

ORIGINAL ARTICLE

CYP1B1 mutations in French patients with early-onset primary open-angle glaucoma

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Introduction: Primary open-angle glaucoma (POAG) is a leading cause of visual impairment worldwide and a complex genetic disorder that affects mostly adults. Mutations in the *MYOCILIN* (*MYOC*) and *OPTINEURIN* genes account for rare forms with a Mendelian inheritance and for <5% of all POAG cases. The *CYP1B1* gene, a member of the cytochrome P450 gene family, is a major cause of primary congenital glaucoma (PCG), a rare and severely blinding disease with recessive inheritance. However, *CYP1B1* mutations have also been associated with cases of juvenile-onset glaucoma in some PCG families or shown to modify the age of onset of glaucoma linked to a *MYOC* mutation in a large family.

Objective: To investigate the role of *CYP1B1* mutations in POAG predisposition, irrespective of the presence of a *MYOC* mutation.

Methods and subjects: *CYP1B1* coding region variation was characterised by denaturing high performance liquid chromatography (DHPLC) and sequencing in 236 unrelated French Caucasian POAG patients and 47 population-matched controls.

Results: Eleven (4.6%) patients carried one or two mutated *CYP1B1* gene(s) and no *MYOC* mutation. They showed juvenile or middle-age onset of disease (median age at diagnosis, 40 years, range 13–52), significantly earlier than in non-carrier patients. Apart from one, all mutations detected in POAG patients were previously associated with PCG.

Conclusion: *CYP1B1* mutations might pose a significant risk for early-onset POAG and might also modify glaucoma phenotype in patients who do not carry a *MYOC* mutation.

Primary open-angle glaucoma (POAG) is an optic neuropathy characterised by an excavation of the optic disk and a progressive alteration of the visual field. It is a leading cause of visual impairment, affecting 66 million persons worldwide.^{1–2} Genetic factors play a well-established but complex role in POAG predisposition.^{3–4} Six loci with a Mendelian inheritance have been mapped using large multi-case families.⁵ Two of these, including *GLCIA/MYOCILIN* (*MYOC*) and *GLC1E/OPTINEURIN* (*OPTN*), have been identified at the molecular level.^{6–7} In most cases, however, and in spite of clear familial clustering, POAG does not follow a Mendelian pattern of inheritance. Mutations of *MYOC* have been detected in a minor (2–4%) proportion of the most frequent cases.⁸ Altogether, the genetic basis of POAG remains unknown in the majority of patients. Identifying genetic factors associated with this prevalent disease is nonetheless of substantial research and clinical importance.

Primary congenital glaucoma (PCG) is a rare ocular disorder characterised by marked elevation of intraocular pressure (IOP) at birth or in early childhood, leading to ocular enlargement (buphthalmos) and corneal oedema.⁹ If it is not urgently treated, optic nerves are irreversibly damaged and vision may be lost. This severe defect is mostly inherited in an autosomal recessive manner.¹⁰ A major gene, *CYP1B1*, belonging to the superfamily of cytochromes P450 and mapping on chr.2p21, has been identified.^{11–12} A broad diversity of mutations in this gene has been characterised in different populations.^{13–19}

PCG is currently considered as a consequence of a developmental defect of the iridocorneal angle and a clinical and pathogenetic entity distinct from POAG.⁹ In certain pedigrees, however, both PCG and POAG segregate, suggesting that shared or overlapping mechanisms might predispose to both forms of glaucoma. The identification of *CYP1B1* provides an opportunity to test this hypothesis. Indeed, three

studies have reported delayed expression of a *CYP1B1* mutation and co-existence of PCG and POAG in the same pedigree.^{19–20–21} Moreover, in a large family where *MYOC*-linked POAG segregated, a heterozygous mutation of *CYP1B1* was associated with earlier onset of the disease in patients carrying the *MYOC* mutation, indicating that a *CYP1B1* mutation might behave as a modifier of the *MYOC* gene.²¹

Considered collectively, these observations suggested that mutations of *CYP1B1* might be a cause of POAG or at least a risk factor for this disease. In the present study, we report two additional pedigrees with both POAG and PCG patients carrying *CYP1B1* mutations. This led us to investigate *CYP1B1* mutations in a group of 236 French patients with POAG.

