TIGR/MYOC Gene Sequence Alterations in Individuals with and without Primary Open-Angle Glaucoma

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Purpose. To discover sequence alterations in the *TIGR/MYOC* gene associated with primary open-angle glaucoma (POAG) in Chinese subjects.

Methods. Two hundred one unrelated Chinese patients with POAG and 291 unrelated individuals without glaucoma, aged 50 years or more, were screened for sequence alterations in the *TIGR/MYOC* gene by polymerase chain reaction, conformation sensitive gel electrophoresis, and DNA sequencing. Up to 111 more control subjects were screened for some of the alterations.

Results. Fourteen sequence variants that lead to amino acid changes were identified. Seven were novel: Pro16Leu, Ala17Ser, Leu95Pro, Leu215Pro, Glu300Lys, Glu414Lys, and Tyr471Cys. Of these, Glu300Lys and Tyr471Cys were found only in POAG. Arg46Stop was found in 4 patients with POAG (2.0%) and 9 of 402 control subjects (2.2%); one control subject was homozygous. IOP showed a trend ($P = 0.11$) toward a decrease of 1.5 mm Hg among the control subjects, with Arg46Stop compared with matched control subjects without Arg46Stop. Gly12Arg occurred four times as frequently in control subjects as in patients, but the difference was not statistically significant.

Conclusions. Gly12Arg might be negatively associated with POAG, suggesting a protective effect. Three patients with POAG had a sequence change not found in control subjects, for a frequency of possible disease-causing *TIGR/MYOC* mutations of 1.5% (95% confidence interval [CI] = 0.3%–4.3%). Arg46Stop occurred with similar frequency in patients with POAG and control subjects, suggesting that the reduced amount of TIGR/MYOC predicted to result from this truncation does not dramatically increase or decrease risk of glaucoma. (*Invest Ophthalmol Vis Sci.* 2002;43:3231–3235)

Glaucoma, a degeneration of the optic nerve fibers, is one of the major contributors to visual field loss and blindness.[1] Primary open-angle glaucoma (POAG) is the major type of glaucoma, affecting almost 2% of the world's population.[2] Onset of the disease can occur at a young age or later in life, but the prevalence increases with age. The causes of POAG are not known, although risk factors include high intraocular pressure (IOP), positive family history, age, and the presence of diabetes and/or hypertension.[3–6]

The first identified gene with mutations that cause POAG was *TIGR/MYOC*.[7] Approximately 2% to 4% of POAG cases are probably due to *TIGR/MYOC* mutations.[8–11] Most tissues of the eye express TIGR/MYOC protein, including trabecular meshwork, sclera, ciliary body, and retina and nonocular tissues such as the lungs, heart, and pancreas.[12–14] At the cellular level TIGR/MYOC is present in the extracellular matrix of both glaucomatous and normal trabecular meshwork, colocalizing with fibronectin, laminin, and type IV collagen.[15,16] The function of TIGR/MYOC is unknown. In fact, it is not necessary for apparently normal eye development or function, and its absence does not cause glaucoma.[11,17] The mechanism whereby mutations in *TIGR/MYOC* lead to POAG is also unknown, but the finding that mutant TIGR/MYOC proteins form aggregates that are not secreted suggests that mutant TIGR/MYOC accumulates in trabecular meshwork cells and disturbs normal cellular function, resulting in impaired outflow of aqueous humor.[18,19] Administration of glucocorticoids induces expression of TIGR/MYOC and secretion by trabecular meshwork cells, and topical glucocorticoid treatment increases IOP in some individuals.[20] TIGR/MYOC protein with a normal sequence can aggregate and block outflow when injected into the anterior chamber.[21] Thus, one hypothesis is that high levels of normal, secreted TIGR/MYOC protein may be a cause of aqueous outflow obstruction,[20] which subsequently increases IOP and causes POAG.

