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Heterozygous Loss-of-Function Variants in *CYP1B1* Predispose to Primary Open-Angle Glaucoma

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Purpose. Although primary congenital glaucoma (PCG)–associated *CYP1B1* mutations in the heterozygous state have been evaluated for association with primary open-angle glaucoma (POAG) in several small studies, their contribution to the occurrence of POAG is still controversial. The present study was conducted to determine whether heterozygous functionally characterized *CYP1B1* mutations are associated with the disease in a large cohort of German patients with POAG.

Methods. The frequency of *CYP1B1* variants on direct sequencing of the entire coding region was compared in 399 unrelated German patients with POAG (270, POAG; 47, JOAG; and 82, NTG) and 376 control subjects without any signs of glaucoma on ophthalmic examination. In vitro functional assays were performed and relative enzymatic activity of the *CYP1B1* variants embedded in their respective background haplotypes and not previously unambiguously classified were determined, to assess their possible causative role.

Results. Apart from known polymorphic variants, 11 amino acid substitutions in *CYP1B1* reported before, both in PCG and POAG cases, were identified. After in vitro functional assay, variants P52L and R368H showed marked reduction of activity, confirming their role as loss-of-function mutations similar to previously determined variants G61E, N203S, and G329V. In contrast, variants G168D, A443G, and A465V showed no relevant effects and were thus classified as polymorphisms. Overall, seven functionally impaired variants were present in 13 (3.6%) patients and in 1 (0.2%) control subject ($P = 0.002$, OR = 5.4). Reanalysis of previous studies reporting *CYP1B1* mutations in patients with POAG based on updated functional validation showed a significant excess of carriers among patients compared to controls (OR = 3.85; $P = 2.3 \times 10^{-7}$).

Conclusions. Heterozygous *CYP1B1* mutations with absent or reduced relative enzymatic activity can be considered a risk factor for POAG. (*Invest Ophthalmol Vis Sci.* 2010;51: 249–254) DOI:10.1167/iovs.09-3880

Glaucoma represents a heterogeneous group of complex neurodegenerative diseases and is the second leading cause of blindness worldwide.[1,2] The most common form of glaucoma is primary open-angle glaucoma (POAG; OMIM 137760) which affects more than 35 million people worldwide.[3,4] POAG is characterized by loss of retinal ganglion cells, specific atrophy of the optic nerve, progressive loss of the neuroretinal rim of the optic disc, with corresponding progressive visual field loss, and, if untreated, eventual blindness.[2] Elevated IOP appears to be a major risk factor, although glaucoma develops in some patients even in the absence of elevated IOP.[5] In this case, glaucoma is defined as normal-tension glaucoma (NTG). Based on age at onset, a juvenile form (JOAG, age at onset before 40 years) is differentiated from adult-onset POAG.[6] A family history of the disease has long been recognized as a risk factor, suggesting that specific genetic variants contribute to pathogenesis of POAG. In some families, glaucoma segregates as a Mendelian trait with reduced penetrance and variable expressivity, but most cases are sporadic, suggesting a multifactorial contribution to its etiology.[7]

To date, 14 genetic loci for POAG have been identified through linkage studies (*GLC1A*–*GLC1N*). However, only three causative genes have been described: myocilin (*MYOC*/GLC1A), optineurin (*OPTN*/GLC1E), and WD repeat domain 36 (*WDR36*/GLC1G). Altogether, mutations in these genes account for less than 10% of POAG cases.[8] In addition, association studies have implicated more than 20 other genes.[9] Although most of these have been reported in single studies, a few have been investigated in multiple-association studies, but findings have been inconsistent. One of these POAG-associated genes is cytochrome P450 1B1 (*CYP1B1*: MIM 601771 (Mendelian Inheritance in Man; National Center for Biotechnology Information, Bethesda, MD). Cytochrome P450 proteins are monooxygenases, which catalyze many reactions involved in the metabolism of drugs as well as steroids and other lipids.[10] Mutations in *CYP1B1* are the predominant cause of primary congenital glaucoma (PCG), which although generally rare, is the most common form of glaucoma in infants, with more than 80% of cases observed within the first year of life.[11] This disorder is most likely due to developmental defects in the trabecular meshwork and the anterior chamber angle. In PCG, elevated IOP can rapidly lead to axonal loss and permanent loss of vision in untreated individuals. Inheritance is primarily autosomal recessive with variable penetrance.[12] Although the role of CYP1B1 in congenital glaucoma is not well understood, the enzyme is probably responsible for the metabolism of compounds that are critical for the developing eye.[10]

