Human Molecular Genetics, 1999, Vol. 8, No. 5 **899–905**

Analysis of myocilin mutations in 1703 glaucoma patients from five different populations

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Received December 24, 1998; Revised and Accepted February 14, 1999

A glaucoma locus, *GLC1A*, was identified previously on chromosome 1q. A gene within this locus (encoding the protein myocilin) subsequently was shown to harbor mutations in 2–4% of primary open angle glaucoma patients. A total of 1703 patients was screened from five different populations representing three racial groups. There were 1284 patients from primarily Caucasian populations in Iowa (727), Australia (390) and Canada (167). A group of 312 African American patients was from New York City and 107 Asian patients from Japan. Overall, 61 different myocilin sequence variations were identified. Of the 61 variations, 21 were judged to be probable disease-causing mutations. The number of probands found to harbor such mutations in each population was: Iowa 31/727 (4.3%), African Americans from New York City 8/312 (2.6%), Japan 3/107 (2.8%), Canada 5/167 (3.0%), Australia 11/390 (2.8%) and overall 58/1703 (3.4%). Overall, 16 (76%) of 21 mutations were found in only one population. The most common mutation observed, Gln368Stop, was found in 27/1703 (1.6%) glaucoma probands and was found at least once in all groups except the Japanese. Studies of genetic markers flanking the myocilin gene suggest that most cases of the Gln368Stop mutations are descended from a common founder. Although the specific mutations found in each of the five populations were different, the overall frequency of myocilin mutations was similar (~2–4%) in all populations, suggesting that the increased rate of glaucoma in African Americans is not due to a higher prevalence of myocilin mutations.

INTRODUCTION

The glaucomas are a group of diseases that share a clinical phenotype characterized primarily by progressive degeneration of the optic nerve. These degenerative changes cause a gradual loss of visual field and are often accompanied by increased intraocular pressure. Glaucoma is a leading cause of blindness in developed nations. In the USA, glaucoma is the second most common cause of permanent blindness. One to two percent of Americans over the age of 40 are affected with glaucoma, and each year 12 000 are blinded by the disease (1,2). Of the many forms of glaucoma, primary open angle glaucoma (POAG) is by far the most common. The prevalence of POAG, however, varies among populations. African Americans are affected with POAG approximately five times more frequently than the general American population (3).

Although the pathophysiology of the degenerative changes in POAG is poorly understood, there is strong evidence that genetic factors play a role in this process. The prevalence of glaucoma among first degree relatives of glaucoma patients is as much as eight times that of the general population (4). Also, there are reports of pedigrees demonstrating clear Mendelian inheritance of glaucoma (5). In the last 6 years, the chromosomal loci of several genes involved in open angle glaucoma have been identified (6–10). The first of these loci, *GLC1A*, was mapped to chromosome 1q by linkage analysis of a large pedigree affected with juvenile onset open angle glaucoma (6). The *GLC1A* locus was refined by recombination and haplotype analysis of additional glaucoma families (11). Myocilin subsequently was shown to be the glaucoma gene at the *GLC1A* locus (12). Mutations in

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this gene are associated with most if not all familial cases of juvenile onset open angle glaucoma as well as ~3% of all POAG in a Midwestern American population (12,13).

The myocilin gene originally was cloned from eye tissue [trabecular meshwork cell culture (14,15), ciliary body (16) and retina (17)]. It encodes a protein of unknown function and has a broad range of expression not limited to the eye (18). Sequence analysis has shown that the third exon of the myocilin gene encodes a peptide sequence that is homologous to olfactomedin, a protein secreted by nasal epithelium (17). The vast majority of myocilin mutations have been identified in this olfactomedin homology domain.

Mutations in the myocilin gene have been reported in glaucoma patients and pedigrees from many different ethnic backgrounds (12,13,19–28). In this report, 1703 glaucoma probands from Iowa, New York City (African Americans), Australia, Canada and Japan were studied to assess the role of myocilin in these populations.

