



Heterozygous *CYP1B1* gene mutations in Spanish patients with primary open-angle glaucoma

María-Pilar López-Garrido,¹ Francisco Sánchez-Sánchez,¹ Francisco López-Martínez,² José-Daniel Aroca-Aguilar,¹ Cristina Blanco-Marchite,² Miguel Coca-Prados,³ Julio Escribano¹

¹Área de Genética, Facultad de Medicina/Centro Regional de Investigaciones Biomédicas (CRIB), Universidad de Castilla-La Mancha and ²Servicio de Oftalmología, Complejo Hospitalario Universitario de Albacete, Albacete, Spain; ³Department of Ophthalmology and Visual Science, Yale University School of Medicine, New Haven, CT

Purpose: To investigate *CYP1B1* gene mutations in Spanish patients with ocular hypertension (OHT) or primary open angle glaucoma (POAG).

Methods: The two coding exons of *CYP1B1* were screened for sequence alterations by direct PCR DNA sequencing in 37 and 82 unrelated Spanish subjects diagnosed with OHT and POAG, respectively. As a control we used a group of 93 subjects from whom OHT or glaucoma were ruled out.

Results: We found three different predicted amino acid substitutions (Ala189Pro, Ala330Ser, and Ala443Gly) in three (8.1%) OHT subjects, and seven different mutations (Ser28Trp, Gly61Glu, Tyr81Asn, Gln144His, Arg145Trp, Glu229Lys, and Val409Phe) in nine (10.9%) glaucoma patients. These sequence variations showed higher frequencies in cases than in controls (as recently reported in French patients). They are predicted to produce a significant change in the amino acid sequence and affect conserved regions of the protein. All these missense mutations were found as heterozygotes. In addition, four of them have been previously found in PCG and/or POAG patients, whereas the other six mutations (Ser28Trp, Gln144His, Arg145Trp, Ala189Pro, Ala330Ser, and Val409Phe) have not been previously described. Clinically, these mutations are associated with an age at diagnosis ranging from 12 to 58 years (mean 34.3 years) and from 48 to 77 years (mean 59.9 years) among OHT and glaucoma patients, respectively.

Conclusions: Heterozygous *CYP1B1* mutations could confer increased susceptibility to the development of POAG in the Spanish population.

Glaucoma is a complex and genetically heterogeneous disease characterized by the progressive apoptotic death of retinal ganglion cells. This process originates excavation of the optic nerve head, visual field loss, and eventually leads to blindness [1]. Primary open-angle glaucoma (POAG) is the most common form of glaucoma, featured by a juvenile or adult (>40 years) onset, a gonioscopically open angle, and a reduced outflow facility. Although mutations in genes such as *MYOC*, *OPTN*, and *WDR36* have been identified in some POAG cases [2-5], the genetic defect remains unknown in most patients.

Mutations in the *CYP1B1* gene are the predominant cause of primary congenital glaucoma (PCG) [6], a rare and severe form of autosomal recessive glaucoma. This disease is produced by an anomalous development of the trabecular meshwork and anterior chamber angle, which increases resistance to aqueous humor outflow and raises intraocular pressure (IOP).

The human *CYP1B1* gene encodes a member of the cytochrome P450 superfamily, subfamily I, is located on chromosome 2p22-21, and is composed of three exons with the translated region beginning at the 5' end of the second exon. The

CYP1B1 protein is a membrane-bound monomeric mixed function monooxygenase. It has been proposed that this cytochrome participates in iridocorneal angle development [7], thus alteration of *CYP1B1* activity could impair the morphogenesis of the outflow angle, leading to IOP elevation and glaucoma.

CYP1B1 mutations are also present in certain families where PCG and POAG co-exist [8-10], and in sporadic cases of POAG in different populations [11,12]. Recently the association of the common polymorphism N453S with optic disc cupping and visual field alteration in patients with POAG has been described [13]. Herein, we investigated the role of *CYP1B1* mutations in a population of unrelated Spanish individuals with either OHT or POAG. We found that *CYP1B1* heterozygous mutations are increased in these two groups of patients.