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Initially, *CYP1B1* was suggested to be a modifier gene for the expression of *MYOC* in patients with JOAG.[13] However, some studies have indicated that *CYP1B1* may play a causative role in JOAG, with possible monogenic association in French,[14] Indian,[15] and Spanish[16] patients. Furthermore, heterozygous variants in *CYP1B1* have been proposed as potential factors governing severity in patients with POAG.[17] In all these reports, however, heterozygous *CYP1B1* variants have also been found in healthy subjects, which raises questions about their role as disease-causing mutations.

In a recent study, we reported a measurement method for relative enzymatic activity (combining net enzymatic activity and protein abundance) and found variation in activity between the major *CYP1B1* haplotypes as well as either a drastic or an intermediate to mild reduction in activity of four different *CYP1B1* mutations (G61E, Y81N, N203S, and E229K), compared with that of the corresponding background haplotype.[18] A marked decrease of the relative activity was seen for variants N203S and G61E, which were classified as bona fide mutations. In contrast, variants Y81N showed an intermediate and E229K a mild reduction in activity, thus leading to a classification as hypomorphic alleles.[18,19]

In the present study, we evaluated whether heterozygous *CYP1B1* mutations showing absent or reduced relative enzymatic activity are associated with the disease in a large cohort of German patients with POAG.

Materials and Methods

Patients and Control Subjects

The study was approved by the ethics review board of the Medical Faculty of the University of Erlangen-Nuremberg and was conducted in accordance with the tenets of the Declaration of Helsinki. All subjects gave informed consent before entering the study.

The group of patients with glaucoma consisted of 399 subjects of German (European) origin. Two hundred seventy had POAG (high-pressure POAG), 47 had JOAG, and 82 had open-angle NTG. All individuals underwent standardized clinical examinations for glaucoma at the Ophthalmology Department of the University of Erlangen-Nuremberg. These comprised slit lamp biomicroscopy, gonioscopy, automated visual field testing (model G1; Octopus, Interzeag, Switzerland), fundus photography (fundus camera; Carl Zeiss Meditec, Jena, Germany), optional laser scanning tomography (HRT I and II; Heidelberg Engineering, Heidelberg, Germany) of the disc, and a 24-hour Goldmann applanation IOP tonometry profile with five measurements. Manifest high-tension POAG was defined as the presence of glaucomatous optic disc damage (in at least one eye), visual field defects in at least one eye and IOP higher than 21 mm Hg in one eye without therapy. According to Jonas et al.[20] a stage 0 optic disc is defined as normal, stage I as having vertical elongation of the cup and neuroretinal rim loss at the 12- and 6-o'clock positions, stage II as showing focal rim loss, stage III and IV has having advanced rim loss, and stage V as absolute optic disc atrophy.[21] Disc area was measured with HRT or estimated with Goldmann lens and slit lamp (Haag-Streit, Köniz, Switzerland). A pathologic visual field was defined by a pathologic Bebie curve: three adjacent test points with more than 5-dB sensitivity loss or at least one point with more than a 15-dB loss.