Many sequence alterations have been found in *TIGR/MYOC*, some occur exclusively in patients with POAG and segregate with disease in POAG-affected families, suggesting they are disease-causing mutations.[7–10,13,23–32] These are mainly missense alterations in exon 3, although many exon 3 sequence alterations have also been found that are not associated with disease. However, the majority of patients with sporadic POAG do not have mutations in *TIGR/MYOC*.[8–10] Discovery of the range of mutation types and locations may shed light on the role of *TIGR/MYOC* in POAG and normal eye function. Because some mutations may be ethnicity specific, it is important to conduct case–control mutation studies in various populations. Only one large study on *TIGR/MYOC* mutations in the Chinese has been published, in which we examined 91 patients with POAG, 132 control subjects, and family members of some patients.[11] To uncover further detail of the function of *TIGR/MYOC*, we examined an additional 110 patients with POAG, 270 control subjects, and some family members and analyzed them in combination with the earlier subjects.

Materials and Methods

Study Subjects

Two hundred one unrelated patients with POAG were recruited from the Eye Clinic of the Prince of Wales Hospital, Hong Kong. These included 91 patients as previously reported.[11] POAG was diagnosed on the fulfillment of all the following criteria: exclusion of secondary causes (e.g., trauma, uveitis, or steroid-induced or neovascular glaucoma), open anterior chamber angle, IOP more than 21 mm Hg,

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TABLE 1. Number of Study Subjects with Sequence Alterations in *TIGR/MYOC*, Probabilities for Association with POAG, and Previously Unreported Alterations

Sequence Alteration	Location	POAG Patients (n = 201)	Control Subjects (n = ≥291)	P	Novel
1–83 (G→A)	Promoter	28 (14.0)	70/388 (18.0)	0.20	
Gly12Arg (34G→C)	Exon 1	1 (0.5)	8/388 (2.1)	0.17	
Pro16Leu (47C→T)	Exon 1	0 (0.0)	1/388 (0.3)	1.0	Yes
Ala17Ser (49G→T)	Exon 1	0 (0.0)	1/291 (0.3)	1.0	Yes
Arg46Stop (136C→T)	Exon 1	4 (2.0)	9/402 (2.2)	1.0	
Arg76Lys (227G→A)	Exon 1	29 (14.4)	70/388 (18.0)	0.27	
Arg91Stop (271C→T)	Exon 1	1 (0.5)	0/388 (0.0)	0.34	*
Leu95Pro (284T→C)	Exon 1	0 (0.0)	1/291 (0.3)	1.0	Yes
Thr123Thr (369C→T)	Exon 1	2 (1.0)	5/291 (1.7)	0.71	
Asp208Glu (624C→G)	Exon 2	2 (1.0)	3/388 (0.8)	1.0	
Leu215Pro (644T→C)	Exon 2	0 (0.0)	1/291 (0.3)	1.0	Yes
(730+35A→G)	Intron 2	63 (31.3)	117/388 (30.2)	0.77	
Ala260Ala (780A→G)	Exon 3	2 (1.0)	1/291 (0.3)	0.57	
Ile288Ile (864C→T)	Exon 3	0 (0.0)	4/291 (1.4)	0.15	
Glu300Lys (898G→A; 900G→A)	Exon 3	1 (0.5)	0/291 (0.0)	0.41	Yes
Gln309Gln (927G→A)	Exon 3	0 (0.0)	1/291 (0.3)	1.0	
Thr353Ile (1058C→T)	Exon 3	4 (2.0)	6/388 (1.6)	0.74	
Glu414Lys (1240G→A)	Exon 3	0 (0.0)	1/291 (0.3)	1.0	Yes
Tyr471Cys (1412A→G)	Exon 3	1 (0.5)	0/291 (0.0)	0.41	Yes
1515+73 (G→C)	3′UTR	2 (1.0)	10/291 (3.4)	0.13	

Data are number of subjects, with the percentage of the total group in parentheses.
* Previously reported only in our study on a subset of these subjects.[11]

characteristic optic disc changes (e.g., vertical cup-to-disc ratio more than 0.3, thin or notched neuroretinal rim or disc hemorrhage), and characteristic visual field changes with reference to Anderson's criteria for minimal abnormality in glaucoma.[32]

Some relatives of the index patients were also examined and genotyped, but were not included in statistical analyses. Four hundred two unrelated control subjects were recruited from people at least 50 years of age who attended the clinic to accompany patients or to obtain treatment for conditions other than glaucoma, including cataract, refractive errors, and itchy eyes. All study subjects were given a complete ocular examination, and blood was collected. Approval for the study protocol was obtained from the Ethics Committee for Human Research and the Chinese University of Hong Kong, and the protocol followed the tenets of the Declaration of Helsinki. Informed consent was obtained from the study subjects.