METHODS

Subjects: Eighty-two and 37 unrelated patients diagnosed with POAG and OHT, respectively, were studied retrospectively for *CYP1B1* mutations. All the individuals were Spanish Caucasians recruited and regularly followed-up in the "Servicio de Oftalmología, Complejo Hospitalario Universitario de Albacete", Spain. None of the subjects were immigrants. Four families of *CYP1B1* mutation carriers were also studied. The criteria for a diagnosis of OHT were pressure greater than 21

Correspondence to: Dr. Julio Escribano, Área de Genética, Facultad de Medicina, Avda. de Almansa 14, 02006 Albacete, Spain; Phone: +34 967 599200, ext. 2928; FAX: +34 902 204130; email: julio.escribano@uclm.es

mmHg on two or more occasions in the absence of a field defect and open angles on gonioscopy, with no history of angle closure and absence of any ocular disease contributing to the elevation of pressure. All the following conditions were required to diagnose POAG: exclusion of secondary causes (e.g., trauma, uveitis, steroid-induced- or neovascular glaucoma); open anterior chamber angle (grade III-IV gonioscopy); IOP higher than 21 mmHg in the absence of medication, characteristic optic disc changes (e.g., vertical cup-to-disc ratio higher than 0.3); and an alteration of the visual field, tested by automated perimetry (with Humphrey's perimeter). The global indices such as mean deviation (MD) and pattern standard deviation (PSD) of the baseline visual fields were analyzed for all cases. Some patient's partners who did not have glaucoma were also included in the study as control subjects (42.8% of controls). Other unrelated controls were recruited from patients who attended the clinic for conditions other than glaucoma, including cataracts, floaters, refractive errors, and itchy eyes. All study subjects underwent a complete ocular examination. The study protocol was approved by the Ethics Committee for Human Research of the Albacete University Hospital, and followed the tenets of the Declaration of Helsinki. Informed consents were obtained from all the study subjects.

Patients were classified as having early (MD better than -6 dB), moderate (MD between -6 and -12 dB), or severe (MD worse than -12 dB) visual field alteration, according to the classification by Hodapp et al. [14]. Medical treatment included primarily topical beta-blockers and prostaglandin.

Mutation screening: Genomic DNA was extracted from the peripheral leukocytes of all subjects with the Perfect gDNA Blood Mini kit (Eppendorf, Madrid, Spain) according to the manufacturer's protocol. The translated genomic regions of the *CYP1B1* gene (exons II and III) were PCR amplified in three different fragments using primers shown in Table 1. The exon I and the promoter region of *CYP1B1* (-1 to -867) were also amplified in all carriers of *CYP1B1* mutations, using intronic primers designed to allow analysis of splicing consensus sequences (Table 1). PCRs were performed in a 50 μ l volume containing 50-100 ng of genomic DNA, 20 pmol of forward and reverse primers, 2 mM MgCl₂, 200 μ M of each dNTP, and 1 U of Taq DNA polymerase (Biotools, Madrid, Spain). Dimethylsulfoxide (10%) was added to reactions performed with primer sets 1 and 2. Thermocycling included an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation, annealing and extension (Table 1). A final cycle was performed at 72 °C for 7 min. Terminator cycle sequencing was carried out using the BigDye® (version 3.1) kit (Applied Biosystems, Foster City, CA) and the products of sequencing reactions were analyzed in an automated capillary DNA sequencer (ABI Prism 3100-Avant genetic analyzer, Applied Biosystems, Foster City, CA).

Statistical analysis: Significance of the difference in sex and mutation proportions between patients and control subjects was determined by χ^2 test when all expected values were 5 or more, and Fisher's exact test was used when expected values were less than 5. Significance of difference in age, IOPs and C/D ratios among the different groups of subjects was

determined by the t-test. Data were statistically treated by using SigmaStat 2.0 software (SPSS Inc., Chicago, IL).

RESULTS

Phenotype of patients: A total of 119 unrelated cases were investigated, comprised of 37 persons diagnosed with OHT and 82 with POAG. The control group included 93 individuals from whom glaucoma was ruled out. Table 2 shows the main clinical features of these subjects. The three groups were homogeneous with respect to age, gender, and iridocorneal angle. The two groups of patients were under medical treatment to reduce IOP, therefore their IOP mean values were below 21 mmHg. The mean intraocular pressure and C/D ratios in both eyes of OHT and POAG patients were significantly higher than in controls (Table 2). The visual field status among POAG patients was severe in 18 (10.9%) eyes, moderate in 43 (26.21%) eyes, early in 83 (50.6%) eyes, normal in 14 (8.5%) eyes, and in six eyes (3.6%) from six different patients the visual field could not be determined. The normal eyes were from 14 patients (17%) who showed monolateral visual field alterations. No visual field damage was detected in OHT patients.

Identification of CYP1B1 mutations and genotype-phenotype correlation: Genomic DNA from each individual included in the study was screened for mutations in the two coding exons of the *CYP1B1* gene by direct PCR sequencing. As a first approach, sequence variations were classified as disease-causing mutations when they met any of the following criteria: (1) presence of a predicted amino acid substitution altering the physicochemical or structural properties of the *CYP1B1* polypeptide chain in at least one OHT or glaucoma patient, and an absence of the change in the control group, and/or (2) previous report of the sequence variation being involved in glaucoma.

