Mutations in the Myocilin Gene in Families With Primary Open-angle Glaucoma and Juvenile Open-angle Glaucoma

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Objectives: To investigate the prevalence of myocilin (*MYOC*) mutations in Italian families with glaucoma and to determine the relationship of these mutations to primary open-angle glaucoma (POAG), juvenile open-angle glaucoma (JOAG), and pigmentary dispersion glaucoma.

Methods: Twenty-six patients with POAG were selected based on a positive family history of glaucoma. All patients and 210 relatives had an accurate clinical characterization.

Main Outcome Measure: Each index patient was screened by single-stranded conformational polymorphism analysis for mutations in the *MYOC* gene.

Results: A *MYOC* gene mutation was found in 2 families. In one family, a previously reported p.K423E mutation was transmitted from the index patient with POAG to the 2 sons with JOAG. In the second family, a p.C25R change, affecting the signal peptide, was transmitted from the index patient with POAG to the son with JOAG, but not to the son with pigmentary dispersion glaucoma.

Conclusions: Clinical characterization of 2 families with *MYOC* gene mutations indicates that POAG and JOAG are the 2 sides of a continuum phenotypical spectrum due to a common molecular defect. On the other hand, our results confirm the different origin of pigmentary dispersion glaucoma.

Clinical Relevance: Because *MYOC* gene mutations may be responsible for a fraction (2 [8%] of 26) of families with POAG/JOAG, a molecular genetic diagnosis should be included in the management of patients with glaucoma.

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PRIMARY OPEN-ANGLE glaucoma (POAG) is the most frequent cause of irreversible blindness in white people, with a 2% prevalence in persons older than 40 years.[1] A positive family history for the glaucomatous disease must be considered an important risk factor for POAG.[2] Some POAG-associated variables (intraocular pressure [IOP], outflow facility, optic nerve head cupping, and a hypertensive response to corticosteroids) are believed to be inherited.[3] The term primary glaucoma includes various clinical entities, such as glaucoma with an elevated IOP (POAG) and normal-tension glaucoma, the relationship of which remains to be elucidated. Ocular hypertension (OH) has to be considered as suspected glaucoma. In young people, glaucoma may be the expression of a late variant of congenital glaucoma, characterized by anterior chamber angle malformations, or an early form of POAG. This latter form is named juvenile open-angle glaucoma (JOAG), and it is believed to be

different from the more common adult-onset glaucoma, which usually affects people older than 35 years.[4] This form is frequently transmitted as an autosomal dominant trait.[5]

The first locus for autosomal dominant JOAG, named *GLC1A*, has been mapped by linkage analysis to chromosome 1q21-q31.[5] Subsequently, several other loci named, from *GLC1B* to *GLC1E*, and associated with either late-onset glaucoma or normal-pressure glaucoma have been identified.[6-10] An additional locus (*GLC1F*), on chromosome 7q35-q36, has been associated with pigmentary dispersion syndrome (PDS).[11] The patients with JOAG linked to the *GLC1A* locus are characterized by an age of onset of younger than 35 years and an increased IOP, which can be resolved by surgery. In 1997, a gene associated with *GLC1A* was identified and found to code for a 57-kDa protein called trabecular meshwork–induced glucocorticoid response (TIGR) protein,[12] originally described by Polansky et al.[13] This

protein, recently renamed myocilin (MYOC) (information available at: http://www.gene.ucl.ac.uk/hugo/), is present in many human tissues.[14] Although the exact mechanism is unknown, this gene has been implicated in the pathophysiological characteristics of glaucoma by causing obstruction of the aqueous outflow through the trabecular meshwork, resulting in an increased IOP.[15] Until recently, when the gene of the *GLC1E* locus was cloned (optineurin), the *MYOC* gene was the only known gene for familial glaucoma.[16]

Several mutations that lead to different characteristic forms of JOAG have been described in the *GLC1A* gene, which can produce open-angle glaucoma with different clinical findings.[14] Approximately 2% to 4% of patients with POAG carry a mutation in the *MYOC* gene. All of these mutations are located in the coding region, predominantly in the third exon, which encodes a 250–amino acid domain homologous to olfactomedin.[17,18] Recent articles[14,19] suggest that the clinical spectrum of the disease, due to *MYOC* gene mutations, can range from juvenile glaucoma to typical late-onset POAG.

