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A polymorphism in the *CYP1B1* promoter is functionally associated with primary congenital glaucoma

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Primary congenital glaucoma (PCG) is a childhood autosomal-recessive disorder caused by developmental defects in the trabecular meshwork and anterior chamber angle. These defects cause raised intraocular pressure (IOP) that damages the optic nerve and if left untreated, results in irreversible blindness. Mutations in *CYP1B1* gene at the *GLC3A* locus (2p21) are associated with PCG. However, there has been very limited exploration of its promoter region. We resequenced the *CYP1B1* promoter in a large cohort ($n = 835$) that included patients with PCG ($n = 301$), other primary glaucomas (primary open-angle glaucoma: $n = 115$ and primary angle closure glaucoma: $n = 100$) and unaffected controls ($n = 319$). We functionally characterized one associated variant by luciferase reporter assay using the trabecular meshwork (TM3) cell line. We found evidence of strong ($P = 6.01 \times 10^{-4}$) association of rs2567206 (T2805C) SNP in PCG and not in other primary glaucomas. Luciferase assay indicated a ~90% reduction in CYP1B1 promoter activity in the risk-allele (C) compared to the other allele (T). The association of the risk allele was stronger in cases harboring homozygous *CYP1B1* mutations ($P = 3.42 \times 10^{-12}$). The risk haplotype 'C-C-G' in the promoter had a strong non-random association to the previously characterized risk haplotype 'C-G-G-T-A' in the coding region. The independent effect of genotype at the promoter T2805C locus ($P = 0.001$), and the interaction effect of genotypes at the promoter and coding region mutations loci ($P = 0.001$) were significant for the presenting IOP of the worst affected eye. This is the first study that unequivocally shows the functional involvement of a *CYP1B1* promoter variant in PCG.

INTRODUCTION

Primary congenital glaucoma [PCG (MIM 231300)] is an autosomal-recessive disorder of the eye, caused by developmental defects in the trabecular meshwork (TM) and anterior chamber angle (1,2). It has an onset in the neonatal or early infantile period. The developmental defects cause obstruction of aqueous outflow leading to raised intraocular pressure (IOP) that damages the optic nerve and if left untreated, leads to irreversible blindness (3,4). The disease prevalence is very high among the inbred populations [1 in 1250 among the Slovakian Gypsys (5), 1 in 2500 among the Saudi Arabians (6) and 1 in 3300 among the Indian population in Andhra Pradesh (7)] compared with the western (1 in 10 000 to 1 in 20 000) populations (3,5).

Three chromosomal loci, viz. *GLC3A* [2p22–p21 (MIM 231300)] (8), *GLC3B* [1p36.2–p36.1 (MIM %600975)] (9) and *GLC3C* [14q23 (10)], have been identified by linkage analysis, and the human cytochrome P450 gene *CYP1B1* (MIM 601771) on *GLC3A* has been widely implicated in PCG (11,12). Allelic heterogeneity of *CYP1B1* is the hallmark of

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Table 1. Distribution of mutations in the coding region of *CYP1B1* in patients with different glaucoma phenotypes

Patients with different types of glaucoma (*n*)	Overall frequency of *CYP1B1* mutations (*n*, %)	Patients with the same variant nucleotides at the same nucleotide position [true homozygotes (*n*, %)]	Patients with variant nucleotides, but at two different nucleotide positions [compound heterozygotes (*n*, %)]	Patients heterozygous for a variant nucleotide (*n*, %)	Cases without mutations (*n*, %)
PCG ($n = 301$)[a]	133 (44.2)	74 (24.6)	17 (5.6)	42 (14.0)	168 (55.8)
POAG ($n = 134$)[b]	25 (18.6)	3 (2.2)	–	22 (16.4)	109 (81.4)
PACG ($n = 100$)[b]	10 (10.0)	–	–	10 (10.0)	90 (90.0)

[a]Based on the present study.
[b]Based on our earlier study (19).

PCG, and a wide spectrum of mutations has been observed in the coding region of *CYP1B1* worldwide (13). Globally, these mutations are clustered on specific intragenic haplotypes irrespective of their geographical locations that have provided evidence of strong founder effects (14,15). Rarely, *CYP1B1* is associated in other primary glaucomas: both primary open-angle glaucoma [POAG (MIM 137760)] (16–18) and primary angle closure glaucoma [PACG (19)]. Interestingly, the mutation spectrum in the primary glaucomas was similar to that found in PCG and occurred on the same haplotype backgrounds (19).