Our analysis revealed 10 different heterozygous mutations among the two groups of patients (Table 3). These mutations were detected in three (8.1%) OHT and nine (10.9%) POAG cases.

Location of the predicted amino acid changes in the coding region of the gene is shown in Figure 1. The existence of additional mutations in exon 1, in the promoter region of *CYP1B1* (-1 to -867), and in the coding regions of *MYOC* and *OPTN* of these patients were ruled out by direct PCR sequencing (data not shown). Mutations Gly61Glu, Tyr81Asn, Glu229Lys, and Ala443Gly were previously reported in glaucoma cases, as will be discussed later. As far as we know, our study is the first to identify the remaining six mutations. Mutations Ala189Pro, Ala330Ser, and Ala443Gly were identified in three OHT individuals, whose ages at diagnosis were 12, 58, and 33 years, respectively (mean, 34.3 years), and showed bilateral elevated IOP values, ranging from 16 to 20 mmHg with medical treatment (Table 3). Since this is a retrospective study IOP values without treatment were not always available. The correct control of IOP required more than one drug in two of these patients (6 and 99) who additionally showed cup:disc ratios higher than 0.5, after at least 12 and four years of evolution, respectively (Table 3). Patient 99 had elevated

IOP and altered C/D ratios, indicating that he is at risk of developing POAG.

Mutations Ser28Trp, Gly61Glu, Tyr81Asn, Gln144His, Arg145Trp, Glu229Lys, and Val409Phe were identified in POAG patients who, in general, required two or three drugs for appropriate control of IOP. In addition, patient 128, who carried mutation Val409Phe, showed the most severe phenotype identified in this study, characterized by intense bilateral visual field alteration, a high cup:disc ratio (0.8), and resistance to medical treatment of IOP, necessitating surgery (Table 3). Interestingly, the mean IOP value with medical treatment among POAG patients who carried *CYP1B1* mutations (18.8 ± 2.2 mmHg) was higher than among noncarriers (17.2 ± 3.3 mmHg; $p=0.047$). No differences in C/D ratios were found between these two POAG groups (Table 3). The most prevalent mutation found in this study was Tyr81Asn. It was detected in three POAG patients (patients 5, 11, and 115; Table 3). In addition, this mutation was identified in two subjects (patients 63 and 84) who presented no visual field alterations

but showed C/D ratios out of the normal range, with normal optic disc sizes, at ages of 42 and 44 years (Table 3). They were classified as glaucoma suspects. We also found an individual with no visual defects (66 years old) carrying the novel variation Pro52Leu (Table 3).

Evaluation of mutated positions by multiple sequence alignment: Cytochrome P450s possess a well conserved tertiary structure. Therefore, to locate mutations in the different structural domains of the family, we compared the amino acid sequences of some mammalian CYP1B1 enzymes and two well characterized members of the CYP1A subfamily (CYP1A1 and CYP1A2; Figure 2).

Mutations previously reported in glaucoma were positioned three amino acids downstream from the hinge region (Gly61Glu); in the A helix, which could form the substrate access channel (Tyr81Asn); in the substrate recognition site 2 (SRS-2; Glu229Lys), and in the meander involved in heme-thiolate ligand binding (Ala443Gly; Figure 2). On the other hand, the novel mutations were located in the transmembrane

TABLE 1.

Primer set	Region	Primer sequence (5'→3')		Annealing temperature/time (°C/s)	Extension time (s)
		Forward	Reverse		
1	Exon II	TCTCCAGAGAGTCAGCTCCG	GGGTCGTCGTGGCTGTAG	57/ 5	45
2	Exon II	ATGGCTTTCGGCCACTACT	CGATCTTGGTTTTGAGGGGTG	57/ 5	45
3	Exon III	AATAAGAATTTTGCTCACTTGCTTTTC	AGCAGCACAAAAGAGGAACTGG	61/20	40
4	Promoter	ATGACTGGAGCCGACTTTCC	GCAAAGTCGAGGTTTCCTCACA	58/20	50
5	Exon I	CCTCCTTCTACCCAGTCCTTAAAA	TAAAGTACCTACCACGCCACCC	57/20	20

Oligonucleotide primers used for amplification of the *CYP1B1* gene

TABLE 2.