RESULTS

Screening 1703 POAG probands from Iowa, New York City (all African Americans), Australia, Canada and Japan for myocilin gene mutations revealed 61 different sequence variations. Twenty one of these variations were judged to be probable disease-causing mutations because they: (i) altered the predicted myocilin amino acid sequence; (ii) were present in one or more glaucoma subjects; (iii) were present in <1% of the general population; and (iv) were absent from the normal controls.

Probable disease-causing mutations were identified in probands from all five glaucoma populations (Table 1). The numbers of probands found to harbor probable disease-causing mutations in each population were: Iowa 31/727 (4.3%), African Americans from New York City 8/312 (2.6%), Japan 3/107 (2.8%), Canada 5/167 (3.0%), Australia 11/390 (2.8%) and overall 58/1703 (3.4%). Most of the myocilin mutations (16/21) were identified in isolated glaucoma probands or kindreds. The Gln368Stop mutation, however, was found in 27 glaucoma probands, and accounted for >40% of the total probable disease-causing mutations detected in this study. Screening results on 716 of 1703 glaucoma probands (mostly from Iowa) in this study have been reported previously (13).

Many myocilin gene sequence variations were judged to be non-disease-causing polymorphisms because they were present in normal controls or because they did not alter the predicted amino acid sequence of the myocilin protein (Tables 2 and 3). All but six of 40 polymorphisms were observed at a frequency of <1%. In fact, more than half (55%) of the polymorphisms were rare, population-specific variations that were detected only once in the entire study of 2496 glaucoma subjects and controls.

Table 1. Probable glaucoma-causing mutations

Sequence change	Exon	Effect of change[a]	Iowan		New York City		Australian		Canadian	Japanese		Caucasian	African American	
			POAG	Normal	African American		POAG	Normal	POAG	POAG	Normal	general	general	
			n = 727	*n* = 91	POAG	Normal	*n* = 390	*n* = 58	*n* = 157	*n* = 107	*n* = 49	population	population	
					n = 312	*n* = 40						*n* = 505	*n* = 50	Total
Arg45Stop	1	Premature termination	0	0	0	0	0	0	0	1	0	0	0	1
17 bp dup 56–72 bp	1	Multiple	0	0	1	0	0	0	0	0	0	0	0	1
Arg82Cys[b,c]	1	Change in charge	1	0	0	0	1	0	0	0	0	0	0	2
Trp286Arg[b]	3	Change in charge	1	0	0	0	0	0	0	0	0	0	0	1
Thr293Lys[b,c]	3	Change in charge	1	0	1	0	0	0	0	0	0	0	0	2
Glu352Lys[c,d]	3	Change in charge	1	0	2	0	0	0	2	0	0	1	0	6
Thr353Ile	3	Change in polarity	0	0	0	0	0	0	0	1	0	0	0	1
Pro361Ser[b]	3	Change in polarity	1	0	0	0	0	0	0	0	0	0	0	1
Gly364Val[b]	3	None	2	0	0	0	0	0	0	0	0	0	0	2
Gln368Stop[b,c]	3	Premature termination	16	0	1	0	8	0	2	0	0	1	0	28
Thr377Met[b,c]	3	Change in polarity	1	0	0	0	1	0	0	0	0	0	0	2
Ser393Arg	3	Change in charge	0	0	1	0	0	0	0	0	0	0	0	1
Tyr437His[b]	3	Change in charge	4	0	0	0	0	0	0	0	0	0	0	4
Ala445Val[b]	3	None	0	0	0	0	1	0	0	0	0	0	0	1
1 bp del codon 453	3	Multiple	0	0	1	0	0	0	0	0	0	0	0	1
Ile465Met	3	None	0	0	0	0	0	0	0	1	0	0	0	1
Arg470Cys[b]	3	Change in charge	1	0	0	0	0	0	0	0	0	0	0	1
Ile477Asn[b]	3	Change in polarity	1	0	0	0	0	0	0	0	0	0	0	1
Pro481Thr	3	Change in polarity	1	0	0	0	0	0	0	0	0	0	0	1
Pro481Leu	3	None	0	0	1	0	0	0	0	0	0	0	0	1
Glu483Stop	3	Premature termination	0	0	0	0	0	0	1	0	0	0	0	1
Total			31	0	8	0	11	0	5	3	0	2	0	60

[a]Non-conservative changes were defined as sequence changes that altered the charge, size or polarity of the predicted myocilin protein.
[b]Previously reported mutations (13).
[c]Mutations identified in multiple populations.
[d]This mutation was reported previously as a polymorphism (13).