TIGR/MYOC Sequence Analysis

Genomic DNA was extracted from 200 μL of blood using a kit (Qiamp Blood Kit; Qiagen, Hilden, Germany). The three coding exons and adjacent sequences of *TIGR/MYOC* were screened for sequence alterations by polymerase chain reaction in seven amplicons, as previously reported,[11] followed by conformation-sensitive gel electrophoresis.[33–35] Samples corresponding to bands of altered mobility were sequenced on an automated DNA sequencer (model 377; Applied Biosystems, Foster City, CA).[36] For some sequence alterations, 97 or 111 additional control subjects were screened by PCR, followed by restriction endonuclease assays or direct sequencing.[11]

Statistical Analysis

The computer and several software programs (SPSS; SPSS Inc., Chicago, IL; Epi6, Centers for Disease Control, Atlanta, GA; and Excel; Microsoft, Redmond, WA) were used for statistical calculations. Significance of the difference in distribution of each sequence change between patients and control subjects was determined by χ^2 tests when all expected values were 5 or more and by the Fisher exact two-sided test otherwise. Significance of difference in IOPs between subjects with and without Arg46Stop was determined by both unpaired and paired t-test. Samples were paired by age, with the average of three samples without Arg46Stop being paired with each sample with Arg46Stop. In patients, the IOP used was the level measured before treatment.

RESULTS

Twenty sequence alterations were identified in our study subjects (Table 1): 2 were nonsense sequence changes, 11 were missense changes in encoded amino acid residues, 4 were synonymous codon changes, and 3 were changes in noncoding sequences. Seven were novel: Pro16Leu, Ala17Ser, Leu95Pro, Leu215Pro, Glu300Lys, Glu414Lys, and Tyr471Cys.

No sequence changes showed a significant difference between patients and control subjects. However, three sequence changes were found only in POAG, in one patient each: Arg91Stop (previously reported), Glu300Lys, and Tyr471Cys. These patients' clinical features are shown in Table 2. No relatives of these three patients were available for examination. Gly12Arg was observed four times as frequently in control subjects as in patients, yet this difference was not significant. The lone patient with POAG with Gly12Arg had an age of first examination of 80 years, highest IOP of 26 in both eyes, and cup-to-disc ratio of 0.3 in the right eye and 0.8 in the left eye. Eight other sequence changes were also found at least twice as frequently in control subjects as in patients with POAG in our sample. The promoter polymorphism 1-83 (G→A) usually occurred with the Arg76Lys polymorphism. The most common polymorphism was 730+35 (A→G), in intron 2.

Arg46Stop was found in four patients with POAG (2.0%; Table 2). One has been reported before,[11] and the other three were detected in this study. The sequence change was also identified in 9 of 402 control subjects (2.2%), one of whom was homozygous. She was a 77-year-old woman with no eye disease except cataracts. The maximum observed IOP was 11 mm Hg in both eyes (Table 3). The average of the IOPs recorded in the right and left eyes are plotted for the 9 control subjects and 4 patients with Arg46Stop and for 27 control subjects and 12 patients who did not have Arg46Stop and who were age- and sex-matched to each of those 13, or 3 matching individuals without Arg46Stop for each one with Arg46Stop (Fig. 1). The average age of the control subjects with Arg46Stop was 74.7 ± 8.4 years (SD), and that of the matched control subjects without Arg46Stop was 74.7 ± 7.8 years. The control subjects with Arg46Stop consisted of 8 women and 1 man, and the matched control subjects without Arg46Stop consisted of 24 women

TABLE 2. Clinical Features of Patients with POAG Who Had *TIGR/MYOC* Sequence Alterations

Codon Change	Subject	Sex	Age at Diagnosis (y)	Visual Acuity		Highest Known IOP (mm Hg)		Cup-to-Disc Ratio	
				OD	OS	OD	OS	OD	OS
Arg91Stop*	P184	M	50	20/20	20/50	34	32	0.3	0.3
Glu300Lys*	P387	M	75	20/30	20/40	20	23	0.6	0.4
Tyr471Cys*	P394	M	66	20/200	20/100	25	25	0.6	0.6
Arg46Stop	P161	M	4	LP	20/50	35	18	1.0	0.3
Arg46Stop	P475	F	48	20/50	20/50	34	28	0.3	0.4
Arg46Stop	P512	M	73	20/40	20/40	23	19	0.8	0.8
Arg46Stop	P515	F	61	7/200	7/200	25	25	0.3	0.3