METHODS

Patients

POAG patients were recruited at the Glaucoma Institute of Saint-Joseph Hospital and at the Quinze-Vingts Hospital in Paris, and at the Lille University Hospital as described.²² Proband of PCG families were reported previously.¹⁸ A protocol approved by the Ethics Committee of Necker Medical School and in keeping with European legislation was followed. Written informed consent was obtained from all patients or, for children, from their parents. Ophthalmologic examination included slit-lamp biomicroscopy, optic nerve examination, measurement of IOP by applanation tonometry, and visual field assessment by automated perimetry. POAG was defined by the conjunction of a normally open iridocorneal angle, characteristic cupping of the optic disk, and alteration of the visual field. Patients

Abbreviations: DHPLC, denaturing high performance liquid chromatography; DMSO, dimethylsulfoxide; IOP, intraocular pressure; PCG, primary congenital glaucoma; POAG, primary open-angle glaucoma; TEAA, triethylammonium acetate

with cataract or media opacities and those with a cause of secondary glaucoma, notably including exfoliation, pigment dispersion, history of trauma, surgery, and glucocorticoid exposure, were excluded. Elevation of IOP was not an inclusion criterion. Final POAG diagnosis was made at the time of inclusion after review of inclusion and exclusion criteria, and before genotyping was conducted. Genotyping was performed blind to clinical data. All patients had been screened for *MYOCILIN* mutations by denaturing high performance liquid chromatography (DHPLC). Seventeen of these patients carried a *MYOC* mutation.²²

CYP1B1 mutation search

Two methods were employed when searching for mutations in *CYP1B1* (GenBank accession number: U56438). First, most of the coding region, including exon II and the 3' half of the coding region of exon III, could be screened by denaturing high performance liquid chromatography (DHPLC). Pilot experiments indicated that all the mutations that had been characterised in PCG patients from France,¹⁸ were reliably detected by this method. Overlapping fragments ranging from 302 to 456 base pairs were amplified by PCR using the primers and conditions shown in table 1. The PCR was performed in a 25 µl mixture containing 100 ng of genomic DNA, 0.4 µmol/l of forward and reverse primers, 1.5 mmol/l MgCl₂, 5% dimethylsulfoxide (DMSO), 200 µmol/l of each dNTP, and 0.5 U of Taq DNA polymerase (Invitrogen, Life Technologies, Carlsbad, CA, USA). Cycling conditions for each cycle were: 1 min at 94°C, 1 min at an annealing temperature specific to each amplicon (shown in table 1), and 1 min at 72°C, for 35 cycles. DHPLC analysis was conducted with the Wave Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE). The oven temperature and gradient conditions for heteroduplex detection were predicted with WaveMaker software (version 4.1.40) and specified experimentally as described previously.²² Actual parameters for DHPLC analysis of *CYP1B1* amplicons were established in this work and are listed in table 1. PCR products from DNA to be tested were mixed with an equimolar amount of PCR product from a DNA sample known to be unmutated. This DNA carried the common SNPs forming the most frequent haplotype in the French population (C3947G, G4160T, C8131G, C8184T, A8195G). The mixture was heated at 95°C for 5 min and allowed to cool at room temperature for 30 min to form heteroduplexes. A 5 µl sample was loaded on a DNA Sep Column (Model L-7300+, Transgenomic) and was eluted with a linear gradient of 5% triethylammonium acetate (TEAA) (buffer A) and 25% acetonitrile+5% TEAA (buffer B) at a flow rate of 0.9 ml/min. Elution profiles were compared to those of normal DNA. Samples with altered profiles were considered as potentially having a sequence variation and were sequenced bidirectionally as previously.^{13–14} Representative profiles are shown in the online supplemental fig 1 (available at <http://jmg.bmjournals.com/supplemental/>).