Table 2. Probable polymorphisms

Sequence change	Effect of change[a]	Iowan POAG *n* = 727	Normal *n* = 91	New York City African American POAG *n* = 312	Normal *n* = 40	Australian POAG *n* = 390	Normal *n* = 58	Canadian POAG *n* = 167	Japanese POAG *n* = 107	Normal *n* = 49	Caucasian general population *n* = 505	African American general population *n* = 50	Overall frequency (%)
Promoter: bp –83 (g→a)[b]	None	121	22	33	0	55	–	25	–	–	85	5	15
Phe4Ser	Change in polarity	0	0	1	0	1	1	0	0	0	0	0	0.12
Cys9Ser[b]	None	0	1	1	0	0	0	0	0	0	0	0	0.080
Gly12Arg	Change in charge	0	0	0	0	0	0	0	1	2	0	0	0.12
Gln19His[c]	Change in charge	1	0	0	0	0	0	0	0	1[d]	0	0	0.12
Asn73Ser	None	0	1	0	0	0	0	0	0	0	0	0	0.040
Arg76Lys[b]	None	12	18	1	0	9	0	3	3	0	-	0	2.3
Arg82His	None	0	0	0	0	0	0	0	0	0	0	1	0.040
Val118Leu	None	0	0	0	0	0	0	0	0	0	0	1	0.040
Arg189Gln	Change in charge	0	0	0	1	0	0	0	0	0	0	0	0.040
Intron 1 bp 14 (g→a)	None	0	0	1	0	0	0	0	0	0	0	0	0.040
Intron 1 bp 19 (g→c)	None	0	0	2	1	5	0	0	2	0	0	2	0.48
Ser203Phe[b]	Change in polarity	0	1	0	0	0	0	0	0	0	0	0	0.040
Intron 2 bp 3 (a→g)	None	0	0	0	0	1	0	0	0	0	0	0	0.040
Val329Met	None	0	0	2	0	0	0	0	0	0	0	1	0.12
Lys398Arg[b]	None	7	4	5	0	6	0	2	0	0	3	0	1.1
Val402Ile	None	0	0	0	0	0	0	0	0	0	0	1	0.040
Arg422Cys	Change in charge	0	0	0	0	0	0	0	0	0	1	0	0.040
Ser425Pro	Change in polarity	0	0	0	0	0	0	0	0	0	1	0	0.040
Tyr473Cys	None	0	0	0	0	0	0	0	0	0	1	0	0.040
Val495Ile[b,e]	None	1	0	0	0	0	0	0	0	0	0	0	0.040
Lys500Arg[c]	None	1	0	4	0	0	0	1	0	0	0	1	0.28

[a]Non-conservative changes were defined as sequence changes that altered the charge, size or polarity of the predicted myocilin protein.
[b]Previously reported mutations (13).
[c]These polymorphisms were reported previously as mutations (13).
[d]This control subject is homozygous for the Gln19His variation.
[e]This variation was judged a probable polymorphism because it was found in the same allele as a Thr377Met mutation in two affected members of a single glaucoma family (13).