Other glaucoma-associated candidate genes such as *MYOC* [MIM 601652 (20,21)] and *FOXC1* [MIM 601090 (22)] have exhibited a limited involvement in PCG. Recently, null mutations in *LTBP2* gene (MIM 602091) flanking the *GLC3C* locus has been characterized in a few PCG families (23). However, the mutation spectrum of *LTBP2* across diverse PCG populations is yet to be determined.

CYP1B1 is an estrogen-metabolizing enzyme of the *CYP1* gene family (24,25). It forms metabolites via hydroxylation of CYP450 and catalyzes 4-hydroxylation of the 17β-estradiol (26). It has a ubiquitous expression in multiple tissues that include heart, lung, liver, kidney and reproductive tissues (27). Variants in the CYP1B1 protein coding region have exhibited enhanced activity and altered kinetics (28,29). Although the exact mechanism by which *CYP1B1* causes glaucoma is yet unknown, *Cyp1b1*$^{-/-}$ mice have exhibited structural abnormalities in the ocular drainage structures similar to human PCG (30,31).

The coding region of *CYP1B1* has been screened extensively in PCG patients, but relatively fewer studies have examined the promoter region (21,32). Because of the extensive involvement of *CYP1B1* in PCG, the apparent lack of involvement of this gene in a substantial (~50%) fraction of PCG patients (13,14,20–22) together with a large variability in post-treatment control of IOP in patients harboring *CYP1B1* mutations prompted us to hypothesize that variations in other genomic regions, especially the *CYP1B1* promoter, may be responsible for the disease.

RESULTS

The distribution of *CYP1B1* mutations among the PCG, POAG and PACG cases are provided in Table 1. Resequencing of the *CYP1B1* promoter and the untranslated region revealed three variants, of which two were in the promoter and the other in the non-coding exon 1 (Fig. 1). There was no significant deviation from Hardy–Weinberg equilibrium for these SNPs in the normal controls.

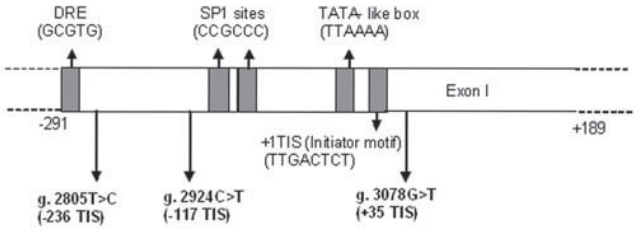

Figure 1. A schematic representation of the *CYP1B1* promoter (44) with locations of regulatory elements and the variants that were observed in PCG.

Among the observed variants, rs2567206 (T2805C) was significantly associated with PCG ($P = 6.01 \times 10^{-4}$), whereas the others were monomorphic (MAF < 0.05; Table 2). The association of the rs2567206 SNP was even stronger [OR = 6.04 (CI: 3.51–10.43)] in PCG cases (Table 3) that harbored homozygous and compound heterozygous *CYP1B1* mutations ($P = 3.42 \times 10^{-12}$). The exclusion of compound heterozygotes did not alter (Table 3) the strength of the association [OR = 5.91 (CI: 3.28–10.64)] significantly ($P = 1.67 \times 10^{-10}$). There was no association between the rs2567206 SNP with PCG cases that had either a heterozygous mutation ($P = 0.503$) or did not harbor any *CYP1B1* mutation ($P = 0.328$). The genotype counts for the rs2567206 SNP in different categories of patients are provided in Supplementary Material, Table S1.

Additionally, we also assayed rs2567206 in a cohort of POAG and PACG but no association was evident with these non-congenital glaucoma cases (Table 3). As the number of primary glaucoma cases with a homozygous *CYP1B1* mutation was very less (Table 1), we did not carry out any further analysis on them.