Persons with secondary glaucoma caused by such disorders as primary melanin dispersion, pseudoexfoliation, or uveitis or by previously raised IOP after trauma or a period of steroid administration were excluded. Glaucomatous optic nerve damage was defined as focal/diffuse loss of neuroretinal rim or nerve fiber layer associated with a specific visual field defect. Patients who showed glaucomatous changes of the optic disc and visual field, but no IOP elevation over 21 mm Hg after 24 hours of IOP measurement (sitting and supine body position) without therapy, received a diagnosis of NTG. In addition, in the patients with NTG, a neurologic examination was performed to exclude an intracerebral expansion or malperfusion. Stenosis of the aorta carotis interna was excluded by means of sonography. Patients were classified as having JOAG when age at onset in the index case was below 40 years and no other ocular reason for open-angle glaucoma was identifiable. At the time of examination, the ages of the patients ranged from 14 to 96 years with a mean of 66.9 ± 13.4. In total 178 (44.4%) patients had a family history of glaucoma.

The 376 control subjects were all of German origin and recruited from the same geographic regions as the patients. In addition, control subjects underwent ophthalmic examination and were age and sex matched. Thus, at the time of examination and inclusion in this study the ages ranged from 51 to 92 years with a mean of 73.9 ± 6.4. They had IOP below 20 mm Hg, no glaucomatous disc damage, and no family history of glaucoma. Visual acuity was at least 0.8 or 20/25 and the media were clear for examination.

All patients were screened for the myocilin (*MYOC*), optineurin (*OPTN*) (data not shown), and WD repeat domain 36 (*WDR36*)[22] mutations, as determined by direct sequencing of all coding regions.

Mutation Screening

Genomic DNA was prepared from peripheral blood samples by a standard salting-out protocol. Individual coding exons of the *CYP1B1* gene, including flanking intronic/UTR sequences were amplified by polymerase chain reaction (PCR) with appropriate amplification protocols. Primer sequences were selected using Primer3 software and are available on request. Purified PCR fragments were sequenced with big dye termination chemistry (ver. 3.1; Applied Biosystems, Inc., [ABI], Weiterstadt, Germany) on a capillary automated sequencer (3730 Genetic Analyzer; ABI). Each variant was confirmed by a second independent analysis. GenBank for *CYP1B1* accession NM_022184 was used as cDNA reference sequence and NT_000104 as genomic reference sequence. We used Q16678 (CYP1B1_HUMAN), from the Swiss-Prot/TrEMBL Database as reference protein sequences. URLs of all data sources are provided in the Appendix.

Plasmids Expression

Based on wild-type human *CYP1B1* cDNA cloned into the pYeDP60 expression plasmid[23] various *CYP1B1* constructs were generated by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA) according to the instructions of the manufacturer. Several rounds of site-directed mutagenesis were needed to establish the different SNP haplotypes and the variants embedded in their original haplotype (for more details please refer to Ref. 32).

All plasmids were sequenced using the dye-termination cycle sequencing kit (Prism Big Dye; ABI) and analyzed (Genetic Analyzer 3730; ABI) to ensure that the constructs were correct.

Expression of Human *CYP1B1* Variants in Yeast

The different human *CYP1B1* variants were coexpressed with human reductase in a *Saccharomyces cerevisiae* strain, INVSc1-HR MAT αhis3Δ1 leu2 trp1–289 ura3–52 (pFL-35 human reductase). Yeast cells were transfected with the pYeDP60-CYP1B1 expression plasmids. Single colonies were inoculated into selective medium (lacking uracil) and grown, followed by microsome isolation according to a previously established protocol.[24] Microsomes were stored at −80°C until use. Microsomal protein concentration and total P450 content were determined with previously described methods.[25,26] The p450 content is essentially accounted for by the overexpressed CYP1B1.