LP, light perception.
* Sequence alterations not found in control subjects.

and 3 men. The average IOP was 11.8 ± 2.7 mm Hg in the control subjects with Arg46Stop and 13.3 ± 2.2 in those without Arg46Stop. Comparison of IOPs of these groups by two-tailed unpaired *t*-test resulted in $P = 0.11$ or $P = 0.17$ by paired *t*-test (each triplet without Arg46Stop was averaged for pairing). The average age of patients with Arg46Stop was 46.5 ± 30 years and 46.8 ± 24 years without. They consisted of two men and two women with Arg46Stop and six men and six women without. The average IOP (measured before treatment) was 25.9 ± 4.1 with Arg46Stop and 27.6 ± 4.8 without. Comparison of IOPs of these groups by two-tailed unpaired *t*-test resulted in $P = 0.52$ or $P = 0.58$ by paired *t*-test. When the patients and control subjects were combined, the average age was 66 ± 21 years with Arg46Stop and 66 ± 19 years without. The average IOP was 16.2 ± 7.4 with Arg46Stop and 17.7 ± 7.4 without. Comparison of IOPs by two-tailed unpaired *t*-test resulted in $P = 0.52$ or $P = 0.15$ by paired *t*-test.

DISCUSSION

Three patients with POAG in this study each had a unique sequence change—Arg91Stop, Glu300Lys, and Tyr471Cys—not found in control subjects, for a frequency of possible disease-causing *TIGR/MYOC* mutations of 1.5%. Two of these sequences changes are novel and one, Arg91Stop, has been reported.[11] The 95% CI for this proportion, 0.3% to 4.3%,[37] is consistent with the range of 2% to 4% reported in various ethnic groups.[9] Mutations are disease-causing sequence alterations. However, these three sequence changes have not been reported in other populations and have not been linked with glaucoma by statistics, segregation in families, or biochemical studies.[18,19] Therefore, they cannot yet be conclusively termed mutations. Because the nearby truncation Arg46Stop may not affect disease risk, Arg91Stop may also not affect the risk for POAG.

Asp208Glu and Thr353Ile, which have been reported as possible glaucoma-causing mutations,[9,38] were found in control subjects in this study. Therefore, these are not disease-causing mutations, although it remains possible that they affect the risk for POAG. It is possible that other such assignments of perhaps harmless sequence changes as disease-causing mutations have occurred, suggesting that reported frequencies of disease-causing *TIGR/MYOC* mutations may be somewhat overstated. This study has identified seven predicted protein sequence alterations not reported elsewhere, including our earlier study of a subset of these subjects.[11] Five of these alterations were found in control subjects, lending some support to the above assertion that many rare sequence alterations in TIGR/MYOC may not be disease-causing mutations.

TABLE 3. Average IOP of Control Subjects with *TIGR/MYOC* Sequence Alteration Arg46Stop

Subject	Sex	Age (y)	IOP (mm Hg)
C57	F	78	10.5
C155	F	66	17.5
*C177	F	77	11.0
C182	F	79	11.5
C203	F	61	15.0
C221	M	70	11.5
C238	F	80	9.5
C311	F	89	10.0
C362	F	72	10.0

* This subject is homozygous for Arg46Stop.

