3\%) were located in exon 3,\textsuperscript{14} suggesting the olfactomedin-like domain to be important for POAG pathogenesis. The most common MYOC mutation is Gln368Stop, reported in 1.65\% of probands with POAG. Our previous screening of MYOC gene in 201 POAG probands and 402 control subjects revealed no Gln368Stop among Chinese but found 3 missense mutations, Arg91Stop, Glu300Lys, and Tyr471Cys, all of which have not been reported in other populations.\textsuperscript{15,16} Here, we report a novel MYOC missense mutation in a Chinese family with JOAG.

**METHODS**

**FAMILY RECRUITMENT**

A 3-generation family with JOAG was recruited from the day clinic of the Hong Kong Eye Hospital (Figure 1). The study protocol was approved by the Ethics Committee for Human Research, Chinese University of Hong Kong, Hong Kong. In accordance with the tenets of the Declaration of Helsinki, informed consent was obtained from the study subjects after explanation of the nature and possible consequences of the study. Juvenile-onset open-angle glaucoma was defined as meeting all the following criteria: exclusion of secondary causes (eg, trauma, uveitis, or steroid-induced glaucoma); open anterior chamber angle (grade 3 or 4 gonioscopy); intraocular pressure greater than 22 mm Hg in both eyes; characteristic optic disc damage and/or typical visual field loss. Subjects with intraocular pressure greater than 22 mm Hg in both eyes but no characteristic optic disc damage or visual field impairment were diagnosed with ocular hypertension (OHT). Intraocular pressure and visual field were measured by applanation tonometry and Humphrey perimeter with the Glaucoma Hemifield Test, respectively. In this family, 5 members had JOAG while 2 others were diagnosed with OHT (Figure 1). Blood samples and complete ophthalmic examination were obtained from 6 family members. Clinical information for the other family members was obtained through previous medical records.

**MUTATION SCREENING**

Genomic DNA was extracted from 200 µL of whole blood using a QIamp Blood Kit (Qagen, Hilden, Germany). The coding regions of MYOC, exons 1 through 3, and of OPTN, exons 4 through 16, including their intron-exon boundaries, were screened for sequence alterations using polymerase chain reaction and direct DNA sequencing\textsuperscript{16,17} on an ABI 3730XL automated DNA sequencer (Applied Biosystems, Foster City, Calif). Sequence data were compared with the published MYOC and OPTN gene sequences (GenBank accession numbers SEG_AB006686S and AF420371, respectively). MYOC mtI (-10000C>G) was determined using AlwNI restriction endonuclease assay.\textsuperscript{24} For apolipoprotein E (APOE), the promoter polymorphisms -491A>T, -427G>C, and -219T>G and the exon 4 ε2ε3ε4ε genotype were investigated by polymerase chain reaction and restriction endonuclease assays.\textsuperscript{25}

**SECRETION STUDY**

The Cys245Tyr and Lys423Glu MYOC mutants were generated by site-directed mutagenesis on the prC-MYOC expression vectors coding for human myocilin complementary DNA (cDNA) using the QuickChange mutagenesis kit (Stratagene, La Jolla, Calif). The cDNA sequences were verified using ABI 3730XL sequencing equipment. COS-7 cells (ATCC), plated at a density of 1.5 × 10\(^4\) per 35 mm, were grown in Dubecco Modified Eagle Medium high glucose complemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 200 µmol/L of L-glutamine (Invitrogen, Carlsbad, Calif) and incubated at 37°C in a humidified chamber with 5% carbon dioxide. Transient transfections were performed 16 hours later using FuGENE 6 transfection reagent (Roche, Laval, Quebec). We used 2 µL of FuGENE 6 and 1 µg of total plasmid. After 48 hours, an aliquot of the extracellular medium was taken before the cells were washed twice with ice-cold phosphate-buffered saline and scrapped in lysis buffer (0.5% Triton X-100, 30 mmol/L Tris hydrochloride [pH 7.4], 150 mmol/L sodium chloride), complete protease inhibitor cocktail tablets (Roche), and 0.7 µg/mL pepstatin (Sigma-Aldrich Corp, St Louis, Mo) using a rubber policeman. Before analysis, cellular extracts were sonicated (Sonic Dismembrator 550, Fisher Scientific, Nepean, Ontario) and protein concentrations measured (Bio-Rad Protein Assay, Bio-Rad, Mississauga, Ontario). Culture media and cellular extracts were heated at 70°C for 10 minutes, resolved on NuPAGE Tris-Acetate 7 precast protein gels (Invitrogen), and transferred onto nitrocellulose membrane (BioTrace NT, Pall Corp, Mississauga, Ontario) with a Mini Trans-Blot Module (Bio-Rad). Myocilin proteins were revealed using a well-characterized rabbit polyclonal antimyocilin at a concentration of 50 ng/mL.\textsuperscript{20}