Table 3. Synonymous codon changes

Sequence change	Iowan POAG *n* = 727	Normal *n* = 91	New York City African American POAG *n* = 312	Normal *n* = 40	Australian POAG *n* = 390	Normal *n* = 58	Japanese POAG *n* = 107	Normal *n* = 49	Canadian POAG *n* = 167	Caucasian general population *n* = 505	African American general population *n* = 50	Overall frequency (%)
Pro13Pro	2	1	30	6	0	1	0	0	2	3	6	2.0
Ser69Ser	0	0	0	0	0	0	1	0	0	0	0	0.040
Thr88Thr	0	0	0	0	0	0	0	1	0	0	0	0.040
Glu96Glu	0	0	0	0	0	0	0	1	0	0	0	0.040
Gly122Gly	3	0	1	0	3	0	0	0	0	2	0	0.36
Leu159Leu	0	1	10	0	0	0	0	0	0	2	0	0.52
Val170Val	0	0	0	0	0	0	1	0	0	0	0	0.040
Thr204Thr	0	0	10	1	0	0	0	0	0	1	0	0.48
Lys216Lys	0	0	0	0	1	0	0	0	0	0	0	0.040
Lys266Lys	1	0	0	0	0	0	0	0	0	0	0	0.040
Thr285Thr	9	1	0	0	2	0	0	0	0	0	0	0.48
Thr325Thr	4	1	52	9	0	1	0	0	5	8	8	3.5
Val329Val	1	0	0	0	0	0	0	0	0	0	0	0.040
Tyr347Tyr	40	8	8	0	25	2	0	0	8	23	1	4.6
Thr351Thr	0	0	1	0	0	0	0	0	0	0	0	0.040
Glu396Glu	1	0	13	3	0	0	0	0	0	1	0	0.72
Val439Val	0	0	0	0	0	0	0	0	0	1	0	0.040
Ala488Ala	0	0	0	0	1	0	0	0	0	0	0	0.040