Haplotype analysis indicated that a 'C-C-G' haplotype in the promoter region was significantly associated with PCG ($P = 0.0012$). Similarly, this association was stronger among PCG cases that harbored homozygous *CYP1B1* mutations ($P = 3.42 \times 10^{-12}$) and thus it could be a risk haplotype. The 'T-C-G' haplotype was protective ($P = 8.11 \times 10^{-12}$). Earlier studies have shown that a risk haplotype in the *CYP1B1* coding region 'C-G-G-T-A' harbored the vast majority of mutations across different populations (6,14), except Chinese (21). Based on evolutionary insights and considerations of parsimony, we had demonstrated this to be the ancestral haplotype as majority of the mutations were clustered on this background (14). We observed strong non-random associations of the promoter 'C-C-G' with the 'C-G-G-T-A' risk haplotypes and the protective 'T-C-G' with the 'G-T-C-C-A' coding haplotypes (Fig. 2).

Table 2. Distribution of *CYP1B1* promoter and non-coding variants in PCG cases and controls

Genomic position[a]	Location in the gene	Associated allele	Frequency of associated allele among		χ^2 (d.f. = 1)	*P*-value
			Cases	Controls		
g.2805T>C	Promoter (rs2567206)	C	0.694	0.573	11.914	6.01×10^{-4}
g.2924C>T	Promoter (Novel)	C	0.993	0.992	0.014	0.9052
g.3078G>T	Exon 1 (Novel)	T	0.008	0.007	0.127	0.1448

[a]CYP1B1 GenBank ID: U56438.

Table 3. Distribution of the risk allele ('C') frequency of rs2567206 in PCG, POAG and PACG patients and unaffected controls

Phenotype combinations	Frequency of 'C' allele among		χ^2	*P*-value	Odds ratio (95% CI)
	Cases	Controls			
All PCG cases versus controls	0.694	0.573	11.914	6.01×10^{-4}	1.69 (1.25–2.28)
PCG cases with *CYP1B1* mutation versus controls	0.802	0.573	30.889	2.72×10^{-8}	3.01 (2.03–4.48)
PCG cases who are 'true homozygotes'[a] at *CYP1B1* versus controls	0.888	0.573	40.817	1.67×10^{-10}	5.91 (3.28–10.64)
PCG cases who are 'true homozygotes'[a] or compound heterozygotes for *CYP1B1* mutations versus controls	0.890	0.573	48.428	3.42×10^{-12}	6.04 (3.51–10.43)
PCG cases who are single heterozygotes for a *CYP1B1* mutation versus controls	0.615	0.573	0.449	0.503	1.19 (0.71–2.00)
PCG cases without any *CYP1B1* mutation versus controls	0.612	0.573	0.956	0.328	1.18 (0.85–1.64)
All POAG cases versus controls	0.639	0.573	2.997	0.083	1.32 (0.96–1.80)
All PACG cases versus controls	0.615	0.573	1.07	0.301	1.19 (0.86–1.64)

[a]True homozygotes as defined in Table 1.