The proband (III:1) was affected with advanced glaucoma (Table). He was diagnosed with JOAG at 16 years of age and both eyes underwent trabeculectomy. Receiving topical β-blocker (0.5% timolol maleate twice a day OU), his intraocular pressure control was fair. He was 24 years old on his last visit. He had a cup-disc ratio of 0.9, open grade 3 (Shaffer) angles, and typical glaucomatous visual field loss in both eyes. No other obvious anterior segment dysgenesis was noticeable. His mother

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**Figure 1.** Pedigree structure of the Chinese family segregating juvenile-onset open-angle glaucoma (JOAG). Persons who were examined and analyzed for mutations are indicated with asterisks below their symbols. Squares indicate male subjects; circles, female subjects. Solid symbols indicate affected family members with JOAG; half-filled symbols, patients with ocular hypertension; open symbols, unaffected family members. Arrow indicates the proband (III:1). Slash through symbol (I:1) indicates that the individual is deceased. Cys245Tyr MYOC genotypes are indicated with combined plus or minus; plus indicates the wild-type; minus, the mutant.
Table. Clinical Findings in the Family Members

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex/Age at Inclusion, y</th>
<th>Diagnosis</th>
<th>Age at Diagnosis, y</th>
<th>Highest IOP (Right/Left), mm Hg</th>
<th>Cup-Disc Ratio (Right/Left)</th>
<th>Humphrey 24-2 Visual Field (Right/Left)</th>
<th>Treatment</th>
<th>Cys245Tyr Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>III:1</td>
<td>M/24</td>
<td>JOAG</td>
<td>16</td>
<td>26/19</td>
<td>0.9/0.9</td>
<td>A/A (advanced)</td>
<td>Trabeculectomy β-blocker</td>
<td>Yes</td>
</tr>
<tr>
<td>III:2</td>
<td>F/17</td>
<td>OHT</td>
<td>17</td>
<td>26/24</td>
<td>0.4/0.4</td>
<td>N/N</td>
<td>β-blocker</td>
<td>Yes</td>
</tr>
<tr>
<td>III:3</td>
<td>M/16</td>
<td>OHT</td>
<td>12</td>
<td>26/26</td>
<td>0.5/0.4</td>
<td>N/N</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>II:1</td>
<td>F/50</td>
<td>JOAG</td>
<td>27</td>
<td>NA</td>
<td>0.2/0.2</td>
<td>None</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>II:2</td>
<td>M/48</td>
<td>Asymptomatic</td>
<td>NA</td>
<td>20/20</td>
<td>0.3/0.3</td>
<td>None</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>I:2</td>
<td>F/70</td>
<td>Asymptomatic</td>
<td>NA</td>
<td>20/20</td>
<td>NA</td>
<td>NA</td>
<td>None</td>
<td>No</td>
</tr>
</tbody>
</table>

Abbreviations: A, abnormal; IOP, intraocular pressure; JOAG, juvenile-onset open-angle glaucoma; N, normal; NA, not available; OHT, ocular hypertension.

(II:1) was diagnosed with glaucoma at 27 years of age. Since age 30 years, she has been blind in both eyes. Her eyes became phthtical as a result of a repeatedly ruptured descemetocele. The right eye subsequently required enucleation plus artificial eye implantation because of persistent pain and leakage from the descemetocele. We could not obtain any information regarding her optic nerve or intraocular pressure because of her phthitious eyeballs.

The proband’s grandfather (I:1) was deceased at the time of study but was known to have glaucoma since a young age. His grandmother (I:2) never had glaucoma. Last year, the proband’s younger sister (II:2) was diagnosed with OHT but with normal open Shaffer grade 3 angles. Receiving topical β-blocker (0.5% timolol maleate twice a day OU), she achieved a median intraocular pressure of 17 mm Hg and below. The proband’s younger brother (III:3) was also diagnosed with OHT with normal open Shaffer grade 3 angles. He chose a conservative treatment and hence was not put on any glaucoma medications at the time. The father (II:2) did not have any eye disorders, and he did not recall any glaucoma cases among his lineage or relatives. In addition, according to old medical records, the mother’s younger sister (II:3) and brother (II:4) were previously diagnosed with JOAG.

One missense mutation, 734G>A: Cys245Tyr (GenBank accession number AY599062), was identified in exon 3 of the MYOC gene in the mother (II:1) and 3 offspring (III:1, III:2, III:3), all heterozygous. It was not detected in the grandmother (I:2) and father (II:2). In addition, 3 common polymorphisms (-83G>A, Arg76Lys, and IVS2 + 35A>G) were found in this family. Genotype at MYOC.mt1 was normal with a C at position −1000. None of the 200 unrelated controls carried the mutant MYOC allele of Cys245Tyr. Also in this family, no OPTN sequence alterations were detected, and the APOE polymorphism frequencies were similar to those for controls.