Determination of Enzymatic Activity

CYP1B1 activity in microsome extracts was quantified (P450-Glo CYP1B1 Assay Kit; Promega, Madison, WI) according to the instructions of the manufacturer. To measure enzymatic activity, 1 picomole of each CYP1B1 variant was incubated with a luminogenic substrate and NADPH regeneration system. As a result, a luciferin product (D-luciferin) was generated, and luminescence (proportional to cyto-

TABLE 1. Identified *CYP1B1* Mutations and Polymorphisms

Exon	Nucletotide Change	Amino Acid Change	Patients (n = 399)	Controls (n = 376)	Functional Classification
E2	c.155C>T	Pro52Leu	1	0	Mutation
E2	c.171G>A	Trp57X	1	0	Mutation
E2	c.182G>A	Gly61Glu	1	0	Mutation
E2	c.241T>A	Tyr81Asn	6	1	Hypomorphic allele
E2	c.608A>G	Asn203Ser	1	0	Mutation
E2	c.986G>T	Gly329Val	1	0	Mutation
E3	c.367G>A	Arg368His	2	0	Mutation
E2	c.503G>A	Gly168Asp	1	0	Polymorphism
E2	c.685G>A	Glu229Lys	8	10	Polymorphism
E3	c.1328G>C	Ala443Gly	1	1	Polymorphism
E3	c.1394T>C	Val465Ala	1	0	Polymorphism

chrome P450 activity) was measured on a microplate reader (GENios; Tecan, Maennedorf, Switzerland). The assays were performed in triplicate for all variants, original background haplotypes, and the negative control (microsomal extract from yeast transformed with the empty vector). The luminescence values for the negative control were subtracted from values of the rest of the samples. The mutants were compared against the corresponding background haplotype.

Statistical comparisons were performed with a one-way ANOVA, followed by the least-significant difference (LSD) test (for more details please refer to Ref. 18).

Statistical Analysis

Probabilities were calculated with Fisher's exact test; $P < 0.05$ was considered statistically significant. Odds ratio (OR) and 95% confidence interval (CI) were calculated with the free available software written by David J. R. Hutchon.

RESULTS

An extensive screening for mutations using direct sequencing of the complete coding sequence of *CYP1B1*, including untranslated 5′, 3′, and intronic flanking regions was performed on a well-studied, previously characterized cohort of 399 unrelated patients with glaucoma (270 with POAG, 47 with JOAG, and 82 with NTG) and 376 healthy subjects of German (European) origin.[22] Apart from known polymorphic variants, this systematic mutation screen led to the identification of 11 amino acid substitutions in CYP1B1 (Table 1). These 11 variants have been reported before, both in PCG and POAG cases, and some of them have also been found in healthy subjects.[27,28] To delineate variants impairing enzymatic function, we performed in vitro functional assays for CYP1B1 amino acid changes, P52L, G168D, R368H, A443G, and V465A not previously unambiguously classified. These variants were generated in vitro by site-directed mutagenesis, embedded in their respective background haplotypes. The relative enzymatic activity of each variant was calculated by multiplying its molar enzymatic activity and relative enzyme amount.[18] Variants P52L and R368H presented a drastic decrease in relative enzymatic activity (Fig. 1), confirming their role as loss-of-function mutations similar to the previously reported variants G61E, N203S,[18] and G329V.[29] In contrast, relative enzymatic activity of the variants G168D, A443G, and V465A was unchanged or even slightly increased in respect to that of their respective haplotypes, but within the normal range seen in wild-type haplotypes resulting in a classification as polymorphisms.