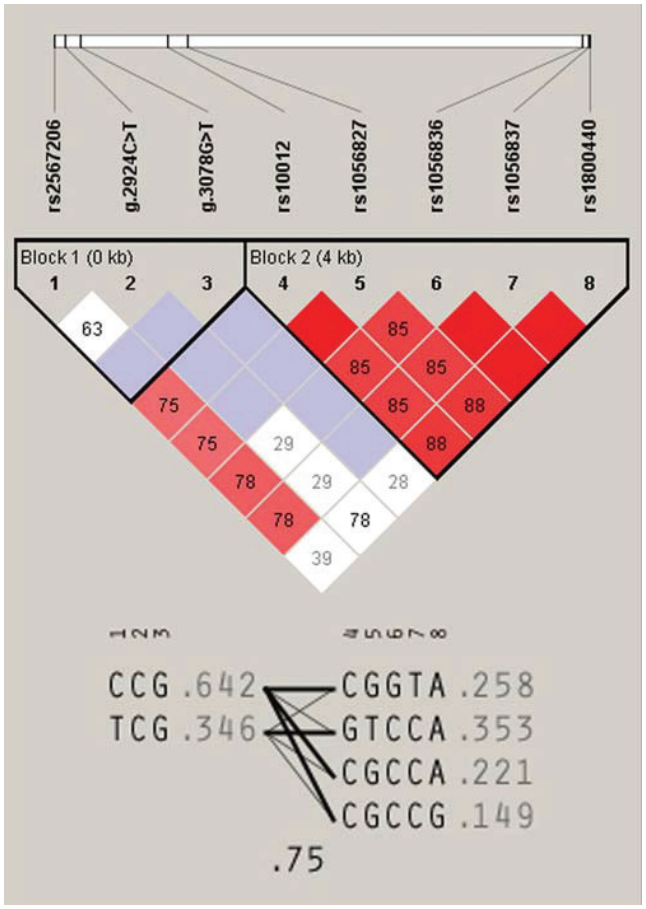

Figure 2. A linkage disequilibrium (LD) map based on the promoter and coding region SNPs in *CYP1B1*.

Extended haplotype analysis with the T2805C variant and the coding region variants indicated that the C-C-G-G-T-A was the risk haplotype that harbored majority of the *CYP1B1* mutations under each phenotype category (Table 4). Interestingly, this haplotype was strongly associated with PCG cases that harbored homozygous (and compound heterozygous) mutations, but not among single heterozygotes or among those who did not harbor any mutations.

As rs2567206 (T2805C) was the most important SNP in the promoter that influenced the risk in PCG cases with *CYP1B1* mutations, we investigated the functional significance of this polymorphism and its effect on the *CYP1B1* promoter activity. The choice of the TM3 cell line was based on the pathophysiology of PCG, which is primarily due to raised IOP caused by abnormalities in the drainage structures in the outflow pathway, particularly the trabecular meshwork (2–4).

The CYP1B1 promoter was active in TM3 cells, with 30–40-fold increase in the luciferase activity in cells transfected with CYP1B1 promoter with the 'T' allele compared with cells transfected with the vector alone. Within each replicate, there was a high reduction in the average luciferase activity (averaged over the two duplicate values within a replicate) in cells transfected with the CYP1B1 promoter with the C allele compared with cells transfected with the T (wild-type) allele. Across the three replicates, relative luciferase activity, averaged over the two duplicate measurements within a replicate, varied from 15 to 47 for the T allele promoter and from 0.7 to 5.0 for the C allele in the promoter. The fold reduction in promoter activity from T to C varied between 9 (Fig. 3A) and 21.

Genotype–phenotype correlation based on the linear [analysis of variance (ANOVA)] model with fixed and interaction effects showed, as expected, that the effect of coding mutation genotype on presenting IOP was significant ($P = 0.022$). In addition, also

Table 4. Distributions of the estimated frequencies of *CYP1B1* haplotypes based on promoter and coding region SNPs across different types of PCG patients and unaffected controls

Phenotype combinations	Haplotypes	%Cases	%Controls	*P*-value	Odds ratio (95% CI)
PCG cases with any *CYP1B1* mutation versus controls	**C-C-G-G-T-A**	**47.1**	**11.9**	**2.46×10^{-19}**	**6.68 (4.30–10.37)**
	C-C-G-C-C-A	20.4	15.6	0.148	1.37 (0.88–2.13)
	C-C-G-C-C-G	8.6	13.5	0.068	0.61 (0.35–1.05)
	T-G-T-C-C-A	18.8	29.7	0.003	0.55 (0.37–0.82)
	C-G-T-C-C-A	2.6	15.1	3.66×10^{-7}	0.15 (0.10–0.35)
PCG cases who are 'true homozygotes'[a] at *CYP1B1* versus controls	**C-C-G-G-T-A**	**56.6**	**11.9**	**1.27×10^{-22}**	**9.62 (5.90–15.70)**
	C-C-G-C-C-A	22.3	15.4	0.079	1.61 (0.97–2.68)
	C-C-G-C-C-G	7.4	13.2	0.070	0.54 (0.27–1.09)
	T-G-T-C-C-A	11.5	29.2	3.96×10^{-5}	0.32 (0.18–0.56)
	C-G-T-C-C-A	0.09	15.5	2.76×10^{-6}	0.04 (0.01–0.27)
PCG cases who are 'true homozygotes'[a] or compound heterozygotes for *CYP1B1* mutations versus controls	**C-C-G-G-T-A**	**53.0**	**11.9**	**1.31×10^{-21}**	**8.41 (5.26–13.45)**
	C-C-G-C-C-A	23.3	15.5	0.036	1.65 (1.02–2.66)
	C-C-G-C-C-G	9.1	13.3	0.166	0.64 (0.35–1.19)
	T-G-T-C-C-A	10.8	29.3	2.73×10^{-6}	0.30 (0.18–0.51)
	C-G-T-C-C-A	1.8	15.4	2.27×10^{-6}	0.09 (0.03–0.30)
PCG cases who are single heterozygotes for a *CYP1B1* mutation versus controls	**C-C-G-G-T-A**	**33.7**	**11.6**	**2.05×10^{-6}**	**3.90 (2.17–7.03)**
	C-C-G-C-C-A	13.7	15.2	0.739	0.85 (0.41–1.72)
	C-C-G-C-C-G	7.0	13.3	0.122	0.51 (0.21–1.25)
	T-G-T-C-C-A	34.6	28.7	0.304	1.03 (0.61–1.75)
	C-G-T-C-C-A	5.7	16.0	0.016	0.33 (0.13–0.86)
PCG cases without any *CYP1B1* mutation versus controls	**C-C-G-G-T-A**	**16.1**	**11.3**	**0.089**	**1.50 (0.93–2.41)**
	C-C-G-C-C-A	23.6	16.1	0.022	1.61 (1.07–2.42)
	C-C-G-C-C-G	15.1	13.9	0.665	1.11 (0.71–1.75)
	T-G-T-C-C-A	34.6	29.7	0.197	1.26 (0.90–1.77)
	C-G-T-C-C-A	4.1	14.5	6.20×10^{-6}	0.25 (0.13–0.48)

