RESEARCH NOTE

Complex genetics of glaucoma: defects in *CYP1B1*, and not *MYOC*, cause pathogenesis in an early-onset POAG patient with double variants at both loci

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Introduction

Glaucoma is the second largest cause of blindness worldwide, affecting almost 60 million people (Quigley and Broman 2006). Defects in *MYOC* and *CYP1B1* have been implicated in primary open angle glaucoma (POAG) and primary congenital glaucoma (PCG), respectively. Variants in both the genes have been detected in both the diseases indicating a higher complexity in the pathogenesis of the disease. In this context, we present here the case of a POAG patient who is found to be a compound heterozygote for *CYP1B1* mutations and a homozygote for *MYOC* variant. None of the three non-synonymous changes were found in 170-unrelated controls with matched age and ethnicity. Analysis of the family members reveal that proband's younger sister, not affected, was homozygous for the *MYOC* variant but lacked one variant in *CYP1B1*. This is in contrast to other reports that showed implication of *CYP1B1* in POAG. This case is unique, since both parents of the proband do not show any symptom of POAG, despite of having one defective allele for both *MYOC* and *CYP1B1*.

Glaucoma is an anterior optic neuropathy with characteristic changes of the optic disc due to loss of retinal ganglion cells. The major risk factor of this disease is elevated intraocular pressure (IOP). A large fraction of the patients are sporadic, pointing to a multifactorial etiology. When familial, glaucoma can be autosomal dominant (POAG) or autosomal recessive (PCG). POAG (MIM (Mendelian inheritance in man), 137760) is the major subtype and to date three genes, namely, *MYOC* (Stone *et al*. 1997), *OPTN* (Rezaie *et al*. 2002) and *WDR36* (Monemi *et al*. 2005) have been reported to be primarily responsible for POAG. Among them *MYOC* (MIM, 601652) is the most frequently mutated gene in POAG, accounting for 3%–4% of all the cases.

On the other hand, defects in *CYP1B1*, have been reported to be the major cause of PCG, as summarized in a recent review (Vasiliou and Gonzalez 2008). Interestingly, it has been demonstrated as a modifier locus for POAG that together with *MYOC* mutation expedites the disease progression from adult onset to a juvenile form by a digenic mode of inheritance (Vincent *et al*. 2002). A large study conducted on French POAG patients reported that the *CYP1B1* mutations in 4.6% (11 out of 236) of the cohort, who were tested negative for *MYOC* mutations (Melki *et al*. 2004). Recently, our study also showed that *CYP1B1* alone could be responsible for juvenile-onset POAG (Acharya *et al*. 2006). This evidence points toward a functional interaction between *CYP1B1* and *MYOC*, and gets further support from the evidence that PCG could be caused by *MYOC* mutations (Kaur *et al*. 2005). All these studies point to the fact that although defects in *MYOC* or *CYP1B1* alone can cause POAG or PCG, respectively, both the genes should be screened routinely in POAG and PCG patients for a better understanding of the complex genetic interactions in glaucoma pathogenesis.

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Materials and methods

Selection of subjects and collection of blood samples

The patient for this study was already recruited in our ongoing study on POAG from the glaucoma department in Regional Institute of Ophthalmology, Kolkata, followed by the tenets of the Declaration of Helsinki and approved by the institutional Review Board. The diagnosis involved clinical ocular and systemic examinations. The ocular examinations involved measurement of intraocular pressure by applanation tonometry (Goldmann Ocular Instrument, Bellevue, USA). Gonioscopy by Goldmann 3-mirror gonioscope (Shaffer's grading Ocular Instrument, Bellevue, USA) revealed the angles of the anterior chamber, and was also used for optic disc evaluation and fundoscopy. Optic disc was further evaluated with +78-D lens and visual field was assessed by Humphrey automated perimeter Cart Zeirs, Dublin, USA. Controls without any history of ocular disease were selected from the general population and tested negative for POAG using the same tests that were used to recruit patients.

