



Association of a common coding polymorphism (N453S) of the cytochrome P450 1B1 (*CYP1B1*) gene with optic disc cupping and visual field alteration in French patients with primary open-angle glaucoma

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Purpose: To investigate a role of common polymorphisms of the *CYP1B1* gene in French patients with primary open-angle glaucoma (POAG).

Methods: Six common *CYP1B1* variants, 5 coding and one in promoter, were compared in 224 unrelated French Caucasian POAG patients, excluding those with a *CYP1B1* mutation, and in 47 population-matched controls with a normal ophthalmic examination. Allelic associations were assessed with the D' and r^2 parameters. An effect of the representative variants on subphenotypes, including the age and the intraocular pressure at diagnosis, the cup to disk ratio, and the visual field alteration, was tested by multivariate analyses.

Results: Allele and haplotype frequencies were similar in patients and in controls. Five variants formed two groups with tightly correlated alleles while the sixth one, N453S, was independent. The age and the intraocular pressure at diagnosis were not influenced by any of the variants. In contrast, the 453*Serine allele was associated with decreased cupping of the optic disk (Odds ratio=0.32, 95% CI: 0.15-0.70; $p=0.0036$) and with a milder alteration of the visual field ($p=0.025$).

Conclusions: The common N453S coding variant of *CYP1B1* is potentially a factor of severity in POAG patients.

Primary open-angle glaucoma (POAG) is the most common form of glaucoma and one of the leading causes of irreversible blindness worldwide [1]. It is characterized by cupping of the optic nerve head, degeneration of ganglion cells, and progressive visual field damage [2]. The condition is frequently associated with an increase in intraocular pressure (IOP) which, however, is neither necessary nor sufficient for the onset or the progression of the disease.

Genetic factors play an important but complex role in POAG predisposition. Seven loci were mapped by linkage analysis of large families [3,4]. Three of these loci, *MYOCILIN* (*GLC1A/MYOC*), *OPTINEURIN* (*GLC1E/OPTN*), and *GLC1G/WDR36* were identified at the molecular level [4-6]. Typically, mutations of *MYOC* are associated with juvenile-onset glaucoma, and markedly elevated IOP [5,7], whereas mutations of *OPTN* are associated with adult onset and normal or moderately elevated IOP [6]. *WDR36* mutations were identified in both high and low pressure adult-onset POAG [4].

In addition to *MYOC* mutations that account for a small proportion of POAG cases in the French population (7%) [8], we recently reported that mutations in a fourth gene, *CYP1B1*, a member of the cytochrome P450 superfamily that is strongly inducible by dioxins [9], could be observed in 4.6% of French

POAG patients, preferentially with an early onset of disease [10]. Prior to our report, this gene was demonstrated to be the major gene, recessively mutated, in patients from different populations with primary congenital glaucoma, a rare but severe form of glaucoma [11-14]. The pathogenesis of *CYP1B1* mutations is presently unknown.

Apart from mutations, the *CYP1B1* gene harbors several common single-nucleotide polymorphisms (SNPs), five of which are coding [13,14]. Some of them have been associated with cancer predisposition in different populations [15-17]. Here, in light of these findings, we have investigated whether common SNPs in the *CYP1B1* gene, including an SNP in the upstream region and the 5 coding SNPs, might influence susceptibility to POAG or modify the patient phenotype.

METHODS

Patients and controls: The study included 224 unrelated French Caucasian POAG patients previously described [8,10] and was approved by the Ethics Committee of the Necker Medical School. Informed consent was obtained from all subjects according to the European Legislation. Clinical assessment included slit-lamp biomicroscopy, IOP measurement with a Goldman applanation tonometer, and automated perimetry with a Humphrey visual field analyzer, Octopus, or Moniteur Ophtalmologique. Diagnosis and clinical classifications were done prior to the genetic analysis. POAG was defined with the following criteria; normally open iridocorneal angle (grade III or IV gonioscopy), characteristic optic disc cupping, and