[a]True homozygotes as defined in Table 1.

significant on IOP were (i) the independent effects of genotype at the promoter variant locus ($P = 0.001$) and (ii) the interaction effect of genotypes at these loci ($P = 0.001$). These results were similar when the IOP was analyzed separately for each eye. The data on presenting IOP in the patients with respect to their *CYP1B1* mutation status and variation at the promoter locus are provided in Supplementary Material, Table S2. Consistent with these results, the IOP control in PCG patients with *CYP1B1* mutations was poorer compared with patients who did not harbor any mutations, as evident from the 1 year post-surgery Kaplan–Meier survival analysis (Fig. 4).

DISCUSSION

The results of the present study indicated a strong functional association of the rs2567206 SNP in PCG cases harboring homozygous *CYP1B1* mutations. This SNP did not exhibit any association in a Chinese cohort with PCG (21) and in a French cohort with POAG (32). This could be attributed to the small sample sizes in both these cohorts as well as the low frequencies of *CYP1B1* (homozygous) mutations in them. Screening of the *CYP1B1* promoter did not reveal any mutation in the Spanish PCG cohort (33). No association was observed with rs2567206 in patients with colorectal and prostate cancers (34,35).

The association of the rs2567206 SNP in our cohort was further supported by haplotype analysis, wherein the presence of the risk allele 'C' in the extended haplotype (C-C-G-G-T-A) was strongly associated with PCG cases with homozygous mutations (Table 4). Further evidence of strong association of the 'C' allele was determined by mapping the homozygous mutations under different haplotype backgrounds harboring this allele (Table 5). As evident from this table, multiple mutations with varying frequencies were present in the background of the haplotypes harboring the 'C' allele. Also, the 'C' allele did not mark any specific haplotype and was not in LD with any specific mutation.

The T2805C variant led to a significant reduction in the promoter activity of CYP1B1 in cells from the trabecular meshwork. These results suggest that the rs2567206 polymorphism regulates levels of CYP1B1 *in vivo* and can obviously be involved in the PCG pathogenesis. This is in contrast to an earlier report on rs2567206 in which the investigators did not observe any difference in the promoter activity between the 'T' and 'C' alleles (36). This discordant finding may be attributed to the use of a different cell line (human bronchial epithelial cell line) used in the study (36). Analysis of the *CYP1B1* promoter using the MatInspector software (http:// www.genomatix.de) and TFsearch (http://www.cbrc.jp/research/db/TFSEARCH.html) suggests that the promoter has putative binding sites for transcription factors such as Nuclear Transcription Factor Y (MIM 189903), *Pbx-1* (MIM 176310), *GATA-1* (MIM 305371), *Nkx-2* (MIM 604612) and Growth Factor-Independent1 [*Gfi1* (MIM 600871)], encompassing the rs2567206 polymorphism (Fig. 3B). It is interesting to note that some of these transcription factors such as *Nkx-2*, *GATA-1* and *Pbx-1* regulate patterning during development (37–39) and could be involved in regulating the development