Variable	OHT (n=37)	POAG (n=82)	Control (n=93)	OHT versus control	POAG versus control
Age in years (mean±SD)	56.0±11.7	62.7±11.0	59.6±12.3	0.130	0.085
Female (%)	18 (48.6)	37 (45.2)	53 (57.0)	0.334	0.095
Male (%)	19 (51.4)	45 (54.8)	40 (43.0)		
IOP (mean±SD; mmHg)					
OD:	17.7± 3.9	17.5± 3.3	15.2± 3.0	<0.001	<0.001
OS:	17.9± 4.5	17.0± 3.1	15.2± 3.1	<0.001	<0.001
Iridocorneal angle (mean)	3.9	3.9	3.9	-	-
C/D (mean±SD)					
OD:	0.3± 0.2	0.4± 0.2	0.2± 0.2	0.006	<0.001
OS:	0.3± 0.2	0.4± 0.2	0.2± 0.2	0.008	<0.001

Clinical characteristics of participants in the study. Since this is a retrospective study, intraocular pressure (IOP) values before medical treatment were not always available. The variable "Age in years" indicates the age at the time of the study. In the table, SD indicates standard deviation; and C/D indicates cup-disc ratio of optic nerve.

anchor region (Ser28Trp), the hinge region (Pro52Leu), helix C (Gln144His and Arg145Trp), β 3-1-sheet (Ala189Pro), I helix (Ala330Ser), and β 2-1-sheet (Val409Phe; Figure 2).

All mutations affected conserved amino acid residues and altered the predicted physicochemical properties of the polypeptide chain, as follows. The amino acid residue Ser28 is conserved in the three mammalian CYP1B1 proteins analyzed (Figure 2), and its substitution for the bulky and nonpolar side chain of Trp, could alter the hydrophobicity of the transmembrane domain. Curiously, the predicted amino acid change Pro52Leu was found in a normal subject who was 66

years old at the time of the study. This amino acid residue matches the second Pro of the conserved sequence Pro-Pro-Gly-Pro (Figure 2) and therefore the nonconservative substitution bears the potential to affect the function of the protein. It has been reported that substitutions of the first and last Pro of this sequence for Ala diminish enzyme activity of cytochrome P450 2C2, but mutations of the second Pro have little effect on the enzyme activity [15]. Whether this is a disease-causing mutation with incomplete penetrance or just a rare variant remains to be investigated.

TABLE 3.

Nucleotide change	Amino acid change	Subject number	Phenotype	Age at diagnosis (years)	Age at time of study (years)	IOP (mmHg; OD/OS)	VFA (OD/OS)	C/D Ratio (OD/OS)	Treatment (number of drugs)
c.936G>C	A189P*	99	OHT	12	24	20/20	N/N	0.9/0.8	3
c.1359G>T	A330S*	6	OHT	58	62	18/18	N/N	0.5/0.8	2
c.1699C>G	A443G	114	OHT	33	38	16/20	N/N	0.2/0.2	1
c.454C>G	S28W*	62	POAG	59	65	20/20	E/N	0.7/0.7	2
c.553G>A	G61E	89	POAG	65	67	17/17	E/E	0.3/0.3	1
c.612T>A	Y81N	5	POAG	77	80	22/22	M/M	0.7/0.7	2
c.612T>A	Y81N	11	POAG	49	54	17/17	E/E	ND/ND	1
c.612T>A	Y81N	115	POAG	58	61	22/20	E/M	0.5/0.3	2
c.803G>T	Q144H*	80	POAG	58	67	18/16	ND/M	ND/ND	3
c.804C>T	R145W*	80	POAG	58	67	18/16	ND/M	ND/ND	3
c.1056G>A	E229K	107	POAG	66	80	18/18	E/N	0.5/0.4	1
c.1056G>A	E229K	127	POAG	48	52	20/21	E/E	0.7/0.7	1
c.1596G>T	V409F*	128	POAG	61	67	20/14	S/S	0.8/0.8	2+surgery
c.612T>A	Y81N	63	POAG SUS	-	42	12/12	N/N	0.6/0.6	0
c.612T>A	Y81N	84	POAG SUS	-	44	13/13	N/N	0.5/0.6	0
c.526C>T	P52L*	86	Normal	-	66	16/15	N/N	ND/ND	0

CYP1B1 genotype-phenotype correlations in patients with OHT, POAG, and control subjects. All mutations are heterozygous. The CYP1B1 GenBank accession number is NM_000104. The IOP values are with medical treatment. Since this is a retrospective study, IOP values before treatment were not always available. The asterisk indicates novel mutations. In the table, POAG SUS indicates POAG suspect; IOP indicates intraocular pressure; OD indicates right eye; OS indicates left eye; VFA indicates visual field alteration; C/D indicates cup-disc ratio of optic nerve; N indicates normal; E indicates early; M indicates moderate; S indicates severe; and ND indicates not determined due to oblique insertion of the optic nerve head.