In contrast, the 5' end of exon III was not amenable to DHPLC analysis. This DNA segment contains three common SNPs. Their various combinations generate a diversity of elution profiles that complicates the detection of mutations. The corresponding amplicon was therefore sequenced directly.

RESULTS

POAG and PCG in two French families with *CYP1B1* mutations

In the pedigree shown in fig 1A, two sisters, including the proband, were affected with PCG whereas the other two sisters developed POAG at ages 35 and 40. The four sisters carried the same two mutations of *CYP1B1*, G232R and

E387K. Similarly, in the pedigree shown in fig 1B, the proband developed juvenile-onset glaucoma at the age of 13. Subsequently, it was found that his younger brother was affected with PCG, which led to investigation of *CYP1B1* in this family. Both brothers carried the same two mutations, 3979delA and N423Y.

CYP1B1 mutation screen in 236 unrelated POAG patients

Sequence variation in the *CYP1B1* gene coding region was then investigated in a group of 236 unrelated Caucasian patients with POAG from France. Altogether, 11 patients carried a mutation of *CYP1B1* (table 2). None of them carried a *MYOCILIN* mutation. One of the *CYP1B1* mutations, Y81N, is new. It was present in the heterozygous state in two unrelated patients. It is likely to be pathogenic because the amino acid change is non-conservative while the tyrosine residue at this position is highly conserved across species in the CYP1 gene family and even among other members of the cytochrome P450 superfamily (see online supplemental fig 2, available at <http://jmg.bmjournals.com/supplemental/>). In addition, one of the two patients (patient C) was the father of two children who developed early-onset glaucoma, with ages at onset of 39 and 44 and who both carried the mutation (fig 2). Finally, the mutation was not detected in 394 control chromosomes (see below).

All other mutations encountered in this group of POAG patients were previously observed in PCG patients, in France or elsewhere. The patient A with juvenile-onset glaucoma and with a compound heterozygous mutation (table 2) was described above (fig 1B). He was included as he was the proband of his family. For all other patients, no family history of PCG was recorded. In particular, they were unrelated to French PCG patients previously studied.¹⁸ One mutation, 269delSNF, was homozygous. The carrier was of French descent, whereas the mutation was initially described in PCG patients from Saudi Arabia and, in France, in families originating from North Africa.^{18–23} All other mutations were heterozygous. The heterozygous E229K mutation was previously associated with PCG in two French cases.¹⁸ Two other mutations, including 7901_7913delGAGTGCAGGCAGA (two cases) and R390H (one case), were previously associated with PCG in conjunction with a *CYP1B1* mutation on the other chromosome.^{13–18} The last mutation, A443G, has been observed in Caucasian PCG patients from different countries.^{15–17} In the three French POAG patients, it was associated with one or two rare synonymous variants, including G4534C (V243V) and T7915C (L360L). The latter variant has not been described before. The A443G mutation has not been observed in Caucasian controls.^{13–14} Interestingly, a recent report indicated that it could be a common variant in an African population, with a frequency of 7%.²⁴ The study, however, included no assessment of vision while POAG is more frequent (5–10%) and occurs earlier in black than in white subjects.^{2–3} With this caveat, the pathogenic property of A443G might be population-specific, perhaps because it depends on other factors, themselves population-specific. Alternatively, the mutation might be in linkage disequilibrium with an actually pathogenic variant in Caucasians.