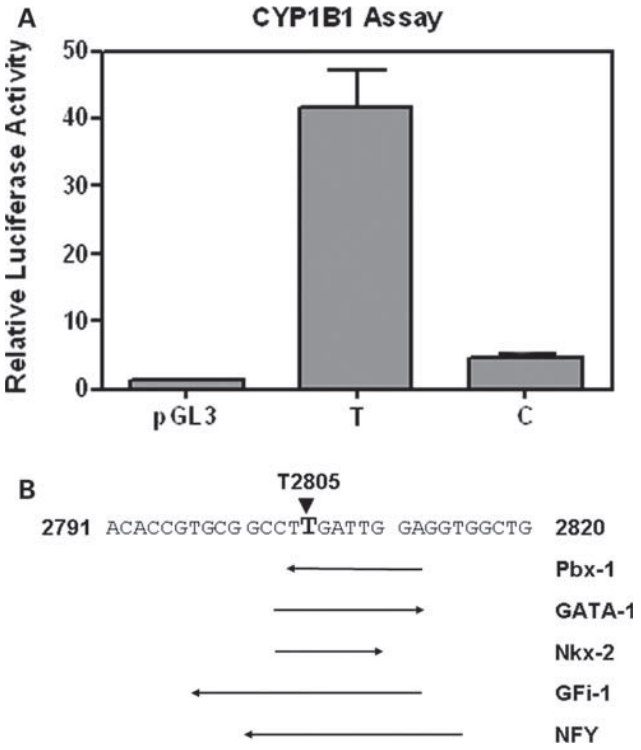

Figure 3. (**A**) Promoter assay of CYP1B1 rs2567206 SNP. The CYP1B1 promoter construct (g.2731–g.3064) with T2805 or C2805 was transfected in TM3 cells. Promoter activity was measured using Firefly luciferase as a reporter and was normalized using Renilla luciferase activity. The experiment was replicated three times, with duplicate assays performed within each replicate. The results of a single replicate, averaged over the duplicate assays, are presented. (**B**) Diagrammatic representation of putative sites for transcription factors in the region harboring rs2567206 SNP after the analysis of the CYP1B1 promoter using MatInspector and TFsearch softwares.

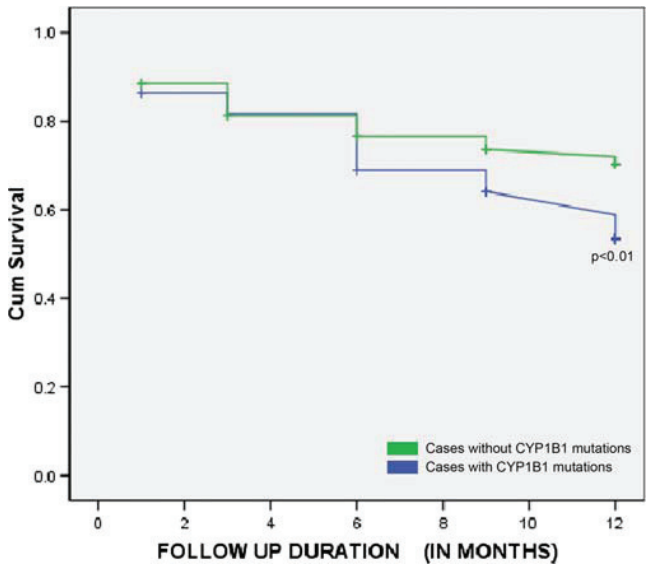

Figure 4. Results of IOP control in PCG patients with and without *CYP1B1* mutations based on 1 year post-surgery Kaplan–Meier survival analysis.