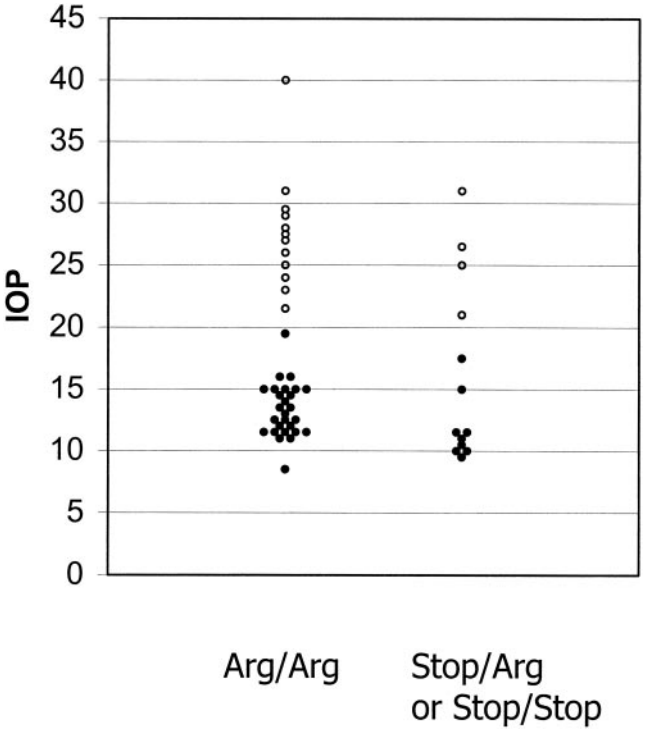

FIGURE 1. IOPs of age- and sex-matched control subjects versus genotype at codon 46. IOPs of patients were measured before treatment. All IOPs in patients are higher than 20 mm Hg (○), whereas those of all control subjects are less than 20 mm Hg (●).

Arg76Lys, the most common protein sequence polymorphism, was not associated with POAG. It usually occurred with the promoter polymorphism 1-83 (G→A), as in a Japanese study[38] (although a few subjects had either polymorphism without the other), suggesting that this haplotype has entered the population recently.

Our earlier study of a subset of these subjects found a trend toward an association of the Gly12Arg sequence alteration with the absence of POAG.[11] The additional subjects reported herein allowed us to reexamine this trend. When unrelated control subjects were compared with patients with POAG (Table 1), there was still a trend toward an association of Gly12Arg with the absence of POAG (Fisher exact two-tailed test, $P = 0.18$). Although the difference in frequency of Gly12Arg between POAG and control subjects was large (odds ratio = 0.24), the low frequency of this sequence change resulted in a power to achieve $P < 0.05$ at these frequencies of only 16%, even with our increased sample sizes. Gly12Arg has been reported only in the Chinese and Japanese. Combining our results with those from two studies of Japanese patients with POAG and control subjects,[9,38] which found Gly12Arg in 1 of 226 patients with POAG and 2 of 149 control subjects, showed a frequency of 0.5% in POAG and 1.9% in control subjects ($P = 0.053$; odds ratio = 0.25). These results suggest Gly12Arg may protect against POAG. Because it is located near the hydrophobic signal sequence, the change to a positively charged amino acid may hinder secretion. Because injection of a nonglycosylated bacterial TIGR/MYOC recombinant protein into the anterior chamber of a perfused anterior segment culture increases IOP,[21] and because glucocorticoids can both stimulate TIGR/MYOC expression and increase IOP,[20] an increase in the level of secreted TIGR/MYOC may increase IOP and cause POAG. Conversely, a decrease in TIGR/MYOC secretion may reduce risk for POAG. However, predictions of the signal sequence and cleavage site locations and probabilities did not differ between TIGR/MYOC sequences with Gly and with Arg at position 12. For both Gly- and Arg-containing sequences, the probability of cleavage between amino acids 32 and 33 was 61%.[39,40] Another possible mechanism for an effect of N-terminal TIGR/MYOC sequence changes on disease risk is suggested by the finding that the region between amino acids 15 and 138 is needed to localize TIGR/MYOC to microtubules inside cells.[41] Sequence changes in this region may alter subcellular localization and function of TIGR/MYOC or of trabecular meshwork cells.

Another missense change that increases the positive charge has been reported in the signal sequence. Gln19His was found in 1 of 1703 patients with POAG and 1 of 238 control subjects in a multiethnic study.[9] The control was homozygous. The frequency is too low to determine statistical significance, but these results are consistent with those of Gly12Arg. Although not found in our original study,[11] this expanded study revealed a total of two other sequence changes in or near the signal sequence Pro16Leu and Ala17Ser. Both of these previously unreported alterations were found only in control subjects, consistent with Gly12Arg. These three signal sequence changes, when combined, show a trend toward a frequency difference between patients and control subjects (1/201 = 0.5% vs. 10/388 = 2.6%, odds ratio = 0.19, Fisher exact two-tailed $P = 0.11$), also suggesting a protective effect. Transfected cell culture studies may show whether these sequence alterations affect secretion of TIGR/MYOC or localization.