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an alteration of the visual field. The IOP level was not a criterion of selection. Because different perimetry devices were used, visual field alterations were grouped by consensus of two ophthalmologists into three robust categories of increasing severity to allow for comparison, including (1) mild alteration (including early defect, arcuate scotoma or nasal step), (2) advanced scotoma, and (3) no light perception. This ordinal variable was concordant with cupping of the optic disc to a highly significant level (Gamma coefficient=0.72, Kendall $\tau=0.44$, $p<10^{-10}$). Cup to disc ratios were estimated by glaucoma specialists and were available for 197 patients and visual field findings for 201 patients. Individuals with a cause of secondary glaucoma including glucocorticoid treatment, history of trauma or surgery, media opacity, pigment dispersion, and exfoliation syndrome were excluded. In addition, patients with a *CYP1B1* mutation were excluded from the data set. The control group consisted of 47 unrelated Caucasian spouses with a normal ophthalmic examination from *GLCIA/MYOC* linked-glaucoma French families.

Genotyping: Alleles of the common coding SNPs shown in Table 1 were determined, blindly to the clinical data, as a result of the exhaustive characterization of the polymorphism of the *CYP1B1* coding region in both the patients and the controls, as reported in our previous work [10]. In addition, an SNP (T>C) in the upstream region, situated at 265 bp before the transcription start site [18,19], at position 2,805 (EMBL/GenBank U56438) was typed by PCR-RFLP. A 328 bp fragment was amplified with the following primers: 5'-GGT TGT ACC GAG CGT GGT TC-3' (forward) and 5'-TCT CAC AAC TGG AGT CGC AG-3' (reverse). The reaction was performed in a 25 μ l mixture containing 100 ng of genomic DNA, 0.4 μ mol/l of forward and reverse primers, 1.5 mmol/l $MgCl_2$, 5% dimethylsulfoxide, 200 μ mol/l of each dNTP, and 0.5 U of Taq DNA polymerase (Invitrogen, Life Technologies, Carlsbad, CA). Cycling conditions were for each cycle, 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, for 35 cycles. The alternative 2805C allele creates a unique Taq I restriction

site, yielding two fragments of 288 and 40 bp following cleavage of the PCR product, which were detected after migration on a 2% agarose gel. All SNPs were in Hardy-Weinberg equilibrium.

Data analysis: Allele frequencies in patients and in controls were compared with the COCAPHASE program of the UNPHASED suite [20]. Haplotypes were deduced from unphased genotype data and compared between cases and controls with similar findings using COCAPHASE and the PHASE v2.0 program [21,22]. Measures of pairwise linkage disequilibrium (LD) between SNPs, including Lewontin's standardized disequilibrium coefficient (D') and the squared correlation coefficient (r^2), were computed with the LDMAX software provided with the Graphical Overview of Linkage Disequilibrium (GOLD) package [23].

The effect of representative SNPs on subphenotypes was assessed through multivariate analyses. The age and IOP at the time of diagnosis were continuous variables following a normal distribution. Their dependency on representative SNPs was tested with a multi-way analysis of variance with the Statistica 6.0 software (Statsoft Inc., Tulsa, OK). The cup to disk ratio was a discrete variable taking values between 0.4 and 1 and with a skewed distribution. Its median was 0.8 and its mode was 0.9. It was therefore transformed into a binary variable, allowing for multivariate analysis. Patients were grouped into two categories, considering 0.8 as the threshold for severe cupping. Findings were very similar if 0.7 was used instead as the threshold. The dependency of this binary variable on representative SNPs was tested by logistic regression, using the LogXact version 6 software (Cytel, Cambridge, MA). Because the time period between diagnosis and examination influenced the cup to disk ratio (OR=1.05, $p=0.025$), effects of the SNPs were estimated after adjustment for this parameter. The p value produced by this analysis was multiplied by 3, using a Bonferroni correction for the number of subphenotypes tested (age, IOP at the time of diagnosis, and cup to disk ratio). Finally, since the visual field was tightly correlated with cup to disk ratios (see above), its dependency on the genetic polymorphism was assessed secondarily to cup to disk ratios, using the nonparametric Mann-Whitney test.