Table 5. Distribution of homozygous *CYP1B1* mutations on different haplotype backgrounds

CYP1B1 haplotype		Homozygous mutations observed on different haplotype backgrounds[a]	%Frequency of homozygous mutations
Promoter region	Coding region		
C-	-C-G-G-T-A	G61E	6.06
		A115P	1.51
		R368H	48.48
		P437L	1.51
		G466D	1.51
		3905-3927del	1.51
		8037-8046dup	1.51
C-	-C-G-C-C-A	L181P	1.51
		S239R	4.54
		C280X	1.51
		R355X	1.51
		R390C	9.09
		R390S	1.51
		P437L	1.51
		3928delG	1.51
C-	-C-G-C-C-G	M132R	3.03
		R390H	3.03
T-	-G-T-C-C-A	Y81N	1.51
		P437L	1.51
		3835insA	1.51
		7900-7901del	1.51
		8148-8152del	1.51
		8214-8215del	1.51

[a]Excluding 17 PCG patients who are compound heterozyqotes for *CYP1B1* mutations.

of trabecular meshwork. Whether any of these or other transcription factors/repressors bind and regulate the activity of the CYP1B1 promoter *in vivo* needs further investigation.

Although the underlying molecular mechanism leading to PCG pathogenesis is yet unclear, *in vitro* studies have hypothesized that CYP1B1 metabolizes an endogenous substrate in order to generate metabolites such as steroids (40), retinoic acid (41) and melatonin (42) that are crucial for development. It has also been suggested that CYP1B1 may completely eliminate a substrate that is required for development (13). Recently, it has been shown that the coding region mutations in *CYP1B1* act either by reducing the enzymatic activity or the abundance of the enzyme or by their combination (43).

In summary, this study indicated the involvement of the rs2567206 SNP with PCG, but not with POAG or PACG; the association was stronger in PCG patients who harbored a homozygous *CYP1B1* mutation. Similarly, the risk haplotype 'C-C-G-G-T-A' exhibited the strongest association among PCG cases harboring homozygous *CYP1B1* mutations. Our results indicate that the rs2567206 SNP can be used as an important diagnostic and predictive marker in PCG cases harboring *CYP1B1* mutations. Genotype–phenotype correlation indicated, in addition to the significant effect ($P = 0.022$) of coding mutations, an independent effect of genotype at the promoter (T2805C) locus ($P = 0.001$), and the interaction effect of genotypes at the promoter and coding region loci ($P = 0.001$) on the presenting IOP in the worse affected eye. Functional studies further confirmed the regulation of CYP1B1 levels *in vivo* due to the T2805C variation.

MATERIALS AND METHODS

Clinical assessment of the cases

The study protocol adhered to the tenets of the Declaration of Helsinki, and prior approval was obtained from the Institutional Review Board. We examined the promoter region of *CYP1B1* in a large cohort of clinically well-characterized subjects ($n = 835$) that included PCG patients ($n = 301$) and ethnically and geographically matched unaffected controls ($n = 319$). We also carried out focused screening of a specific variant in a cohort of primary glaucoma cases that included POAG ($n = 115$) and PACG ($n = 100$). The specific clinical diagnosis and enrollment criteria for the patients in each category and the normal controls are provided in refs (14,19,22). The commonality of all the glaucoma cases was the presence of an elevated IOP of >21 mmHg. Peripheral blood samples were collected from all the subjects by venipuncture with prior informed consent. DNA was extracted from blood using standard phenol–chloroform extraction methods as described earlier (14).

Screening of the *CYP1B1* promoter

A fully active and functional CYP1B1 promoter has been reported by Wo *et al.* (44). Sequence analysis of this region revealed four important regulatory elements, viz. an initiator element, a TATA-like box and two SP1 binding sites (Fig. 1). In deletion analysis of the promoter, a higher activity of the region containing the TATA-like box and the initiator element was observed, indicating that it is the basal promoter for *CYP1B1*. Further mutational analysis of these promoter elements indicated that all the four regions were required for a full promoter activity, and deletion of either or both the Sp1 motifs decreased the activity from 80 to 95% (44).

