Novel Myocilin Mutation in a Chinese Family With Juvenile-Onset Open-Angle Glaucoma

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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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Glaucoma is a leading cause of blindness worldwide. Primary open-angle glaucoma (POAG) is the most frequent form, accounting for more than half of all cases.1 Juvenile-onset open-angle glaucoma (JOAG) appears early in life in autosomal-dominant inheritance.2 Genetic factors play a major role in the cause of POAG, but the precise molecular basis still remains unknown. Inherited forms of POAG have been mapped to 6 chromosomal loci, 1q23-q25 (GLC1A), 2cen-q13 (GLC1B), 3q21-q24 (GLC1C), 8q23 (GLC1D), 10p15-p14 (GLC1E), and 7q35-q36 (GLC1F).3-8 There are recently reported additional linkages to 9 loci on chromosomes 2, 9, 10, 14, 17, 19, and 20.9-11 To date, only 2 genes, myocilin (MYOC; Online Mendelian Inheritance in Man [OMIM] 601652) and optineurin (OPTN; OMIM 602432), were identified from these loci.12,13 MYOC has been reported to mainly contribute to JOAG but also relate to late-onset POAG, whereas OPTN is largely responsible for normal-tension glaucoma.12-17

Mutations in the MYOC gene account for as many as 36% families with JOAG,18 although it is only 2% to 4% in sporadic patients with POAG.19 The GLC1A locus was initially identified by linkage analysis in families with JOAG.3 Fine mapping of this region, together with cellular and functional studies on the trabecular meshwork cells and cytoskeleton of the photoreceptor, led to the eventual identification of the MYOC gene.12,14,20,21 MYOC consists of 3 exons, with lengths of 604, 126, and 782 base pairs (bp), and encodes a 504 amino acid polypeptide. Its sequence homogeneity with olfactomedin and myosin are 40% and 25%, respectively.20-21 More than 70 mutations and a number of polymorphisms have been identified in MYOC from different populations.18 Most mutations cause an early-onset and severe glaucoma (ie, JOAG), whereas a few cause late-onset POAG or...
even normal-tension glaucoma. Among 73 reported MYOC mutations, 63 (86.3%) were located in exon 3, 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. 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 Qiap Blood Kit (Qiagen, 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 on an ABI 377XL automated DNA sequencer (Applied Biosystems, Foster City, Calif). The cDNA sequences were verified using ABI 3730XL sequencing equipment. COS-7 cells (ATCC), plated at a density of 1.5 × 10⁴ 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 scraped 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 (Sonics Dismembranator 530, Fisher Scientific, Nepean, Ontario) and protein concentrations measured (Bio-Rad Protein Assay, Bio-Rad, Mississauga, Ontario).

Cultured 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.

La Jolla, Calif). The cDNA sequences were verified using ABI 3730XL sequencing equipment. COS-7 cells (ATCC), plated at a density of 1.5 × 10⁴ 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 scraped 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 (Sonics Dismembranator 530, 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.

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
remained sequestered intracellularly. To test whether medin homology domain that have been studied to date controls.

morphism frequencies were similar to those for con-

sequence alterations were detected, and the

ting APOE allele of Cys245Tyr. Also in this family, no

IVS2 common polymorphisms (-83G in the grandmother (I:2) and father (II:2). In addition, 3

(III:1, III:2, III:3), all heterozygous. It was not detected

200 unrelated controls carried the mutant

myocilin polypeptide, we transiently transfected Cys245Tyr

MYOC gene in the mother (II:1) and 3 offspring

parts. For instance, the 2 major mutant complexes, one

Lys423Glu mutant under native conditions, all 3 pro-

at about 120 kd and the other at more than 200 kd, were

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 rep-

resented the substitution of the 245 cysteine by a tyro-

sine and the concomitant destruction of a critical disul-

fide bond. Under reducing conditions, all 3 myocilins were

doublets migrating at about 55 and 57 kd and repres-

ented glycosylated and unglycosylated forms of the pro-

tein (Figure 2B).

Regarding secretion, wild-type myo-

cilin was found in the extracellular media (Figure 2C),

but both olfactomedin-homology–domain mutant polype-

ptides were not detected in the COS-7 cell culture media

(Figure 2C). Our data therefore demonstrated that the Cys245Tyr mutant MYOC polypeptide remained se-

questered intracellularly.

One missense mutation, 734G>A: Cys245Tyr (Gen-

Bank accession number AY599652), 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.m1 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 se-

quence alterations were detected, and the APOE poly-

orphism frequencies were similar to those for controls.

Proteins harboring MYOC variations in their olfacto-

medin homology domain that have been studied to date

remained sequestered intracellularly. To test whether the Cys245Tyr mutation also inhibited secretion of myo-

cilin polypeptide, we transiently transfected Cys245Tyr

MYOC cDNA in cultured COS-7 cells. The wild-type and

Lys423Glu mutant myocilin proteins were used as posi-

tive and negative secretion controls, respectively. Three

myocilin proteins were highly expressed in COS-7 cells

(Figure 2). As previously observed for the wild-type and

Lys423Glu mutant under native conditions, all 3 proteins

formed homodimers as well as homomultimers mig-

rating 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-

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>N/N</td>
<td>None</td>
<td>No</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>N/N</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>I:2</td>
<td>F/70</td>
<td>Asymptomatic</td>
<td>NA</td>
<td>20/20</td>
<td></td>
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</tbody>
</table>

Abbreviations: A, abnormal; IOP, intraocular pressure; JOAG, juvenile-onset open-angle glaucoma; N, normal; NA, not available; OHT, ocular hypertension.
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 follistatin domain of myocilin inhibit its secretion.30,37 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 ε2/ε3/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 MYOC 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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Figure 2. Secretion analysis of the myocilin Cys245Tyr mutant. A, Cellular extracts from COS-7 cells transiently expressing the wild-type (WT), Cys245Tyr (C245Y), or Lys423Glu (K423E) myocilin proteins. We analyzed 10 µg of proteins by Western blot under nonreducing conditions. Myocilin was detected with an antimyocilin polyclonal antibody as described in the “Methods” section. B, Detection of cellular myocilin under reducing conditions. Protein samples from COS-7 cells transiently expressing wild-type MYOC, Cys245Tyr MYOC, or Lys423Glu MYOC were treated with 100 mmol/L dithiothreitol (DTT), and 2.5 µg of total proteins were migrated and transferred onto a nitrocellulose membrane. Myocilin was detected using monoclonal anti-Myc antibody. C, Culture media analyzed using the monoclonal anti-Myc antibody. We analyzed 10 µL of extracellular media from COS-7 cells expressing the wild-type, Cys245Tyr, or Lys423Glu myocilin proteins as in A. MW indicates molecular weight.
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REFERENCES


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