In total we saw six bona fide mutations (P52L, W57X, G61E, R368H, N203S, and G329V) in seven patients, but none in control individuals. In addition, we found two variants previously classified as hypomorphic. The more severely impaired variant Y81N was present in six patients compared with one

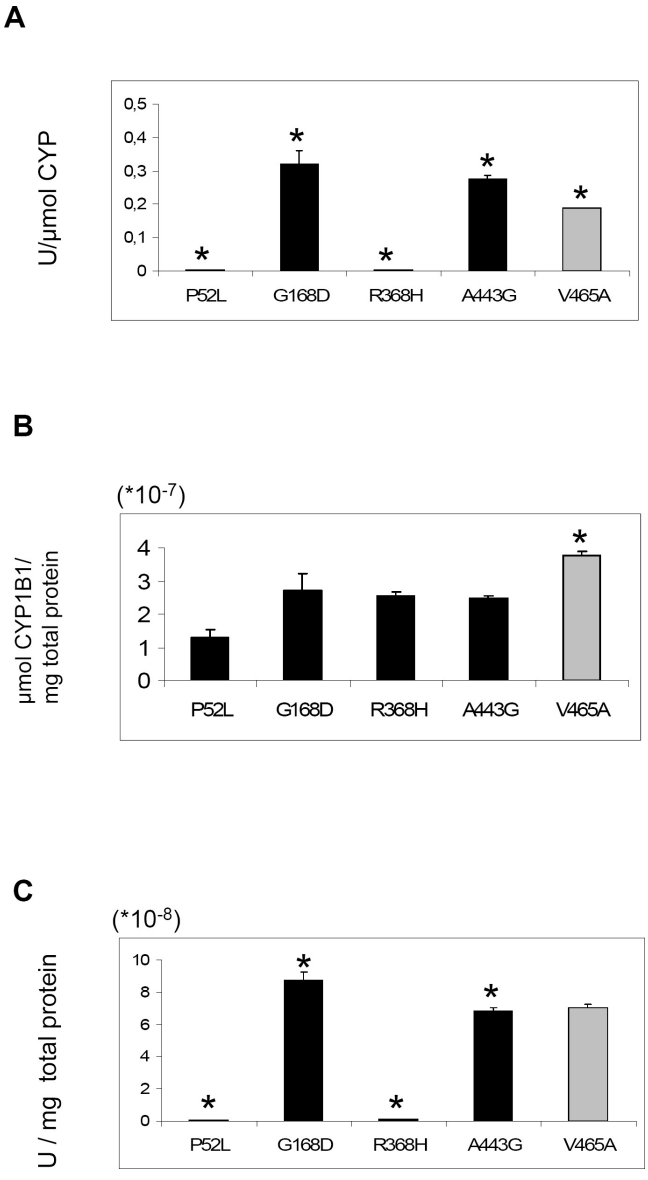

FIGURE 1. Molar enzymatic activity, relative CYP1B1 abundance and relative CYP1B1 activity. (**A**) Molar enzymatic activity of mutations. (**B**) Relative CYP1B1 abundance of the mutations. (**C**) Relative CYP1B1 activity of the mutations. Different column shades correspond to the different background haplotypes. Statistical comparison was performed with a one-way ANOVA, followed by the LSD test. The mutants were compared against the corresponding background haplotype. *Significant at the 5% level.

TABLE 2. Clinical Features of Patients with Functionally Impaired *CYP1B1* Mutations

Subject ID	CYP1B1 Variant	MYOC Variant	Phenotype	Age at Diagnosis (y)	MAX IOP (mmHg; R/L)	Optic Disc (Jonas)	Disc Area (mm^2)
20315	P52L	—	JOAG	6	30/30	ND	ND
99052	W57X	G367R	JOAG	22	46/38	V/III	2.85/2.89
10927	G61E/Y81N	—	POAG	46	43/28	IV/IV	ND
18513	Y81N	—	POAG	74	28/21	ND	2.1/2.1
99184	Y81N	—	NTG	69	21/21	II/II	2.49/2.48
17725	Y81N	—	POAG	54	29/26	ND	2.79/2.97
99004	Y81N	P370L	JOAG	7	30/33	IV/V	ND
99299	Y81N	—	NTG	60	19/19	III/III	2.8/2.8
99590	Y81N	—	POAG	51	24/24	IV/V	2.7/2.0
99168	N203S	E368X	JOAG	39	38/38	ND	1.99/1.87
17687	G329V	—	POAG	47	25/25	ND	2.59/2.59
99195	R368H	—	NTG	56	21/21	I/I	2.68/3.55
11806	R368H	—	POAG	44	32/33	ND	3.6/3.8