Based on the above data, a 481 bp upstream region (g.2753–g.3233) that contained all the four regulatory regions was amplified by PCR using a set of forward (5′-AGCGGCCGGGGCAGGTTGTACC-3′) and reverse (5′-ATTGGGATGGGGACGGAGAA-3′) primers under standard conditions in a thermal cycler (ABI 9700). PCR amplification was performed in 25 μl of a reaction mixture consisting of 50 ng of genomic DNA, 1× PCR buffer (100 mM of Tris–HCl, 500 mM KCl and 0.8% Nonidet P40), 2.5 mM $MgCl_2$, 2.5 μl of 2 mM solution of dNTPs, 10% DMSO, 12.5 pmol of each primer and 1 unit of *Taq* DNA polymerase. An initial denaturation at 94°C for 3 min was followed with 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s) and a final extension at 72°C for 7 min. The amplicons were purified using spin-columns (Ultra Clean PCR clean up, MO Bio Laboratories, Loker Ave West, CA, USA) and subjected to resequencing using Big Dye Terminator Chemistry (version 3.1) according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Both strands were read through an automated ABI 3100 DNA sequencer (Applied Biosystems), and the trace files were analyzed using the Sequencing Analysis Software (version 3.0).

Statistical analysis

Allele frequencies in the patients and controls were calculated by the gene counting method. Test of significance of the difference between allele frequencies was carried out using the chi-square statistic. A *P*-value of <0.05 was considered to be statistically significant. Estimates of Hardy–Weinberg equilibrium in the normal controls, reconstruction of haplotypes from genotypes and estimation of haplotype frequencies were done using Haploview [version 4.0 (45)]. Linkage disequilibria between pairs of loci were estimated using the LD Plot function of this software. Haplotypes were generated with the help of two SNPs in the promoter region (rs2567206 and g.2924C>T), a non-coding SNP (g.3078G>T) and five SNPs in the coding region [rs10012 (R48G), rs1056827 (A119S), rs1056836 (V432L), rs1056837 (D449D) and rs1800440 (N453S)].

Functional analysis of the promoter variant

In order to understand the functional implication of the associated promoter polymorphism (rs2567206), we carried out *CYP1B1* promoter assays in the TM3 cell line derived from the trabecular meshwork. The TM3 cells (46) were a gift from Alcon Biosystems, USA. All tissue culture media, supplements and fine chemicals were obtained from Sigma-Aldrich, USA. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics in humidified incubator at 37°C with 5% CO_2. The region of the *CYP1B1* promoter (g.2731–g.3064), which harbors the basal promoter (44) and an additional upstream promoter sequence, was amplified from genomic DNA of individuals with the homozygous 'TT' and 'CC' genotypes as templates with a set of forward (5′-ACAAGATCTGGCGCGCTCCCAAGTCGAGC-3′) and reverse (5′-CTAAAGCTTCTCCCACTCCCACTCCAGAG-3′) primers using an annealing temperature of 60°C and extension of 30 s in a ABI 9700 thermal cycler (Applied Biosystems). A *Bgl*II site was introduced in the forward primer and a *Hin*dIII site was incorporated in the reverse primer for cloning. The products obtained were cloned into the multiple cloning site of pGL3-Basic vector using *Bgl*II and *Hin*dIII sites and resequenced for confirmation. Five hundred nanograms of each promoter construct were co-transfected with 1 ng of pRL-TK plasmid expressing Renilla luciferase (Promega), in immortalized trabecular meshwork cell line (TM3) using Fugene HD (Roche) according to the manufacturer's protocol. After 24 h of transfection, cells were lysed in passive lysis buffer and the activities of Firefly and Renilla luciferase were measured using Turner Design Luminometer model TD-20/20 (Promega) according to the manufacturer's instructions.

Each transfection and luciferase-reporting assay was done in duplicate. Therefore, from each assay, we obtained duplicate measurements of the promoter activity for each of the T and C alleles. The experiment was replicated three times.

Genotype–phenotype correlation

We aimed to establish whether the phenotypic effects of the promoter variant were independent of the coding mutations in *CYP1B1*. In order to address this, we considered presenting IOP of the worse affected eye as a quantitative phenotype and assessed whether the promoter variant and the coding mutations in *CYP1B1* have independent and/or interacting

effects on IOP. We modeled IOP as the dependent variable, with genotypes at the promoter variant and coding mutations as independent effects and with promoter versus coding variants as an interaction effect, and performed an ANOVA. Additionally, Kaplan–Meier survival analysis was done to understand the prognosis with respect to IOP following a 1 year post-surgical follow-up between PCG cases with and without a *CYP1B1* mutation.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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