If TIGR/MYOC protein containing Arg46Stop were translated and processed, it would be only 13 amino acids long after cleavage of the signal sequence.[20] However, mRNA carrying nonsense mutations often undergoes nonsense-mediated decay. Thus, Arg46Stop may essentially reduce expression of TIGR/MYOC by half in heterozygotes and eliminate expression in homozygotes. To test the hypothesis that blocking TIGR/MYOC secretion lowers IOP and therefore risk of POAG, we may ask whether Arg46Stop is found more frequently in control subjects than in patients with POAG. Our earlier study found 46Stop in 1 of 91 patients and 4 of 132 control subjects.[11] Our expanded study shows no difference in frequency (Table 1, $P = 1.0$, odds ratio = 0.9) and thus does not support the hypothesis, although neither was it disproved, because the power to show a difference was small (7% power to detect odds ratio = 0.5 at $\alpha = 0.05$). The additional subjects acquired since our previous study also allowed examination of the relation between 46Stop and IOP. Although the average IOP did not significantly differ between subjects with or without a 46Stop allele, the truncation was associated with a trend toward a decrease of 1.5 mm Hg (Fig. 1), which would reach significance in control subjects ($P < 0.05$, unpaired *t*-test) if the number with 46Stop could be doubled while maintaining the same IOP distribution.

Our results do not directly address the validity of the complementary hypothesis that elevated levels of secreted TIGR/MYOC increase the risk of POAG, except that such a mechanism is probably not the cause of most cases of POAG, because otherwise Arg46Stop heterozygotes would presumably have a significantly reduced risk of POAG. When truncated TIGR/MYOC similar to the common Gln368Stop mutant form (but terminated at 345) is overexpressed by delivering an adenoviral vector to perfused human anterior segment cultures, the level of secreted full-length TIGR/MYOC protein decreased 30%, whereas outflow facility increased by a similar amount.[42] Although it is unknown whether this decrease in secretion of TIGR/MYOC is the factor responsible for increased outflow, it is possible that reduced levels of secreted TIGR/MYOC may reduce IOP and hence the risk of POAG. Paradoxically, Gln368Stop increases the risk of POAG in humans. Perhaps there are two opposite effects of such exon 3 mutations: a rapid decrease of IOP due to inhibition of secretion of TIGR/MYOC, and an eventual increase of IOP due somehow to an intracellular aggregation of mutant TIGR/MYOC. These effects would correspond with the time scales observed in the present experiment (a few days) and in the development of POAG in people with *TIGR/MYOC* mutations (years).

Other hypotheses have been proposed for the role of TIGR/MYOC in POAG. One of them suggests that secreted TIGR/MYOC is necessary to maintain normal aqueous outflow.[19] The first report of Arg46Stop included a homozygous carrier who had severe POAG,[43] consistent with this hypothesis. However, our Arg46Stop results, especially the existence of an elderly homozygote with normal IOP,[11] demonstrate that TIGR/MYOC is not needed to maintain aqueous outflow. Further undermining this hypothesis are the findings that mice engineered so that they do not express TIGR/MYOC had normal eyes and IOP,[17] and that one person missing one copy of the *TIGR/MYOC* gene (due to a partial chromosome 1 deletion) had normal IOP and no glaucoma.[44]

Another hypothesis holds that mutant TIGR/MYOC aggregates intracellularly and blocks secretory pathways, damaging trabecular meshwork cells and blocking outflow.[19,45] Loss of TIGR/MYOC would not necessarily increase or decrease IOP; therefore, this hypothesis is consistent with our Arg46Stop results. Meanwhile, TIGR/MYOC interacts with the Hep II domain of fibronectin, and such specific protein–protein interactions may affect expression of TIGR/MYOC, the signaling events and even formation of the matrix.[16] These may be mechanisms that affect aqueous outflow. However, an independent mechanism would be needed to explain the possible negative association of Gly12Arg with POAG. Further genetic studies in Asian populations may help to confirm these associations and suggest whether new hypotheses are needed.

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