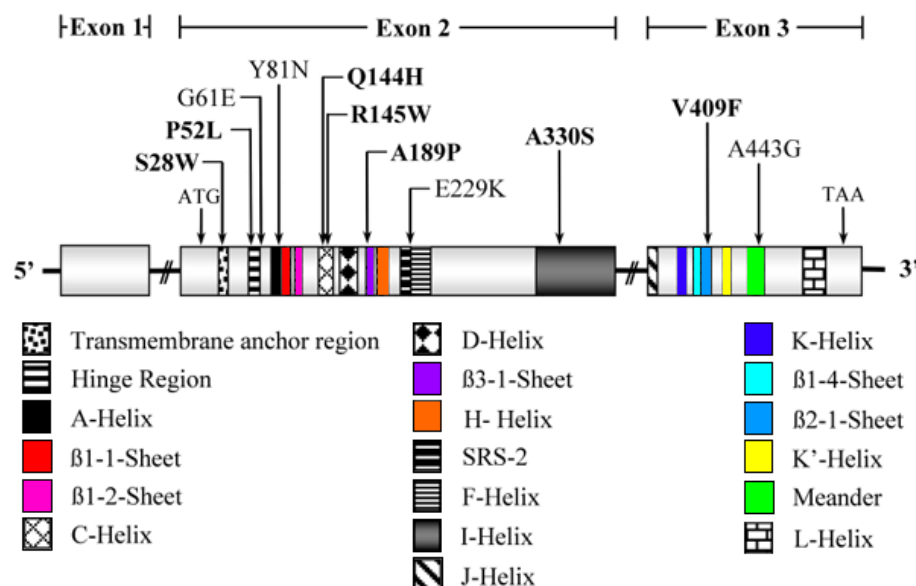


Figure 1. Gene structure of CYP1B1 and location of identified glaucoma mutations. The three exons are represented by boxes, and the conserved structural domains encoded by them are depicted by different patterns and colors as explained in the legend. Novel mutations are indicated by bold face type. All mutations are defined in terms of the one-letter code.