Maximum IOP at time of diagnosis (Goldmann applanation tonometry); optic disc atrophy according to Jonas (from normal 0, moderate cupping I, notching of the neuroretinal rim (NRR) II–III, temporal NRR loss IV, to complete atrophy V); optic disc area of left and right eye, respectively, measured with planimetry or laser scanning tomography (HRT; Heidelberg Engineering, Heidelberg, Germany).

healthy subject, suggesting that it may represent a risk factor for POAG. The milder E229K was found in similar frequency in patients and controls (2% and 3%, respectively) suggesting it should be reclassified as a polymorphism (Table 1). This result is in line with the observation of evolutionary conservation of position Y81 but not of E229 throughout orthologues and paralogues in different mammalian species, as previous reported.[16]

When Y81N was included, we saw a variant with impaired function in a total of 13 patients (3.6%) but only 1 healthy subject (0.2%). Thus, the CYP1B1 mutation rate in POAG patients is significantly increased over that expected (P = 0.0018, Fisher's exact test; OR = 5.4, 95% CI = 1.9–15.5). In fact, considering a prevalence of 2.85×10^{-5} of PCG in Western countries, the expected frequency of heterozygosity in the normal population under Hardy-Weinberg equilibrium is approximately 1%, assuming a recessive mode of inheritance.[30]

The mutation-positive patient group comprised both juvenile- and adult-onset POAG, with age at diagnosis varying from 6 to 74 years (Table 2). Ten patients had elevated (maximum) IOP ranging from 24 to 46 mm Hg, whereas three had IOP measurements in the normal range: 19 and 21 mm Hg, respectively. Among the 13 patients, 3 carried an *MYOC* mutation, but none presented with a *WDR36* or *OPTN* mutation (Table 2). Confirming a previous report,[13] we also observed that patients carrying both *CYP1B1* and *MYOC* mutations presented with a relatively early manifestation of the disease. This finding was also true, however, in those patients carrying only *CYP1B1* mutations with markedly reduced relative enzymatic activity—for example, P52L and N203S. One patient with POAG was found to be compound heterozygous for two *CYP1B1* mutations: the bona fide mutation G61E and the hypomorphic allele Y81N.

We next reanalyzed the *CYP1B1* variants reported in previous studies[14–16,28,31] based on recently published functional tests and those described in this study[18,19,29,32,33] and reclassified them into either functionally impaired, polymorphism, or undetermined (Table 3). In line with our results, we observed a significant excess of patients carrying *CYP1B1* mutations in the combined group as well as in each study (combined OR = 3.85, 95% CI = 2.21–6.70; $P = 2.3 \times 10^{-7}$; two-tailed Fisher's exact test).

DISCUSSION

Different *CYP1B1* heterozygous variants have been implicated as a risk factor for POAG in studies of different population groups.[14,16,28,31] Moreover, in all these studies, it has been seen that the frequency of the mutations, their nature, and their diversity vary greatly with the population studied. This fact combined with the relatively small groups of patients available, and the unknown role of the variants detected had made it difficult to clearly show the association of *CYP1B1* with disease.