Proteins harboring MYOC variations in their olfactomedin homology domain that have been studied to date remained sequestered intracellularly. To test whether the Cys245Tyr mutation also inhibited secretion of myocilin polypeptide, we transiently transfected Cys245Tyr MYOC cDNA in cultured COS-7 cells. The wild-type and Lys423Glu mutant myocilin proteins were used as positive and negative secretion controls, respectively. Three myocilin proteins were highly expressed in COS-7 cells (Figure 2B). As previously observed for the wild-type and Lys423Glu mutant under native conditions, all 3 proteins formed homodimers as well as homomultimers migrating above 180 kd (Figure 2A). Interestingly, Cys245Tyr MYOC formed complexes that had slower electrophoretic mobility migrating at molecular weights that were higher than those of their wild-type counterparts. For instance, the 2 major mutant complexes, one at about 120 kd and the other at more than 200 kd, were migrating at molecular weights 5% to 10% higher than wild-type complexes. Such slower migration patterns represented the substitution of the 245 cysteine by a tyrosine and the concomitant destruction of a critical disulfide bond. Under reducing conditions, all 3 myocilins were doublets migrating at about 55 and 57 kd and represented glycosylated and unglycosylated forms of the protein (Figure 2B).

 Regarding secretion, wild-type myocilin was found in the extracellular media (Figure 2C), but both olfactomedin-homology–domain mutant polypeptides were not detected in the COS-7 cell culture media (Figure 2C). Our data therefore demonstrated that the Cys245Tyr mutant MYOC polypeptide remained sequestered intracellularly.

This novel missense MYOC mutation Cys245Tyr accounts for JOAG in this Chinese family. It is located in exon 3 of the MYOC gene, where most mutations are detected, and causes its second amino acid to change from a cysteine to a tyrosine. This change deprives the sulfhydryl of cysteine and thus hinders the formation of covalent disulfide bridges between cysteine residue pairs within the same polypeptide chain and/or in different polypeptide chains. Five cysteine residues, at positions 47, 61, 185, 245, and 433, are encoded by the mature myocilin protein. One cysteine mutation, the Cys433Arg variation, has been reported. The Cys245Tyr variant is the second mutation reported to alter a cysteine.

We previously demonstrated that wild-type MYOC polypeptides formed homo-oligomeric complexes ranging in size from 116 kd to more than 200 kd. The smallest of these complexes resulted from dimerization be-

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tween 2 MYOC monomers while those above were generated by interaction of at least 2 MYOC moieties. Wild-type homo-oligomeric complexes were secreted in the extracellular media of COS-7 cells whereas the Gln368Stop and Lys423Glu mutant/mutant homomultimers and heteromeric wild-type/mutant oligomers remained sequestered intracellularly.26 The mutated Cys245Tyr protein also formed in nonreducing conditions high molecular weight complexes (Figure 2), most likely generated by multimerization of mutant monomers that remained sequestered intracellularly. These complexes migrated at positions higher than those of their wild-type counterparts. Although cysteine 245 did not impede the homo-oligomerization process, such slower migration patterns may reflect some misfolding resulting from the destruction of the 245 disulfide bridge, thereby changing the structure of the protein complexes. These findings are in agreement with a recent study, which demonstrated that significant migration changes occurred when cysteine 245 is altered to an alanine in addition to cysteines 61, 185, and 433.29 The presence of a tyrosine, as in the Cys245Tyr mutation, may have a more profound effect on migration than a change to alanine.29 Mutations located within the olfactomedin domain of myocilin inhibit secretion.30,31 Cys245Tyr is one such mutation as the cysteine-to-tyrosine change also prevents it from secretion. We hypothesize that the cysteine-to-tyrosine mutation may cause the protein to fail to fold or oligomerize correctly, as observed by its slower migration pattern. This misfolded protein may be retained within the endoplasmic reticulum. Several studies are in agreement with this model. In particular, the amino acid 1-344 truncated form of myocilin was not processed correctly in the endoplasmic reticulum and accumulated in insoluble aggregates.27 Mutant myocilin has been observed to concentrate in fine punctate aggregates in the endoplasmic reticulum.30 Liu and Vollrath31 also recently showed that several disease-causing myocilin mutants accumulated in the endoplasmic reticulum and were prone to aggregate. Further biochemical studies will help decipher the mechanisms by which myocilin mutants cause glaucoma.

Cys245Tyr occurred in all 4 family members affected with JOAG or OHT but not in 200 unrelated normal subjects. It segregated with JOAG in this family in an autosomal-dominant inheritance mode. It is clear that all 3 offspring (III:1, III:2, III:3) obtained the mutant allele from their mother (II:1) because their father (II:2) was free of this mutation. The OHT patients (III:2, III:3) were only teenagers, and thus they might progress to JOAG in the future. We excluded the role of the OPTN gene in this family by finding no sequence changes in the OPTN coding regions. The APOE gene has been suggested as a potential modifier for POAG because its variant −491A>T interacted with the MYOC polymorphism, MYOC.mt1.32 We determined the genotypes for all 4 informative APOE polymorphisms (−491A>T, −427T>C, −219T>G, and ε2ε3/ε4). The frequencies were similar to controls, indicating no association between APOE and JOAG in this family. Moreover, the genotype of MYOC.mt1 was normal and had no contribution to glaucoma. We concluded that the mutation Cys245Tyr in the MYOC gene was the genetic cause of JOAG in this family. This novel mutation confirms the key role of the MYOC gene in JOAG and enriches our understanding of the molecular genetic basis of this disease.

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