TABLE 1. ALLELE FREQUENCIES OF *CYP1B1* SNPs IN 224 POAG PATIENTS AND 47 CONTROLS

Variant	Nucleotide change	Sample sizes (%)	
		Patients	Controls
2805T>C	2805T>C	340 (75.9)	74 (78.7)
R48G	3947C>G	112 (25)	21 (22.3)
A119S	4160G>T	122 (27.2)	21 (22.3)
L432V	8131C>G	202 (45.1)	39 (41.5)
D449D	8184C>T	194 (43.3)	38 (40.4)
N453S	8195A>G	98 (21.9)	22 (23.4)

Six common variants were genotyped in the *CYP1B1* gene, one (2805T>C) in the promoter region and the other five in the coding region. They show similar allele frequencies in patients and in controls. The first allele and the position correspond to the consensus sequence (*CYP1B1* EMBL/Genbank: U56438). Except for the 2805 position, the variant allele is the minor one.

TABLE 2. *CYP1B1* HAPLOTYPE FREQUENCIES ESTIMATED IN 224 POAG PATIENTS AND 47 CONTROLS

Haplotypes	Sample sizes (%)	
	Patients	Controls
C-C-G-C-C-A	35 (8)	12 (13)
C-C-G-C-C-G	88 (20)	22 (23)
C-C-G-G-T-A	185 (41)	38 (40)
T-G-T-C-C-A	99 (22)	20 (21)

Haplotypes formed by the 6 SNPs indicated in Table 1 were reconstructed with the COCAPHASE and PHASE 2 programs. Only common haplotypes with a relative frequency >5% are reported. Their frequencies are similar in patients and in controls.

RESULTS

Allele frequencies at the six common SNPs of the *CYP1B1* gene did not differ significantly between the POAG patients and the controls (Table 1). Other alleles that had been detected in our exhaustive analysis of *CYP1B1* sequence were too rare (frequency less than 2%) to contribute to a statistical finding [10]. Therefore they were not included in the present study.

Haplotypes were reconstructed from the genotypic data. Four major haplotypes, among the 18 estimated by COCAPHASE, showed a frequency >5% (Table 2). They accounted for 91% of estimated haplotypes in patients and for 97% in controls. Their frequencies were similar in both groups. All other haplotypes had frequencies less than 2%. Because of the small size of the control group, we cannot rule out that we did not detect a small difference in allele or haplotype frequencies between patients and controls. Therefore larger samples will have to be tested to observe such differences.

There were fewer haplotypes than expected ($2^6=64$), indicating a strong linkage disequilibrium across the *CYP1B1* locus. As shown in Figure 1, the D' coefficient was equal or close to 1 for all pairs of SNPs, both in patients and in controls (Figure 1A), strongly suggesting that there has been no or little historical recombination in the region over time. The r^2 parameter that measures the correlation between alleles showed that 5 of the 6 SNPs formed two groups of tightly correlated SNPs (Figure 1B). The first group included the SNP of the upstream region, 2805C>T, and the two coding SNPs in exon II, R48G and A119S. The second group included another two SNPs in exon III, L432V and D449D. The remaining SNP, N453S, also in exon III, was weakly correlated to the other five SNPs and was therefore independent. This pattern of association was very similar in patients and in controls.