Collection of blood samples and genomic DNA preparation

Genomic DNA was extracted using routine methods (Sambrrok *et al*. 1989) from 10 ml peripheral blood sample (in EDTA) collected from the individuals included in the study with informed consent.

PCR and sequencing

PCR used to detect potential causal variants in the coding region was carried out for *MYOC, OPTN, WDR36* and *CYP1B1*. In addition, due to some interesting finding in a recent study from our group (Acharya *et al*. 2007), we have also screened the *OPTC* gene in this family. For screening all the five candidate genes, standard PCR reaction was carried out in a thermocycler (GeneAmp-9700, PE Applied Biosystems, Foster City, USA). Primers and PCR conditions for *MYOC* (Mukhopadhyay *et al*. 2002), *CYP1B1* (Acharya *et al*. 2006), *OPTN* (Mukhopadhyay *et al*. 2005) and *OPTC* (Acharya *et al*. 2007), were used as described before. Primers for *WDR36* are available on request.

The PCR products were column-purified using Qiagen PCR-purification kit (Qiagen, Hilden, Germany), and bidirectional sequencing was performed using the dye-termination chemistry in ABI 3130XL capillary DNA sequencer (Applied Biosystems, Foster City, USA) following manufacturers protocol. The data were analysed using Sequencing Analysis software version 5.2. The observed nucleotide sequences in the patients were compared to the *MYOC* (NC_000001.9) or *CYP1B1* (NC_000002.10) wild-type sequence for variations using pairwise BLAST.

Results and discussion

We detected a homozygous variant (c.1196 G>A; p.Gly399Asp) in *MYOC* from a 29-year-old man (figure 1,A) who was diagnosed to have POAG when he was 20-year-old. According to the clinical records, at his first visit to the clinic, he was found to have lost 70% of his vision. The details of the phenotypic findings are provided in table 1. We did not find this variant in 170 controls (340 chromosomes) from the same ethnic group. Moreover, Gly at this position was completely conserved among mammals (data not shown). Although we do not have any additional proof to establish p.Gly399Asp as a mutation, a heterozygous mutation involving the same codon, with a predicted milder substitution (p.Gly399Val) has been shown to cosegregate with POAG in a Guyanese family (Vincent *et al*. 2002). The same study also suggested hastening of the pathogenesis in combination with heterozygous p.Arg368His mutation in *CYP1B1*. However, effect of homozygous *MYOC* mutation (p.Gly399Val) on POAG remained uninvestigated as no individual in the family was reported to be homozygous for the change. In another study, Morissette *et al*. (1998) described a *MYOC*-mutation (p.Lys423Glu) in a French–Canadian family, which resulted in a dominant-negative effect when present in single dosage, but had no phenotypic effect when present in both copies of the gene (Morissette *et al*. 1998). This points to a molecular mechanism known as metabolic interference, where the mutant gene product gains a deleterious function due to the faulty interaction with its wild-type allele. Recently, in an Australian family, a homozygote for the well-known founder mutation in *MYOC* (p.Gln368Ter) has not been found to show any signs suggestive of glaucoma (Hewitt *et al*. 2006). We made a similar observation, as the younger sister of the proband (III-3 in figure 1), homozygous for the identified *MYOC* variant (p. Gly399Asp), had normal vision, despite attaining the age of diagnosis of the disease when compared with the proband (figure 1; table 1). However, in this study, unlike the previous reports, both parents (age 53 and 45 years) of the proband are heterozygous for the mutation in *MYOC* (p.Gly399Asp) and neither of them manifest any symptom of POAG on clinical examination. It is possible that this *MYOC* variant allele has reduced penetration thereby not showing any phenotype by itself. Also, we cannot rule out the possibility of the parents getting affected at a later stage.