In comparison, our work first includes a large number of patients and control subjects, thus providing a stronger power for the association analysis and second assesses the functional

TABLE 3. Combined Analysis of Different Studies on Functionally Validated *CYP1B1* Mutations in Patients with POAG

Study Groups	Ancestry	Patients	Mutations	Undetermined	Controls	Mutations
Melki et al.[14]	French	236	6	1	47	1
Acharya et al.[15]	Indian	200	2	6	100	0
Lopez-Garrido, et al.[16]	Spanish	119	7	4	92	0
Kumar et al.[28]	Indian	251	10	3	100	2
Chakrabarti et al.[31]	Indian	224	11	6	200	0
This study	German	399	13	—	376	1
Total		1429	49	20	915	4
Percentage		100	3.4	1.4	—	0.4

Data from larger studies that reported *CYP1B1* mutation frequencies in patients with POAG was reanalyzed based on updated functional validation of mutations (Refs. [18,19,33], and this study). Undetermined refers to mutations, whose functional relevance has not been determined as yet. Overall, 1429 patients of different ancestry were investigated. In each study and overall, patients were more likely to carry a functionally impaired mutation with an associated OR = 3.85 (95% CI = 2.21–6.70), $P = 2.3 \times 10^{-7}$ (two-tailed Fisher's exact test).

effects of many variants found, thus presenting an unequivocal classification of them. In these aspects, the CYP1B1 mutation rate in our POAG patient cohort was significantly increased over that expected ($P = 0.0018$, Fisher's exact test). Further, our reanalysis of the previous studies about *CYP1B1* association to POAG[14–16,28,31] takes into account the functional classification of the *CYP1B1* mutations, determined in this and other works,[18,19,29,33] and combined the number of all patient groups screened together against all control subjects. As expected we observed an increase in significance ($P = 2.3 \times 10^{-7}$; two-tailed Fisher's exact test), thus strengthening the association of functionally characterized *CYP1B1* mutations in POAG.

Our results emphasize once more both the importance of functional classification of variants, especially in highly polymorphic genes, and the large number of patients necessary to achieve sufficient power when assessing the contribution of a gene to the pathogenesis of a complex disease such as POAG.

Previous data[7,9] support the hypothesis that POAG is characterized by a high locus and allelic heterogeneity with many different rare variants in numerous genes. In fact, rare mutations with low frequencies have also been reported for the other known glaucoma genes such as *MYOC*[34] and *WDR36*.[22] Taken together, these observations suggest that POAG belongs to the same category of traits under the frequent disease–rare variant hypothesis, such as epilepsy[35] and macular degeneration.[36] Characteristics of these diseases are rare, with highly penetrant variants found in numerous genes. Some of these variants are familiar, but most occur sporadically.[37,38] This sporadic occurrence could explain the so far elusive quest to identify more glaucoma genes and has important consequences for designing future studies aimed at unraveling the molecular basis of this devastating disease.

To our knowledge, this is the largest association study linking POAG and rare *CYP1B1* variants to date and the first to systematically classify variants based on relative enzymatic activity. Our study and the reanalysis of previous studies supports the hypothesis that *CYP1B1* has a broader significance for glaucoma pathogenesis than initially thought, ranging from a causal effect in autosomal recessive PCG and other anterior segment dysgenesis disorders, to a risk factor in POAG.

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APPENDIX

URLs of Data Sources

National Center for Biotechnology Information (NCBI), Bethesda, MD; http://www.ncbi.nlm.nih.gov/

Online Mendelian Inheritance in Man (OMIM), NCBI; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM

Primer3 software, Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA; http://frodo.wi.mit.edu/primer3/

Genome Browser of the University of California Santa Cruz (UCSC), Santa Cruz, CA; http://genome.ucsc.edu/cgi-bin/hgTracks (reference sequences used: NT_ 022184 and NM_000104)

Swiss-Prot/TrEMBL Database, Sanger Centre, Hinxton, UK; http://www.sanger.ac.uk/

Expert Protein Analysis System (ExPasy) proteomic server, Swiss Institute of Bioinformatics, Geneva, Switzerland; http://www.expasy.org (Reference sequence Q_Human)

ClustalW, European Bioinformatics Institute, European Molecular Biology Laboratory, Heidelberg, Germany; http://www.ebi.ac.uk/clustalw/

Calculator for Odds Ratios and Confidence Intervals, written by David J. R. Hutcheon; http://www.hutchon.net/ConfidOR.htm