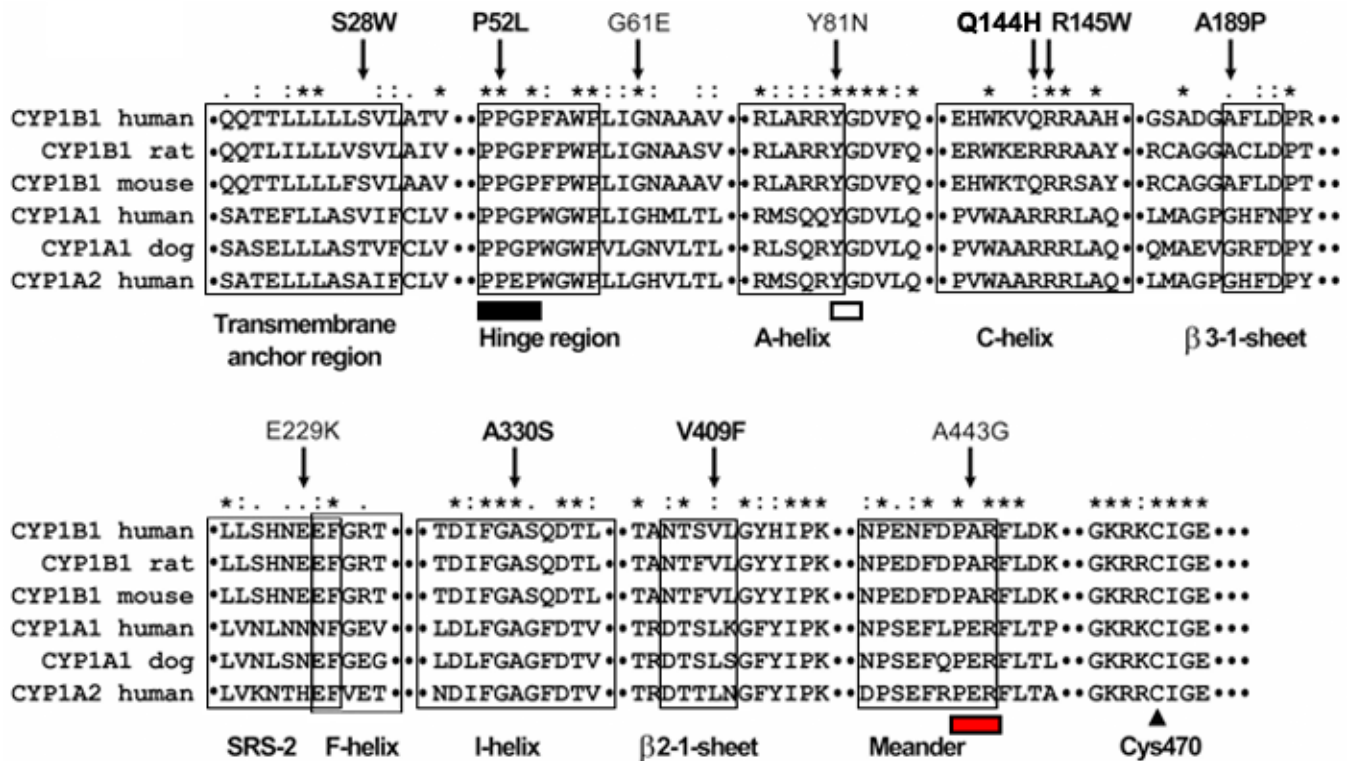


Figure 2. Multiple amino acid sequence alignment of CYP1B1, CYP1A1, and CYP1A2 from different species. Sequence alignment was generated by ClustalW. Residues affected by mutations are indicated by arrows. Different structural domains of the cytochrome P450 superfamily are boxed. Pro-Pro-Gly-Pro, Pro-X-Arg/His, and X-G motifs are indicated by black, white, and red rectangles below the sequences, respectively. The invariant cysteine residue in the heme-binding region is denoted by an arrowhead. Asterisks represent amino acid positions at which all query sequences are identical. Amino acid positions at which all analyzed sequences have amino acids that are chemically similar are denoted by colons (:). One dot denotes amino acid positions with weak chemical similarity. Novel mutations are indicated by bold face type.

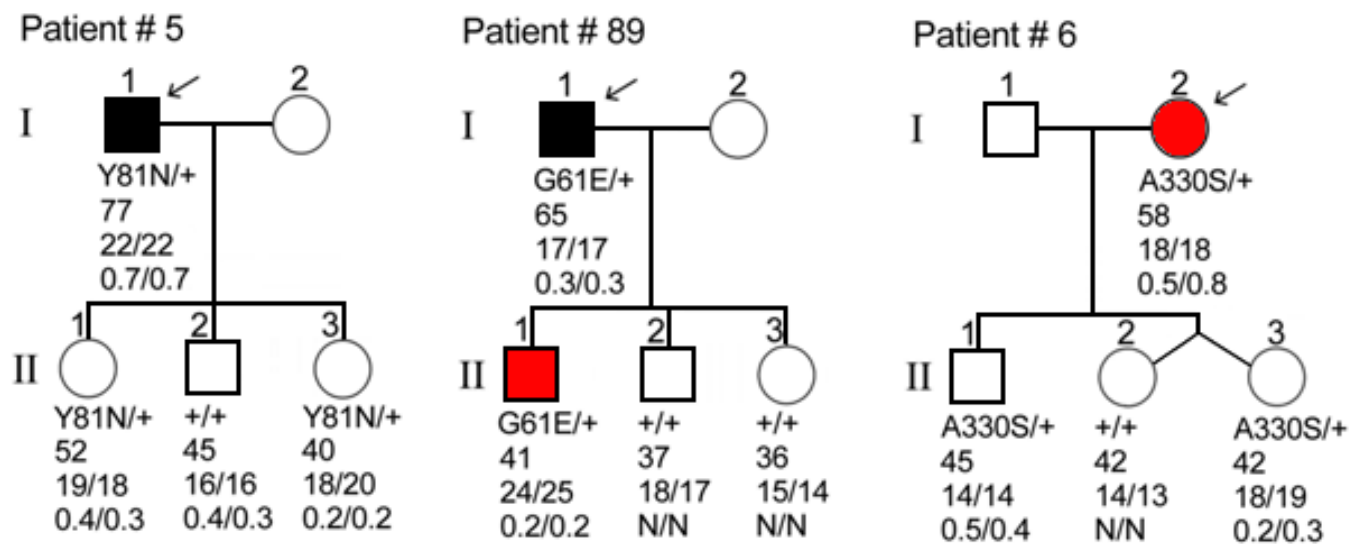


Figure 3. Pedigrees of two cases diagnosed with POAG and 1 case diagnosed with OHT. The genotypes, ages (years) at the time of the study, IOP (mmHg OD/OS), and C/D ratios (OD/OS) are indicated below the symbols. The arrows show the probands. Black and red symbols indicate POAG and OHT phenotypes, respectively.

The two novel contiguous sequence variations, Gln144His and Arg145Trp, were found in the same patient. We were unable to determine whether they were placed on the same or in different chromosomes. The amino acid residue 144 was either Gln or Arg in the polypeptide chains of the aligned cytochrome P450 molecules, while the adjacent Arg145 residue remained conserved (Figure 2). A comparison of amino acid residue 144 in different species showed a His residue in some of them (data not shown), indicating that the Gln144His substitution does not impair enzyme activity. Therefore, we considered that this substitution could represent a rare normal variant of the protein, although this point requires additional verification. In contrast, mutation Arg145Trp, holds the potential to impair the active site, since the side chain of this conserved Arg is involved in heme propionate coordination [16].