Besides the above described mutations, the six common SNPs previously reported including T3793C, R48G, A119S, V432L, D449S, and N453S were also observed in POAG patients.^{14–17–18–23} None of these SNPs nor a particular haplotype formed by them was preferentially associated with the mutations (not shown). The rare synonymous variant G4534C (V243V) was detected by itself in one case. No other *CYP1B1* variant was detected in the POAG group. Previous reports of Caucasian controls totalling 150 persons from three populations, in the United Kingdom, Turkey, and Saudi

Table 1 Primer sequences of *CYP1B1* amplicons, PCR conditions, and DHPLC analysis parameters

Amplicon	Primer sequences (5' to 3')	PCR product size (bp)	MgCl ₂ (mM)	Annealing temperature (°C)	Acetonitrile gradient (%B), start-end	Oven temperature (°C)
Exon 2_A	F: CGCTCTGTCTCTGCACCCC R: GGCGCGGCGCTACGGCGACG	302	0.5	60	57–63 53–59	65 69
Exon 2_B	F: TGGCCACTGATCGGAAACGC R: CCAACGTCATGAGTGCCGTG	456	1	57	61–67 59–65	67 70
Exon 2_C	F: GCCACGTGCTGAGCGAGGCG R: CATCTGGACAAGTCTTGA	329	1	57	58–64 57–63 52–58	65 66.5 71
Exon 2_D	F: GGTGCGCACCGTTTTCCGCG R: GGGTTGGGGTTTCGCTCCA	395	1	55	59–65 57–63	62 64.5
Exon 3_B	F: GAGAACTTTGATCCAGCTCG R: GCAAGAGGCAAGCTGAAATT	343	1	55	58–64 55–61	58 60.5

F, Forward, R, Reverse.

Arabia, indicated an absence of *CYP1B1* variants apart from the six common SNPs.^{13, 14} We also searched for *CYP1B1* variants in 47 French Caucasian controls who were spouses belonging to large families of POAG patients carrying a *MYOCILIN* mutation. Based on gonioscopy, on assessment of the optic disks, and on an IOP measurement in the normal range, their ophthalmic examination was estimated to be normal. The mutation screening protocol was exactly the

same as that used for the group of patients. One mutation, R368H, which was previously associated with PCG,^{17, 21} was detected on one chromosome (table 3). Three synonymous variants, including C3851T (L16L) (one case), C4369A (G188G) (two cases), and V243V (two cases) were also found.

Altogether, the finding of 11 mutation carriers among 236 patients (4.6%) compared to one carrier in 197 controls (47 French+150 previously reported controls) is significant (two-sided exact $p = 0.008$). Also, if one considers a prevalence of 2.85×10^{-5} for PCG cases in Western countries,²⁵ the expected frequency for heterozygotes is approximately 1%, assuming a recessive mode of inheritance and under the condition of Hardy-Weinberg equilibrium. The finding of 11 mutation carriers among 236 subjects is significant (two-sided p value using a binomial law = 0.0001, with the null hypothesis of a probability value of 0.01). The calculation, however, is conservative because one of the POAG patients had a homozygous mutation while the heterozygous E229K mutation is associated with PCG in France and also because all PCG cases are assumed to be caused by *CYP1B1* mutations.