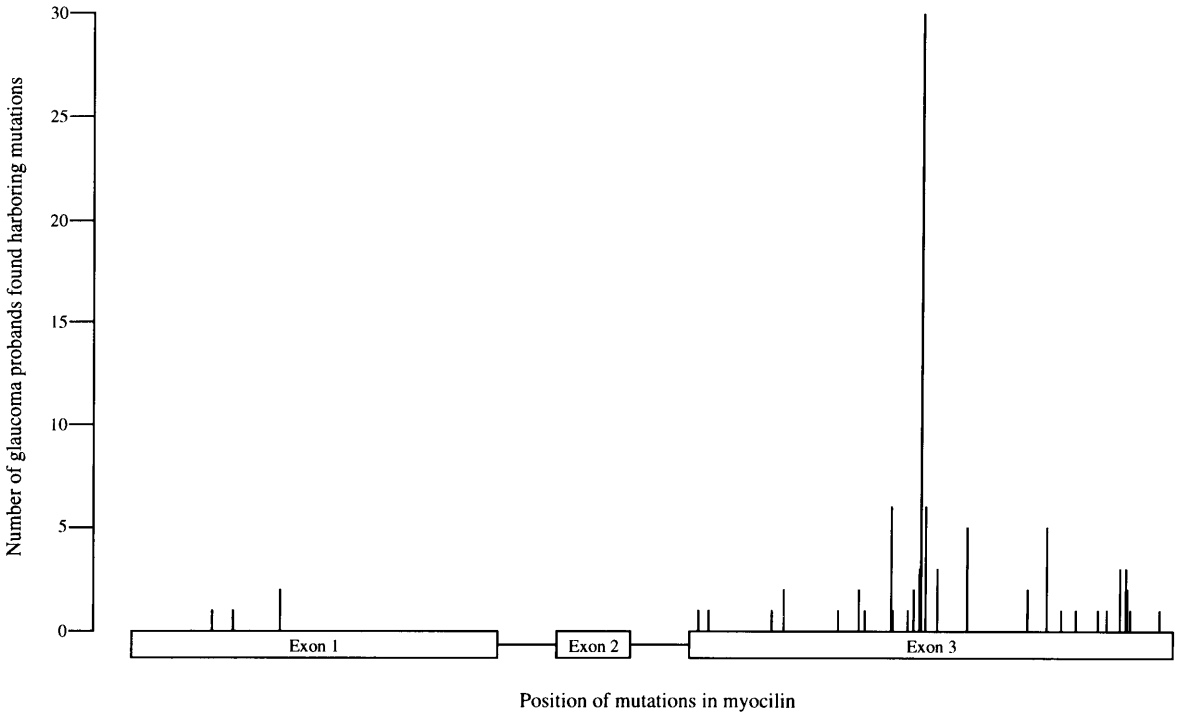

Figure 1. Distribution of mutations across the myocilin gene. The boxes represent the three exons. The location within the myocilin gene of the 23 mutations identified in this study as well as mutations identified in previous studies (12,13,19–28) is indicated by a vertical line. The height of each vertical line represents the total number of glaucoma probands found to harbor a particular mutation.

The effect of the myocilin gene variations on the predicted protein sequence was also evaluated. Sequence changes that altered the polarity, charge or size of the predicted myocilin protein were classified as non-conservative changes. Other variations, including synonymous codon changes and missense changes that encode amino acids of similar character, were classified as conservative changes. Non-conservative variations, which are presumably more injurious to the function of myocilin protein than conservative changes, were found in 57 of 1703 glaucoma probands (3.3%) and 10 of 793 control subjects (1.3%) ($P < 0.01$). Seventeen of the 21 probable glaucoma-causing mutations (81%) were non-conservative sequence changes.

The positions of mutations in the myocilin gene identified in this study as well as in other previous studies (12,13,19–28) are shown in Figure 1. Overall, 27 of 30 mutations (90%) are located in exon 3 (which contains the region of olfactomedin homology), while in the present study, 18 of 21 myocilin mutations (86%) occurred within this exon. Mutations were found predominantly in exon 3 in each of the individual populations.

Although myocilin mutations were detected in similar fractions of all five patient populations (2.6–4.4%), a different set of mutations was identified in each group. The majority of myocilin mutations (77%) were population specific. Five mutations, however, were identified in multiple populations (Table 1). Of these, Gln368Stop, was found in all groups except the Japanese.

The Gln368Stop mutation was identified in POAG probands much more often than any other myocilin mutation. This relatively high frequency suggested the possibility of a founder effect. To explore this possibility, glaucoma probands harboring this mutation were genotyped with short tandem repeat polymorphism (STRP) genetic markers closely flanking the *GLC1A* gene. There was a high degree of allele sharing at all of the markers examined; however, the greatest sharing (100%) was observed at the markers most closely flanking the *GLC1A* gene (Fig. 2). The frequency of the marker alleles observed in the POAG probands was compared with that of ethnically matched normal controls. Although the most closely flanking markers (*MY5* and *MY3*) were of low heterozygosity, the linked alleles of markers *MY5*, *MY3*, *D1S1619* and *D1S2815* were present in the probands at a significantly higher rate than in the controls ($P < 0.05$), providing further support for a Gln368Stop founder effect. Thirty-five sibs harboring the Gln368Stop mutation were genotyped at the flanking markers and also showed a high degree of allele sharing (35/35 at *MY5*). Finally, four of the 27 Gln368Stop probands had affected parents available for study. In all of the four families, the same allele of flanking markers (*D1S1619*, *MY5* and *MY3*) segregated with the Gln368Stop mutation, and a total of six informative meioses were observed. These findings are consistent with a single founder transmitting the Gln368Stop mutation to the 27 POAG probands identified within four different ethnic groups.