METHODS

PATIENTS

Enrolled patients were collected from 7 centers of ophthalmology located throughout Italy and gave their written informed consent. Patients included in the study underwent a complete ophthalmic examination: an anterior segment examination, determination of uncorrected and best-corrected visual acuity, gonioscopy with grading according to the system of Van Herick et al,[20] a fundus examination with a cup-disc ratio evaluation, and a tonometric curve and visual field examination (Program 30-2 on HFA Humphrey Field Analyzer; Humphrey System, Inc, Dublin, Calif). The perimetric defect type and stage evaluation was performed using the Brusini Glaucoma Staging System,[21,22] which is a graphic method using a special diagram that classifies the visual field defects into 5 stages of severity and assigns them to 1 of 3 types (generalized, mixed, or localized) based on the mean deviation and the corrected-pattern SD.

At the end of the examination, patients were placed into 1 of 3 groups: (1) those with healthy eyes (not affected by glaucoma), (2) those with OH or suspected glaucoma (IOP >21 mm Hg and no visual field defects), and (3) those affected by POAG. Patients were defined as affected by POAG when they showed at least 2 of the following criteria: glaucomatous visual field defects (based on the corrected-pattern SD and the mean deviation perimetric indexes), OH (IOP >21 mm Hg), and optic nerve head glaucomatous changes (cup-disc ratio >0.7 or notches). Patients included in the study belonged to families in which at least 2 members were affected by POAG. After a clinical examination, a 10-mL blood sample was obtained from each patient. DNA samples of 26 patients (index patients, or probands) and 210 relatives were collected.

MUTATION ANALYSIS

Genomic DNA samples were obtained from the peripheral blood leukocytes of the patients according to standard methods.[23] The DNA samples of the 26 probands were screened for *MYOC* gene mutations using single-stranded conformational polymorphism analysis. The 3 exons of the *MYOC* gene were amplified by dividing the 2 longer exons into 13 overlapping polymerase chain reaction products. The polymerase chain reaction amplicons were obtained using the primer pairs reported by others.[14] The amplification was performed using an amplification system (GeneAmp PCR System 2400; Perkin-Elmer, Norwalk, Conn). For exon amplification, 100 ng of genomic DNA was denatured at 95°C for 5 minutes; mixed with ×10 buffer, 0.5-µmol/L primers, 200-µmol/L deoxyribonucleoside triphosphate, and 0.2 U of *Taq* polymerase (Finnzymes, Espoo, Finland), for a final volume of 25 µL; cycled (×35) at 94°C for 1 minute, 63°C or 66°C for 1 minute, and 72°C for 1 minute; and finally incubated at 72°C for 5 minutes. Amplification products were loaded on precast gradient polyacrylamide gels (GeneGel Exel 12.5/24 Kit; Amersham Pharmacia Biotech, Uppsala, Sweden), after denaturing for 5 minutes at 95°C, and electrophoresed at 250 V for 3 hours 50 minutes, under a controlled temperature of 10°C and/or 20°C. Following the electrophoresis, gels were stained with the silver nitrate method.[24] Abnormal polymerase chain reaction products identified by single-stranded conformational polymorphism analysis were sequenced using a genetic analyzer (ABI PRISM 310) and a terminator cycle sequencing kit (BigDye) (PE Applied Biosystems, Foster City, Calif).

RESULTS

Twenty-six patients with POAG (index cases, or probands), belonging to families in which there were at least 2 affected individuals, were screened for mutations in the *MYOC* gene by single-stranded conformational polymorphism and direct sequencing combined methods. In all families, the most likely transmission of the disease was autosomal dominant. A careful clinical analysis of the families revealed that in 10 of them there was at least 1 affected individual who may be classified as having JOAG (young adults ≤35 years). The *MYOC* gene mutation analysis revealed 2 different mutations in 2 probands belonging to this last group. Segregation analysis of mutations in both families and a clinical examination revealed interesting features, which will be described in detail.