One SNP from each group of associated SNPs, including 2805T>C and L432V, and the independent SNP, N453S, were

A:

SNP	2805T>C	R48G	A119S	L432V	D449D	N453S
2805T>C		0.92/1	0.97/1	0.93/1	0.92/1	0.91/1
R48G			0.95/1	0.84/1	0.82/1	0.92/1
A119S				0.92/1	0.91/1	0.62/1
L432V					0.98/1	1 /1
D449D						1 /1
N453S						

B:

SNP	2805T>C	R48G	A119S	L432V	D449D	N453S
2805T>C						
R48G	0.81/0.94					
A119S	0.80/0.94	0.80/1				
L432V	0.23/0.19	0.19/0.2	0.26/0.2			
D449D	0.21/0.18	0.17/0.19	0.24/0.19	0.89/0.96		
N453S	0.07/0.08	0.08/0.09	0.04/0.09	0.23/0.22	0.21/0.21	

Figure 1. Groups of allelic correlations among *CYP1B1* common SNPs. Pairwise linkage disequilibrium between *CYP1B1* SNPs was measured with two coefficients: D' (A) and r^2 (B) in 224 POAG patients (first value) and 47 controls (second value). Findings were similar in patients and in controls. The D' parameter is close or equal to 1, indicating that few historical recombinations have occurred in the locus. In contrast, the r^2 coefficient, which measures the correlation between alleles, varies broadly. Based on r^2 , SNPs form three groups represented with the colors red (2805T>C, R48G, A119S), blue (L432V, D449D), and green (N453S). Within each group, alleles of SNP pairs are tightly correlated, as indicated by an r^2 value greater or equal to 0.8. Alleles of two SNPs belonging to different groups are poorly correlated.

selected and their collective effect on important clinical parameters was assessed by means of multivariate analyses. There was no significant effect of the three representative SNPs on the IOP and the age at diagnosis, tested by a three factor ANOVA. In contrast, severe cupping of the optic disk was significantly altered by the SNPs, as assessed by logistic regression ($p=0.0016$; corrected $p=0.005$, for 3 phenotypes tested). Parameter estimation indicated that this effect was associated with the N453S SNP ($p=0.0036$) and not with the other two SNPs (Table 3). The 453*Serine allele was observed in 76 of the 197 patients with measured cup to disk ratios. It was associated with a decreased cupping (60 cases with cup to disk ratio less than or equal to 0.8 and 16 cases with cup to disk ratio >0.8; odds ratio=0.32, 95% confidence interval: 0.15-0.70). There were too few Ser/Ser homozygous patients ($n=8$) to efficiently discriminate between a dominant and a partially dominant effect of the Serine allele.

As seen in Table 4, the 453*Serine allele was also associated with a milder alteration of the visual field (Mann-Whitney test, one sided $p=0.025$), which was somehow expected given the concordance between alteration of the visual field and cupping of the optic disc.

DISCUSSION

Identification of SNPs in the genes that are associated with glaucoma or with glaucoma severity should contribute to better understand the disease mechanisms. In addition, one potentially important application of such information is an improved management of the patients, and hopefully rationalized and individualized treatments. Accordingly, SNPs in the *MYOC*, *OPTN*, *APOE*, and *OPA1* genes were associated with POAG predisposition or with subphenotypes, including visual-field damage, IOP control, or age at onset of the disease [24-29]. Our present study reveals an association of a common coding polymorphism of *CYP1B1* as a potential factor of severity in POAG patients for the first time. Remarkably, the N453S variant is functional and is therefore a good candidate to explain our findings. A recent study demonstrated that the

change of the asparagine into a serine at position 453 resulted into a three fold shorter half-life and a two fold lower cellular level of the *CYP1B1* protein, as a consequence of a higher rate of proteasomal degradation [30]. The Serine allele was recently associated with a decreased risk of endometrial cancer [17]. In our study, it was also associated with less severe cupping of the optic disc and milder alteration of the visual field. This protective association, however, is intriguing in light of the effect of deleterious *CYP1B1* mutations on predisposition to primary congenital glaucoma and to early-onset POAG [10,14,31]. Most of these mutations also cause a lower level or even an absence of functional protein and therefore an SNP causing a decrease in protein level would be expected to be associated with more severe symptoms. We propose that this discrepancy could be explained by the fact that, contrary to gene-inactivating mutations, the N453S variant is unlikely to affect the regulation of the *CYP1B1* gene. The *CYP1B1* gene is highly inducible by dioxins [9], and probably also by other still unknown factors, endogenous or environmental. Inability to upregulate *CYP1B1* appropriately in response to such factors might be a critical mechanism in glaucoma pathogenesis. In contrast, a low steady-state level of *CYP1B1* could exert a long-term protective effect on ocular tissues.