DISCUSSION

There are at least two reasons why one might wish to screen a gene for the presence of disease-causing mutations. The first is to try to implicate the gene as a cause of a particular disease, and the second is to identify as many mutations as possible for the purpose of identifying genotype–phenotype relationships, comparing mutation profiles in different populations or constructing practical genetic tests for clinical use. In the first case, one usually compares the frequency of sequence variations in the gene in patients and controls. A statistically significant preponderance of

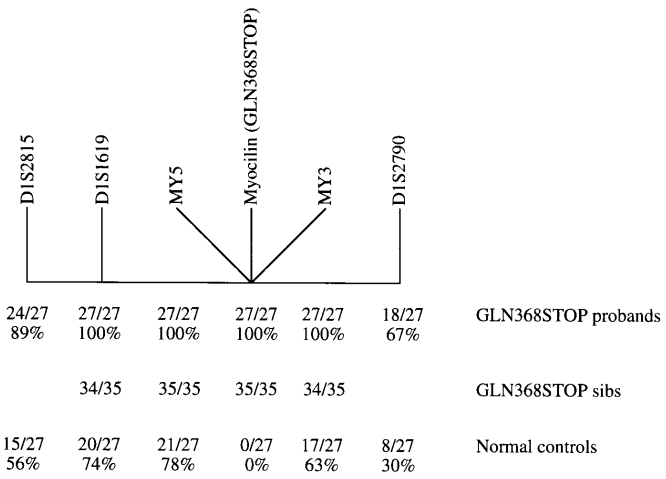

Figure 2. Genotypic evidence of a founder harboring the Gln368Stop mutation. Twenty-seven probands and 35 sibs harboring the Gln368Stop mutation as well as 27 ethnically matched controls were genotyped at five markers closely flanking the myocilin gene. Markers *MY5* and *MY3* are located <1 kb upstream and downstream of the myocilin gene, respectively. The fraction of subjects that shared a common allele of each of the markers is indicated.

mutations in the patients provides evidence for an association between the disease and the mutant gene. In this situation, it is important that the sequence variations included in the statistical analysis are not selected on the basis of their distribution in patients versus controls because such a selection would bias the analysis in favor of finding an association with the disease. Thus, in this and a previous paper (13), we assessed the distribution of all non-conservative sequence variants (regardless of their frequency in patients or controls) when our purpose was to try to infer pathogenicity of the gene itself. In both of these studies, non-conservative myocilin mutations were identified in a significantly higher fraction of glaucoma probands than normal control subjects, thereby providing additional support for the role of the myocilin gene in the pathogenesis of POAG.

A different situation exists when a gene's association with a disease has already been established and one is trying to identify as many clinically relevant mutations as possible. In this case, it is desirable to consider the relative distribution of the mutation in patients and controls because one is looking for mutations that have a sufficiently high penetrance that they will often cause disease by the time a patient reaches the age of the individuals in the study. In this study, we defined such 'probable disease-causing' mutations as those that: (i) altered the myocilin amino acid sequence; (ii) were present in glaucoma patients; (iii) were not commonly observed in the general population; and (iv) were absent from a glaucoma-free control group. Of course, these criteria are not foolproof and would fail to 'recognize' a low-penetrance glaucoma susceptibility allele that is common in both controls and glaucoma patients. These criteria also have the potential to inappropriately assign pathogenicity to a rare, non-disease-causing polymorphism that just happens to alter the amino acid structure of the myocilin protein product. This type of uncertainty is inherent in any assignment of pathogenicity without a functional assay. However, for the type of population comparisons reported herein, any such errors would be expected to be evenly distributed among the populations and would not be expected to affect our interpretations significantly.

As already noted, any one of the specific sequence variations we observed in glaucoma patients may actually be a polymorphism that is skewed randomly between the POAG and control populations. One method of reducing the chances of falsely categorizing a sequence variation as a disease-causing mutation is to increase the size of the population studied. For example, two sequence variations (Gln19His and Lys500Arg) that were each identified in isolated POAG probands and in no Caucasian controls were reported previously by our group as probable disease-causing mutations (13). Since that report, more POAG patients, general population subjects and normal controls have been screened for these variations. In addition to identifying more POAG subjects with the Lys500Arg variation, a single normal control subject (Japanese) was found to harbor a Gln19His variation, and a single general population subject (African American) was found to harbor a Lys500Arg variation. With the addition of these new data, the Lys500Arg variation is present in 1 of 50 (2%) African American general population subjects and no longer meets our criteria for being a probable disease-causing mutation. Similarly, the Gln19His variation no longer meets the criteria for being a probable disease-causing mutation because of its presence in a normal control subject. The probable non-pathogenic nature of the Gln19His polymorphism is supported further by examination of the mouse ortholog of myocilin. The nineteenth residue of the human myocilin protein is a glutamine, while the corresponding residue in the mouse is a histidine, suggesting that a substitution of a histidine for the glutamine residue at this position in the human myocilin gene (Gln19His) is not likely to be associated with disease (18).