FAMILY 1

This is a 3-generation family, in which the proband, aged 74 years, is affected by POAG, with an age of onset of 50 years (**Figure 1** and **Table**). His deceased mother was reported to have glaucoma. His son and daughter, aged 40 and 35 years, have JOAG, with an age of onset of glaucoma of 30 and 27 years, respectively. In this family, an A>G transition at the first base of codon 423 in exon 3 of the *MYOC* gene was found. This substitution caused a missense mutation, changing a lysine at position 423 to a glutamic acid (p.K423E). This mutation is certainly pathogenic because it involves the highly conserved lysine at position 423 in the olfactomedin homologous domain. The same mutation was previously described in patients from a French Canadian family.[25,26] Segregation analysis in our family demonstrated that the mutation is present in all affected individuals: the proband with POAG and the 2 sons with JOAG (Figure 1).

FAMILY 2

In this 2-generation family, 5 subjects underwent a clinical examination (**Figure 2** and Table). The proband, aged
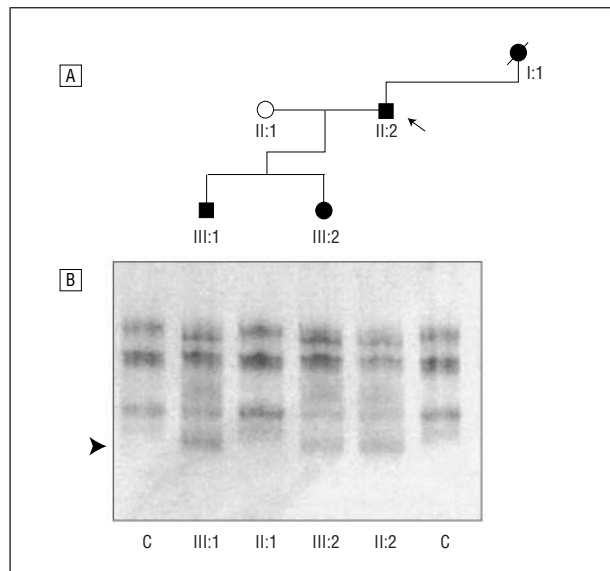
Figure 1. Segregation analysis in family 1. A, Pedigree of the family. Circles indicate females; squares, males; black symbols, individuals with primary open-angle glaucoma or juvenile open-angle glaucoma (the proband is indicated by an arrow); white symbol, unaffected individual; and slash mark, deceased individual. B, Single-stranded conformational polymorphism analysis of segment 3E of exon 3. The primers used are as follows: 5′-GAACTCGAACAAACCTGGGA-3′ and 5′-CATGCTGCTGTACTTATAGCGG-3′. The 4 living members of the family were analyzed. The arrowhead indicates the shifted band present in the individuals with mutations; C, control.

62 years, is affected by POAG. The first son, aged 38 years, was observed the first time at the age of 36 years and was diagnosed as having JOAG; his visual field damage at that time showed that it was a late diagnosis. The second son, aged 30 years, developed OH at the age of 20 years. An examination of his anterior segment revealed signs of PDS. The daughter, aged 28 years, has no signs of glaucoma or OH. Mutation analysis performed in the proband revealed a T>C transition at the first base position of codon 25 in exon 1 of the *MYOC* gene, resulting in a missense mutation, changing the cysteine at position 25 to an arginine (p.C25R). To our knowledge, this mutation has not been previously described. It affects a cysteine within the signal peptide for secretion of the protein, which covers the first 32 amino acids. Extracellular localization of the native protein is correctly predicted by a computer program (PSORT), with 0.50 of certainty (information available at: http://psort.nibb.ac.jp). If the cysteine at position 25 is substituted by an arginine, the certainty score falls to 0.37, suggesting that in vivo the mutation may lead to a reduction of the protein secretion and indicating a pathogenic role of this novel mutation. Segregation analysis in the family showed that the p.C25R mutation is present in the proband, affected by POAG, and in the first son, affected by JOAG. The mutation is not present in the son with OH and signs of PDS. The combined approach of clinical and molecular analysis suggests that this second son has a different disease.