Mutation Ala189Pro could disrupt the structure of the β 3-1 sheet, whereas Ala330Ser could impair the activity of CYP1B1 since it affects a conserved amino acid potentially involved in the catalytic mechanism of the enzyme [17] (Figure 2). A different pathogenic mutation affecting amino acid 330 (Ala330Phe) has been reported in PCG patients [18,19]. Finally, mutation Val409Phe affects a conserved aliphatic residue and introduces a large and aromatic amino acid in the mutant protein (Figure 3).

Rate of CYP1B1 mutations: As all the nonconservative mutations are presumably injurious to the function of the protein, we compared the total frequency of these mutations between cases and controls. A similar approach has previously been followed to analyze the association of *MYOC* and *CYP1B1* mutations with POAG [3,12]. The *CYP1B1* mutation rate is significantly increased in POAG patients (10.9% in POAG compared to 1% in controls; Fisher's exact test, $p=0.011$). The two glaucoma suspects were not included in this calculation. A similar result was obtained comparing the percentage of *CYP1B1* heterozygotes found in this study with the expected frequency for heterozygotes in the Spanish population (approximately 1%), which was estimated based on the prevalence of PCG in Spain (2.85 10⁻⁵) [20], and assuming that the population is in Hardy-Weinberg equilibrium [12]. This is in accordance with a previous report in French patients [12], and also supports the role of the identified mutations in the development of POAG. In any event, larger groups of patients should be studied to confirm these data.

POAG in families with CYP1B1 mutations: To test for cosegregation of POAG with *CYP1B1* mutations we were able to study families of 2 POAG and 1 OHT cases (Figure 3). POAG patient 5 had two daughters heterozygous for the Tyr81Asn mutation (II:1 and II:3), who showed IOPs higher than those of their non carrier brother (II:2). In addition, the oldest Tyr81Asn carrier (II:1), had C/D ratios out of the normal range. The optic excavation of sibling II:2 was considered physiologic, since it was associated with a large optic disc and a normal visual field.

POAG patient 89 had a 41-year-old son carrying the mutation Gly61Glu as a heterozygote (II:1), who was diagnosed with bilateral OHT and required medical treatment. Although

his visual fields and optic discs were normal, he was considered a glaucoma suspect. His noncarrier siblings (II:2 and II:3) had normal IOPs.

OHT patient 6 had two daughters who were dizygotic twins (II:2 and II:3). One of them (II:3) was heterozygous for the novel mutation Ala330Ser, and presented higher IOPs and optic nerve excavations than her noncarrier twin. Sibling II:1 was also a heterozygous carrier with normal IOP. However he presented disc excavations out of the normal range, associated with the normal size of the optic discs.

Patient 127's family was also studied. The mutant allele was absent in his offspring (data not shown), but he reported that his mother had glaucoma. Although she could not be examined, we considered that this data also indicate the pathogenicity of mutation E229K.

DISCUSSION

Studies in different populations are required to clearly support the role of candidate genes in complex diseases, such as POAG. We herein show for the first time an increased proportion of heterozygous *CYP1B1* mutations in Spanish POAG (10.9%) and OHT (8.1%) cases. Previous population studies have also found carriers of heterozygous *CYP1B1* mutations in POAG patients from France (4.6%, $n=236$) [12] and Canada (5%, $n=60$) [11]. French cases showed a median age of 40 years at diagnosis (range 13-52). In our study this age was 59.9 years (range 48-77). This discrepancy could be due to differences in either the etiology of the disease or in the management of patients. The median age at diagnosis in the Canadian study was 23.6 years (range 8-36), reflecting that it was performed with early-onset glaucoma patients. Three reasons prompted us to analyze the presence of *CYP1B1* mutations among OHT subjects: (1) elevated IOP is one of the major risk factors for the development of glaucomatous visual loss [21]; (2) it is the main clinically treatable factor; and (3) high IOP was used as a criterion for diagnosis of POAG. Therefore in all these patients OHT likely was the first stage of the disease. Since the three OHT subjects who carried *CYP1B1* mutations were under medical prophylactic treatment with ocular drops, and two were younger than 40 years, we considered that they were glaucoma suspects who would develop the disease without the proper treatment. Overall these data suggest the role of *CYP1B1* mutations in IOP-raising.