Although the N453S variant is independent of the other SNPs investigated in this study, we cannot rule out that another, yet to be identified, SNP associated with N453S is actually responsible for our findings. Since we have characterized the coding region of *CYP1B1* exhaustively, this putative SNP would have to be noncoding. Importantly, the high values, close or equal to unity, taken by the D' measure of pairwise linkage disequilibrium strongly suggests that the DNA segment lying between the 2805T>C promoter variant and N453S behaves as a single haplotypic block. In this regard, it is striking that the 2805T>C promoter SNP is tightly correlated with the R48G and A119S variants. These variants are associated with altered enzymatic activity of *CYP1B1* [32] and with susceptibility to several cancers [15,16]. Thus, the 2805T>C SNP, or other SNPs recently identified in the upstream region of *CYP1B1* [19],

TABLE 3. ASSOCIATION OF THE 453*SERINE VARIANT WITH DECREASED CUP/DISK RATIO

Allele	CDR<=0.8	CDR>0.8	OR (95% CI)	p value
2805*T	58 (45)	27 (39.7)	0.55 (0.28-1.1)	0.09
432*Val	87 (67.4)	53 (77.9)	1.04 (0.47-2.28)	0.93
453*Ser	60 (46.5)	16 (23.5)	0.32 (0.15-0.70)	0.0036

Three representative *CYP1B1* SNPs, including 2805T>C, L432V and N453S, were selected and an effect of their minor alleles (first column) on the cup/disk ratio (CDR) was assessed by logistic regression. The second and third columns indicate the frequencies (and the corresponding percentages) of cases with a CDR less than or equal to 0.8 (column 2; 129 cases) and >0.8 (column 3; 68 cases). The fourth and fifth column report the estimates of odds ratios (OR) with 95% confidence intervals (95% CI) and the p values for each allele. The overall p value for the logistic model including the 3 alleles, adjusted for the time period between the diagnosis and the examination, was 0.0016. The data show that the 453*Serine allele is associated with a significantly decreased cupping of the optic disk.

TABLE 4. ASSOCIATION OF THE 453*SERINE VARIANT WITH A MILD ALTERATION OF THE VISUAL FIELD

Visual field	Asn/Ser or Ser/Ser number (%)	
	Asn/Asn number (%)	Asn/Ser or Ser/Ser number (%)
Mild alteration	52 (43)	44 (55)
Advanced scotoma	54 (44.6)	32 (40)
No light perception	15 (12.4)	4 (5)

Patients were categorized into three groups depending on the severity of their visual field alteration (first column). In each group, the frequencies (and percentages) of patients without (second column) or with (third column) the 453*Serine allele are reported. Comparison of the severity of the visual field alteration in both genotypic groups with the Mann-Whitney test shows a significant difference (one sided $p=0.025$). The 453*Serine variant is associated with a milder alteration of the visual field.

might also contribute to these reported risks. Recent data from the international HapMap project shed more light on this point and show that the *CYP1B1* gene belongs to a large block of linkage disequilibrium of 99 Kb extending between SNPs rs336035 and rs727631, and covered with a density greater than 1 SNP per 2 Kb, in Caucasian populations. The N453S (rs1800440) and L432V (rs1056836) variants, but not the other four variants of our present study, have been characterized in HapMap (release 16c.1/Phase I June 05). Using the r^2 parameter of correlation between alleles, the N453S variant is not associated with any other SNP of the current dataset. This indicates that the other SNPs currently typed in the HapMap project are unlikely to account for the association reported herein.

In conclusion, this finding is a first observation of an association of *CYP1B1* with POAG subphenotypes in the French population. It needs to be replicated in larger groups and in other populations to clarify the significance of the N453S variant in POAG pathogenesis and to evaluate its usefulness in the clinical management of the patients.

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