Conversely, another myocilin sequence variation (Glu352Lys) was reported previously by our group to be a probable non-disease-causing polymorphism. The Glu352Lys mutation had been identified in a single subject (0.2%) of the Caucasian general population as well as in a single POAG proband (13). Subsequently, another group has reported a Glu352Lys mutation in a POAG proband (26) and, in the current larger study, four additional POAG probands were found to harbor this mutation. The presence of the Glu352Lys mutation in five POAG probands and in no normal controls in our study suggests its association with POAG despite the single general population subject carrying this mutation. These examples underscore the difficulty of assessing the association of rare sequence variations with disease.

Myocilin mutations were found in glaucoma populations from Iowa, New York City, Australia, Canada and Japan. The fraction of glaucoma probands harboring a myocilin mutation ranged from 2.6 to 4.4%, demonstrating that myocilin is a significant cause of disease in these populations. Further, myocilin is associated with approximately the same fraction of glaucoma (~2–4%) in all of these populations. The prevalence of myocilin mutations in the New York City African American glaucoma population was similar to that of the other populations, suggesting that the increased rate of glaucoma in African Americans is not due to a higher prevalence of myocilin mutations.

The vast majority (18 of 21) of probable disease-causing mutations in myocilin occur in exon 3 (olfactomedin homology domain). However, in this study, three probable disease-causing mutations were identified in exon 1. Of these three mutations, two mutations would cause a termination of translation before the domain encoded by exon 3. The remaining mutation is a missense

mutation that was found in two isolated cases of POAG. When the prevalence of non-conservative sequence changes in POAG subjects and controls is compared by exon with Fisher's exact test, there is not a significant difference in exon 1 ($P > 0.05$) but there is a significant difference in exon 3 ($P < 0.01$). These data suggest that mutations in myocilin usually need to affect the portion of the protein encoded by exon 3 in order to cause glaucoma.

Most myocilin mutations (76%) were population specific. However, the Gln368Stop mutation was identified in four of the five glaucoma populations. Overall, this mutation was identified in 27 of 1703 (1.6%) glaucoma probands. If the prevalence of the Gln368Stop mutation observed in this study is an accurate reflection of the prevalence of this variation worldwide, the Gln368Stop mutation is the most common known molecular cause of glaucoma in the world.

A previous study of three Gln368Stop kindreds implied that this mutation arose independently in multiple individuals (29). However, in our study of 27 Gln368Stop probands from four different populations, there was strong evidence of a founder effect for this mutation, suggesting that most POAG families harboring the Gln368Stop mutation did indeed descend from a common ancestor.

Early intervention is critical in the successful treatment of POAG. The loss of vision associated with glaucoma may often be prevented or postponed by existing medical or surgical therapy. The potential benefits of early diagnosis of POAG may someday warrant screening subsets of the general population for mutations in myocilin. For such a large screening to be cost effective, it may be possible to screen for only a few specific myocilin mutations (i.e. the most common ones). This study has identified the most common mutations present in five different populations that may eventually be appropriate to target in such a large population screen.