Clinical Data for 2 Families

| Individual | Age, y | | IOP, mm Hg | | Vertical C/D Ratio | | Visual Field* | | Angle† | | Refraction | | Visual Acuity | | Therapy |
	Present	At Diagnosis	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	
							Family 1								
I:1	69	NA	10	10	0.3	0.2	G3	M2	4	4	M (−3.5 sf)	M (−0.75 sf)	0.03	0.8	NA
II:2	74	50	13	7	0.9	NA	G5	0	3	2	PFK	NA	0.5	HM	Brimonidine tartrate, latanoprost, and timolol maleate in the right eye and trabeculectomy in both eyes
III:1	40	30	6	11	0.7	0.6	L4	M1	2	2	M (−7 sf)	M (−7.5 sf)	0.4	0.6	Trabeculectomy in both eyes
III:2	35	27	17	17	0.6	0.7	L1	0	4	6	emm	emm	1	1	Timolol in both eyes
							Family 2								
I:1	65	NA	18	20	?	?	0	0	?	?	?	?	1	1	NA
I:2	62	?	16	16	?	?	L2	0	?	?	PFK	NA	1	CF	Timolol in the left eye
II:1‡	38	36	22	21	0.6	0.7	L2	L2	4	4	M (−4 sf)	M (−7 sf)	1	1	Timolol in both eyes
II:2	30	20	20	20	0.6	0.6	L1	L1	3	3	M (−8 sf)	M (−11 sf)	1	1	Timolol and dorzolamide hydrochloride in both eyes
II:3	28	NA	15	15	?	?	0	0	?	?	?	?	1	1	NA

Abbreviations: C/D, cup-disc; CF, counting fingers; emm, emmetropic; HM, hand movements; IOP, intraocular pressure; M, myopic; NA, data not applicable; PFK, pseudophakic; sf, spheric lens.
*Assessed with the Brusini Glaucoma Staging System.[21,22]
†Assessed with the grading system of Van Herick et al.[20]
‡A late diagnosis was made in this individual.

Myocilin is significantly homologous to myosin in the N-terminal region coded by the first and second exon of the *MYOC* gene and to olfactomedin in the C-terminal region coded by its third exon.[27] The olfactomedin homologous domain of the *MYOC* gene seems to be the focus of pathogenic mutations in patients with POAG. The homology with the olfactomedin protein and the presence of a signal peptide for extracellular localization at its N terminus suggest that MYOC is an extracellular protein. Myocilin is expressed in the eye, including the retina and the structures involved in aqueous humor regulation, such as the ciliary body and the trabecular meshwork.[28] The elevated IOP in patients with glaucoma is mainly due to increased resistance to the outflow of aqueous humor from the eye through the trabecular meshwork.[28]

To better understand the role of the *MYOC* gene in the pathogenesis of glaucoma, Jacobson et al[29] examined the expression of normal and mutant *MYOC* in cultured ocular and nonocular cells. They demonstrated that, while native *MYOC* is expressed inside the cells and secreted into the medium, mutated *MYOC* with the p.K423E change was not secreted into the medium.[29] This observation strongly supports the pathogenic role of the p.K423E mutation present in family 1. The mutation of family 2, p.C25R, is located at the N terminus of the *MYOC* gene, inside the signal peptide sequence necessary for a correct secretion. The strongly reduced secretion of the protein expected for this second missense mutation, suggested by a computer program (PSORT), supports a pathogenic role of this second mutation and a similar final effect with respect to the first mutation.