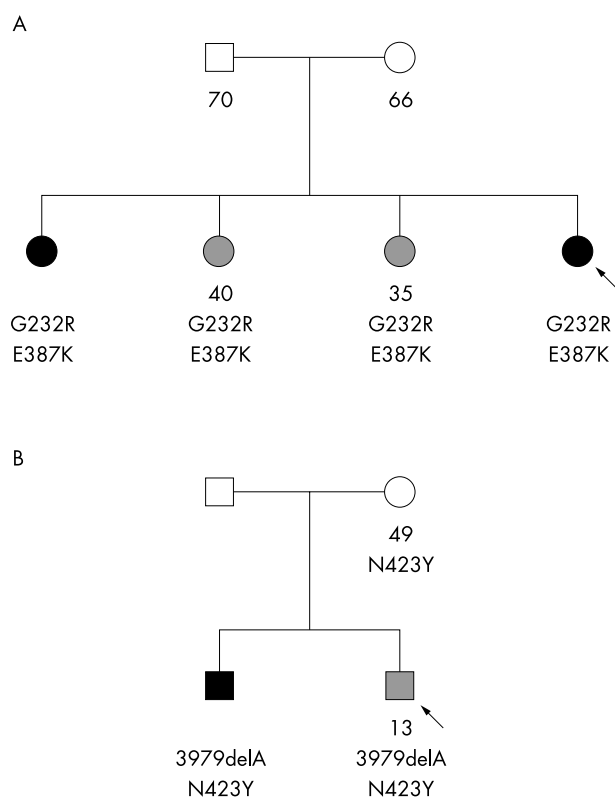


Figure 1 Variable expressivity of *CYP1B1* mutations in two sibships. The arrows show the probands. Available genotypes and ages at diagnosis or at examination are indicated below the symbols. (A) Four sisters, all carrying two missense mutations, G232R and E387K. Two were affected with PCG (dark symbol) while the other two developed POAG (hatched symbol). The parents, who are obligate carriers, showed no glaucoma symptom at the indicated ages. Because they could not be genotyped, the phase of both mutations is unknown. (B) Two brothers, both carrying a deletion mutation (3979delA) and a missense mutation (N423Y). The proband developed juvenile glaucoma at the age of 13, while his younger brother showed PCG. Their mother carried the missense mutation and showed no glaucoma symptom at the age of 49. Their father could not be examined.

Clinical features of *CYP1B1* carriers with POAG

All patients listed in table 2 had definite bilateral POAG with cupping of their optic discs and significant alteration of their visual field. Evolution of glaucoma was severe enough to require either surgical therapy on both eyes (four patients), argon laser trabeculoplasty on both eyes (one patient), or surgical therapy on one eye and medical bi- or tri-therapy on the other eye (four patients). Of note, their iridocorneal angle was recorded as being normal. The most remarkable feature was their young age at the time of diagnosis, with a median of 40 years (range: 13–52). Epidemiologic studies indicate that the prevalence of POAG is 5–10 times lower before the

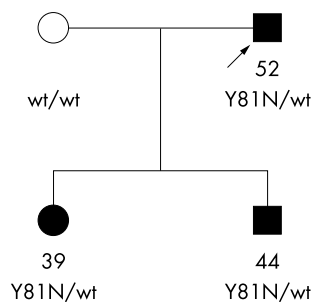


Figure 2 Pedigree showing cosegregation of the heterozygous Y81N *CYP1B1* mutation with POAG (solid symbols). The arrow shows the proband. Available genotypes and ages at diagnosis are indicated below the symbols.

Table 2 *CYP1B1* mutations and rare variants in 12 among 236 unrelated French POAG patients and their clinical initial features at the time of diagnosis

Patient	Mutation	Exons	Effect	Status	Age (years)	IOP (mm Hg) OS/OD	Rare <i>CYP1B1</i> polymorphisms
A	3979 delA+A8104T	2+3	Frameshift and stop at 59+N423Y	Compound heterozygote	13	42/42	
B	T4046A*	2	Y81N	Heterozygous	52	NA	
C	T4046A	2	Y81N	Heterozygous	37	28/28	
D	G4490A	2	E229K	Heterozygous	35	25/21	
E	7901_7913 delGAGTGCAGGCAGA	3	Frameshift+stop at 422	Heterozygous	50	20/20	
F	7901_7913 delGAGTGCAGGCAGA	3	Frameshift+stop at 422	Heterozygous	40	26/36	
G	4611_4619 delGCAACTCA	2	269delSNF	Homozygous	51	40/32	
H	G8006A	3	R390H	Heterozygous	26	25/25	
I	C8165G	3	A443G	Heterozygous	48	30/30	V243V, L360L†
J	C8165G	3	A443G	Heterozygous	36	20/20	V243V
K	C8165G	3	A443G	Heterozygous	31	25/25	L360L
L		2		Heterozygous	50	30/30	V243V

*New mutation; †New polymorphism; NA, not available.

age of 60 compared to that after this age.^{3, 26} Therefore, *CYP1B1* mutations were typically associated with juvenile and middle-age onset POAG. Moreover, although the age at diagnosis in the whole group of patients also was young (median: 48; range: 6–81; interquartile range: 37–58), the age at diagnosis of *CYP1B1* carriers was nonetheless younger than that of non-carriers ($p = 0.023$, Mann-Whitney test).