MATERIALS AND METHODS

POAG subjects and controls

The Human Subjects Review Committee of the University of Iowa approved the study, and informed consent was obtained from all study participants. POAG was defined as the presence of an intraocular pressure >21 mmHg as well as evidence of glaucomatous optic nerve head damage. Visible optic nerve head damage alone was accepted if there was documented enlargement of the optic nerve head cup. Otherwise, both a large optic nerve head cup with a thin neural rim and characteristic optic nerve-related visual field loss were required. Patients were excluded if they had a history of eye surgery prior to the diagnosis of glaucoma or evidence of secondary glaucoma, such as exfoliation or pigment dispersion. Normal volunteers were >40 years of age, had intraocular pressures <20 mmHg, and had no family or personal history of glaucoma. A total of 1703 glaucoma patients was obtained from five different glaucoma populations derived from ophthalmology clinics in Iowa City (727), New York City (312), Australia (390), Canada (167) and Japan (107). The New York City glaucoma patients were all African American. In addition, 238 normal controls were recruited from these populations (91 Iowans, 40 New Yorkers, 58 Australians and 49 Japanese). Finally, 505 Caucasian subjects and 50 African American subjects representing the general population were examined in this study. Of the Caucasian general population subjects, 132 were from Iowa, 210 were from elsewhere in the USA, 79 were from Europe, 19 were from Canada and 13 were from Australia. The African American general population subjects were from New York City. The presence or absence of glaucoma in the general population subjects was not determined. Screening results for 716 glaucoma probands (mostly from Iowa), the Caucasian general population subjects and 91 Iowan normal controls included in this study have been published previously (13).

Mutation screening

Glaucoma patients and control subjects were screened in an identical manner for myocilin mutations using single strand conformation polymorphism (SSCP) analysis. A 12.5 ng aliquot of each patient's DNA was used as template in an 8.35 µl polymerase chain reaction (PCR) containing: 1.25 µl of 10× buffer (100 mM Tris–HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 300 µM each of dCTP, dATP, dGTP and dTTP, 1 pmol of each primer and 0.25 U of Biolase polymerase (ISCBioExpress, Kaysville, UT). The oligonucleotide primer sequences have been reported previously (13). Samples were denatured for 5 min at 94°C and incubated for 35 cycles under the following conditions: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s in a DNA thermocycler (Omnigene, Teddington, UK). After amplification, 5 µl of stop solution (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each sample. Amplification products were denatured for 3 min at 94°C and electrophoresed on 6% polyacrylamide, 5% glycerol gels at 25 W for ~3 h at room temperature. Following electrophoresis, gels were stained with silver nitrate (30). Abnormal PCR products identified by SSCP analysis were sequenced using fluorescent dideoxynucleotides on an Applied Biosystems (ABI, Foster City, CA) model 377 automated sequencer. Mutations were identified by the approximately equal peak intensity of two fluorescent dyes at the mutant base. All sequencing was bi-directional. Deletion, insertion and duplication mutations were confirmed further by cloning mutant PCR products into pGEM T-EASY vector (Promega, Madison, WI) and sequencing multiple clones to identify clones of both the mutant and normal allele sequences.

Genotyping Gln368Stop families

Probands and affected family members with the Gln368Stop mutation were genotyped with five STRP markers that closely flank the myocilin gene. These markers include *D1S2815*, *D1S1619*, *D1S2496* and two markers isolated from the BAC containing myocilin (18) (marker *MY5* is located <1 kb upstream, and marker *MY3* is located <1 kb downstream of the myocilin gene). Using 12.5 ng of each patient's DNA, the STRPs flanking myocilin were PCR amplified (as described above), electrophoresed on 6% polyacrylamide–7 M urea gels at 65 W for ~3 h and stained with silver nitrate. Twenty-seven probands and 35 sibs harboring the Gln368Stop were genotyped at these markers. Twenty-seven ethnically matched normal controls were also studied. Twenty-six of these matched normal control subjects were Caucasians from Iowa (19) and Australia (7), and one of the controls was an African American from New York City.

ACKNOWLEDGEMENTS

We thank L. Affatigato, G. Beck, D. Crouch, B. Rush, L. Streb, C. Taylor and K. Vandenburgh for their excellent technical assistance. This work was supported in part by the Carver Charitable Trust, the Grousbeck Family Foundation, NIH grants EY10564, EY08905, EY02477 and EY02162, and an unrestricted grant from Research to Prevent Blindness, New York, NY.

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