The biological interactions of mutant MYOC protein and its role in the pathophysiological characteristics of glaucoma are still unclear. On the one hand, the mutations previously described seem to suggest that heterozygous individuals produce insufficient amounts of extracellular MYOC protein (haploinsufficiency hypothesis). On the other hand, glucocorticoid-induced glaucoma and an animal model orient toward a gain-of-function hypothesis.[28] In fact, for glucocorticoid-induced glaucoma (in the presence of a normal MYOC protein sequence[30]), an increased amount of the MYOC protein is present and *myoc*-null mice, homozygous and heterozygous, are fertile, are viable, and have normal IOP, trabecular meshwork histological features, and retinal and optic nerve morphological features.[28] Against the haploinsufficiency hypothesis is also the recent identification of a patient with a deletion of chromosome 1, encompassing the *TIGR* or *MYOC* gene, but without clinical signs of glaucoma.[31] The gain-of-function hypothesis is also suggested by the fact that in the previously described large French Canadian family with the p.K423E mutation, only heterozygous siblings had glaucoma, whereas 4 homozygous siblings with mutations were asymptomatic for the disease.[26] Heteromultimerization of mutant and wild-type forms of the *MYOC* gene is probably a critical step in the pathogenesis of *MYOC*-induced glaucoma. These complexes could then lead to an accumulation of aberrant *MYOC* gene products in the cy-
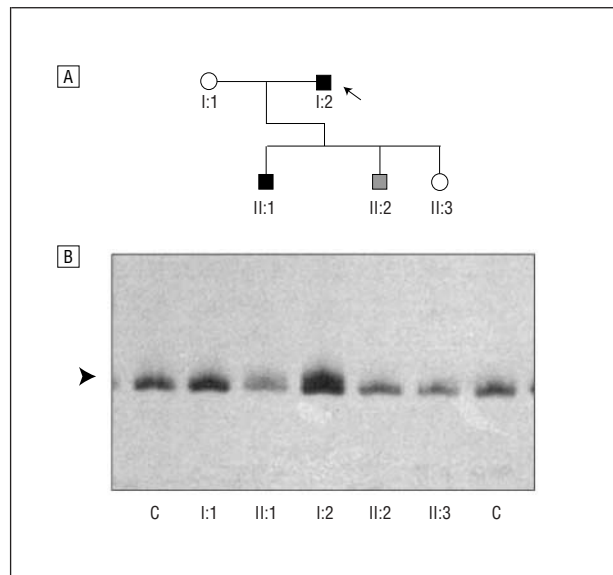

Figure 2. Segregation analysis in family 2. A, Pedigree of the family. Circles indicate females; squares, males; black symbols, individuals with primary open-angle glaucoma or juvenile open-angle glaucoma (the proband is indicated by an arrow); white symbols, unaffected individuals; and gray symbol, individual with pigmentary dispersion glaucoma. B, Single-stranded conformational polymorphism analysis of segment 1B of exon 1. The primers used are as follows: 5'-ACGTTGCTCCAGCTTTGG-3' and 5'-GATGACTGACATGGCCTGG-3'. All 5 members of the family were analyzed. The arrowhead indicates the shifted band present in the individuals with mutations; C, control.

toplasm and/or the extracellular matrix, thereby impairing normal aqueous humor outflow.[28,29]

The p.K423E mutation may be a recurrent mutation in the *MYOC* gene. Alternatively, a founder effect may be hypothesized, as happens for the hot spot p.G368X.[18] Against this hypothesis is the Italian origin of family 1 and the absence of relationships in Canada or in France.

Clinical characterization of the 2 families with the *MYOC* gene mutation indicates that POAG and JOAG are the 2 sides of a continuum phenotypical spectrum due to a common molecular defect. In one of our families, pigmentary dispersion glaucoma (PDS) was also present. In this family, the p.C25R mutation segregated with POAG and JOAG, but not with PDS. One possible explanation of this result is that the p.C25R substitution is not a disease-causing mutation. Supporting this possibility is the recent finding that some patients with pigmentary glaucoma may have *MYOC* gene mutations.[32,33] Alternatively, patients affected by PDS may have a different disease. Supporting this hypothesis is the in silico prediction (ie, computer-based prediction) of reduced secretion of the mutant protein and linkage analysis studies[34-36] demonstrating locus heterogeneity for PDS. In fact, POAG and PDS have long been considered 2 clinically different diseases,[37,38] and it is conceivable that patients affected by PDS in family 2 may have a mutation in another yet uncovered gene.

The results of our study confirm that *MYOC* gene mutations may be responsible for a fraction (2 [8%] of 26) of families with POAG/JOAG with autosomal dominant inheritance. Because of the low detection rate, a *MYOC* investigation can be effective only in a subset of

patients with familial glaucoma. However, within the few families in whom a causative mutation is found, a molecular genetic test may have great relevance in terms of presymptomatic diagnosis for relatives, who may benefit from early treatment. In conclusion, molecular genetic diagnosis could become interesting in the study and management of patients with familial glaucoma.

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