DISCUSSION

Our data demonstrate an increased prevalence of *CYP1B1* mutations among French POAG patients. They extend the previous findings of Vincent *et al*,²¹ indicating that mutations in the *CYP1B1* gene, nearly all of which were previously associated with PCG, are a significant risk factor for early-onset POAG also in patients who do not carry a *MYOC* mutation. Because parents of PCG patients, who carry heterozygous mutations of *CYP1B1*, are apparently not at increased risk of developing glaucoma (see Bejjani *et al*²³ and this work), gene dosage is unlikely to be a general mechanism for delayed expression of heterozygous mutations. Additional factors are probably necessary for development of the glaucomatous process. Apart from a mutation of the *MYOC* gene, a modifier locus influencing penetrance of *CYP1B1* mutations has been described.²³ This locus or a similar one could explain the delayed onset of disease in patients with a homozygous mutation or in compound heterozygotes. Moreover, in an experimental mouse model with a knock-out *cyp1b1* gene, a recent report indicated that an alteration of the dopamine pathway could markedly exacerbate mild ocular symptoms associated with a defective *cyp1b1* gene.²⁷ Alternatively, one of the hallmarks of the *CYP1B1* gene is its inducibility by xenobiotics and by mutagenic chemicals.²⁸ Therefore, onset of glaucoma in *CYP1B1* mutation carriers might be caused by exposure to an environmental factor of this kind.

Intriguingly, the IOPs of *CYP1B1* mutation carriers at the time of diagnosis were variable, ranging from borderline (20 mm Hg) to markedly elevated (40 mm Hg). Given that marked elevation of IOP is a prominent feature of PCG, the observation of normal to moderately elevated IOP measurements was unexpected. However, the recent discovery of expression of *CYP1B1* in the posterior segment of the eye, notably in the neuroretina,²⁹ strongly suggests that mechanisms that do not depend directly on IOP elevation may also explain a role for *CYP1B1* in glaucoma pathogenesis. For example, accumulation of a toxic substrate as a result of a defective *CYP1B1* gene would result in greater sensitivity of neural ganglial cells to other stress factors and notably would lower the threshold necessary for IOP to cause damage to the optic nerve.

Although mutations of the *CYP1B1* gene have been identified in PCG patients from different populations, the frequency of the mutations, their nature, and their diversity vary greatly with the population studied. Similarly, the role of *MYOC* mutations in POAG appears to very much depend on the ethnic origin of the patients.^{22, 30} It will be therefore important to reproduce our findings in different groups of patients, considering both clinical and population factors in their selection, perhaps by means of a multinational collaborative study.

Identifying *CYP1B1* mutations in persons at risk of developing glaucoma, particularly among relatives of POAG patients, is of considerable clinical interest. It should help to monitor vision and to detect early symptoms of POAG in these persons. Because simple treatments are already available and operate optimally when they are given at an early stage of the disease, mutation screening for *CYP1B1* could contribute to preventing the visual impairment caused by glaucoma. Finally, common polymorphisms of the *CYP1B1* gene have been reproducibly associated with varying risks of developing several cancers,^{31, 32} whereas in mice inactivation of *cyp1b1* was associated with an increased resistance to chemically-induced lymphomas.³³ The observation of subjects with null alleles of *CYP1B1* therefore should provide invaluable insight into the role of *CYP1B1* in carcinogenesis³⁴ as well as in other physiological processes involving this gene in the human species.

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Table 3 *CYP1B1* mutation and variants in 47 French controls

DNA variant	Exon	Occurrences (n)	Codon	Effect
G7940A	2	1	368 (Arg)	Change>His
C3851T	2	1	16 (Leu)*	Synonymous
C4369A	2	2	188 (Gly)	Synonymous
G4534C	2	2	243 (Val)	Synonymous

*New variant.

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