Four of the reported mutations (Gly61Glu, Tyr81Asn, Glu229Lys, and Ala443Gly) have been found previously in patients with POAG and/or PCG. One of the interesting findings of this study was that all mutations were present in heterozygosis. Heterozygous *CYP1B1* mutations have been previously reported both in early-onset and primary congenital glaucoma. For instance, mutation Gly61Glu cosegregates with PCG in different populations [9,22-26] and it has been identified in heterozygous subjects with early-onset POAG from Morocco [9]. In addition, mutation Tyr81Asn was previously reported in heterozygous early-onset POAG patients from France [12]. It is interesting that this mutation is the most prevalent one in our study and that it has been previously reported only in two unrelated heterozygous French Caucasian patients

diagnosed with POAG [12]. Whether Tyr81Asn is specifically associated to POAG either as a major genetic factor or as a modifier variant remains to be investigated. In the same way, the amino acid change Glu229Lys has been observed in Indian and French PCG cases in heterozygosis [10,25,26] as well as in compound heterozygotes [24,27]. Some French patients with POAG are also heterozygous for this mutation [12]. Finally, mutation Ala443Gly has been described in heterozygosis in one PCG patient from Brazil [28], and in POAG patients from France [12]. Introduction of these mutations in our population by recent migrations is unlikely since all analyzed individuals were native Spanish, living in Spain for at least four generations.

The identification of 10 different *CYP1B1* mutations in the two groups of patients shows the allelic heterogeneity of this gene. Different pathogenic alleles of *MYOC* and *OPTN* genes have also been described in POAG. We evaluated the effect of *CYP1B1* gene variations on the predicted protein sequence. Mutations considered pathogenic in this study originated nonconservative amino acid changes and affected conserved residues located in structural domains of the protein, holding the potential to reduce the enzyme activity by mechanisms such as the following: (1) incorrect insertion of the protein in the ER membrane (Ser28Trp); (2) alteration of folding and protein stability (Gly61Glu, Ala189Pro, and Val409Phe); (3) modification of substrate binding (Tyr81Asn and Glu229Lys); and (4) impairment of the active center (Arg145Trp, Ala330Ser, and Ala443Gly).

In an effort to confirm the pathogenicity of these mutations, we were able to analyze the cosegregation of four of them (Gly61Glu, Tyr81Asn, Glu229Lys, and Ala330Ser) with POAG in three families or OHT in one family, respectively. Our conclusions were limited by the fact that most of the family members examined were younger than 40 years old. However, we detected some heterozygous carriers who showed phenotypic features suspicious of glaucoma, further suggesting the role of these sequence changes in the disease.

Although the results presented in this study provide the first evidence for the association of *CYP1B1* mutations with POAG in the Spanish population, the size of the samples does not allow a final conclusion. Further investigations are required to confirm these findings.

To explain our findings, we have hypothesized that at least certain *CYP1B1* mutations could determine the onset of the glaucoma phenotype. In this regard, it is well established that homozygous mutations cause congenital glaucoma. Heterozygous mutations might originate a milder glaucoma phenotype characterized by an adult onset. This can be due to haploinsufficiency and/or to the influence of modifier genes. In this regard, it could be possible that the overall enzymatic activity of these heterozygotes is reduced below a "normal" threshold, increasing the susceptibility to POAG. Concerning modifier genes, we have ruled out the presence of mutations in coding regions of *MYOC* and *OPTN* genes of heterozygous patients (unpublished data). In addition, mutations in the promoter region and exon I of *CYP1B1* were not detected. Detailed analysis of the genotype, biochemical, and clinical phe-

notypes, of the different *CYP1B1* pathogenic mutations, as well as search of other modifier genes and population analysis of larger number of individuals are required to unravel the role of this gene in POAG.

The proportion of POAG patients with *CYP1B1* mutations (10.9%) in our sample is around three times higher than that of carriers of *MYOC* mutations (data not shown), highlighting the role of *CYP1B1* in POAG. Further studies with larger samples and different populations are required to precisely define the role of *CYP1B1* sequence variations in POAG.

During revision of this manuscript, a paper entitled "Primary role of *CYP1B1* in Indian juvenile-onset POAG patients" by Acharya et al. [29] appeared in Molecular Vision. They screened *CYP1B1* mutations in 200 unrelated POAG patients comprising 156 sporadic and 44 familial cases. It was found that eight patients (4.0%) carried heterozygous mutations in *CYP1B1* and one patient (0.5%) was homozygous. Six of the heterozygous patients were sporadic glaucoma cases and two had a history of the disease in their families. One of these mutations is the same as we identified in Spanish patients (E229K). Overall the results of this paper further support the role of heterozygous *CYP1B1* mutations in POAG.

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