To understand the molecular basis of POAG in this family, we screened the other candidate genes (*CYP1B1, OPTN, OPTC* and *WDR36*) for causal variants. No such variant was detected in *OPTN, OPTC* or *WDR36*, while two heterozygous variants (c.1057 G>A and c.1060 G>A) were observed in *CYP1B1*. Both these variants resulted in exactly the same alteration of two consecutive amino acid residues (p.Glu229Lys and p.Glu230Lys). The patient (III-1 in figure 1) was heterozygous for both the changes in *CYP1B1*,

Table 1. Comparison of the clinical features of the proband (III-1) and his unaffected sister (III-3).

Clinical features	Proband (III-1 in figure 1)	Unaffected sister (III-3 in figure 1)
Present age (age-of-onset)	29 years (20 years)	23 years
Visual acuity	6/60 (RE) and 6/12 (LE)	6/6 (RE) and 6/6 (LE)
Intraocular pressure (mm of Hg)	30 (RE), 27 (LE) mm of Hg	12 (RE), 14 (LE) mm of Hg
Gonioscopy	Open angle in all quadrants	Open angle in all quadrants
Cup-to-disc ratio	0.9 (RE) and 0.8 (LE)	0.3 (RE) and 0.2 (LE)
Visual field analysis	Superior and inferior arcuate scotoma in both eye	No visual field change

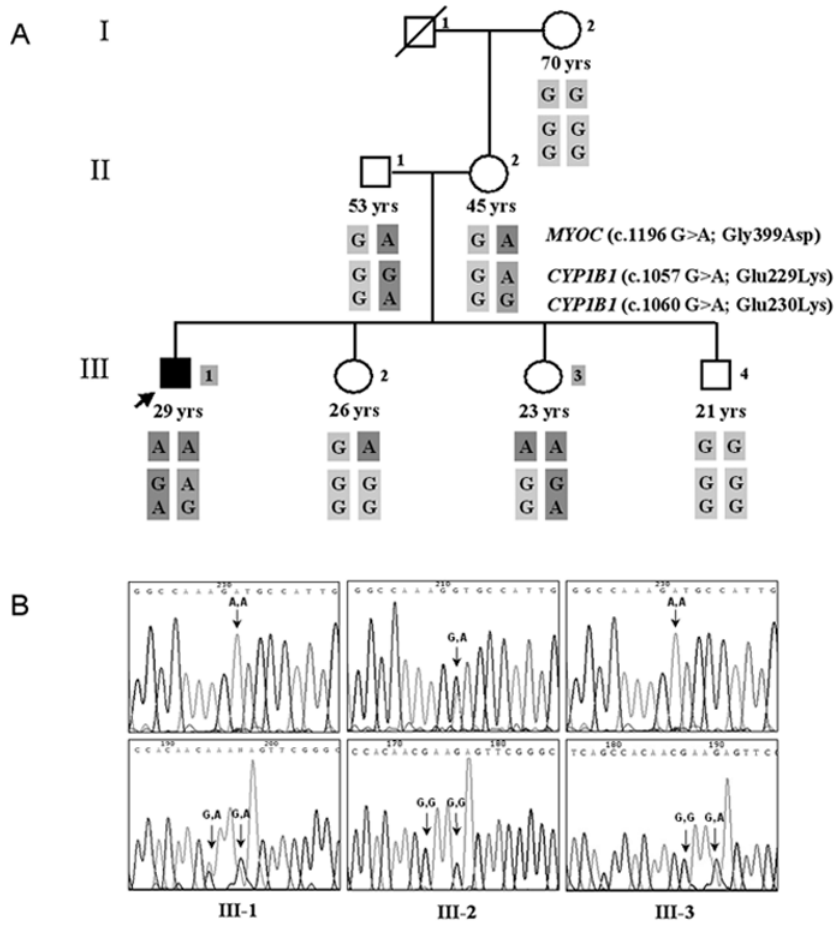

Figure 1. An eastern Indian early-onset POAG patient showing double variants at both *MYOC* and *CYP1B1*. (A) The proband (III-1) is identified by an arrow. Squares and circles represent males and females, respectively, and I-1 represents a deceased individual. Solid and open symbols represent affected and normal individuals, respectively. In the pedigree, age of all the participating individuals and genotypes determined by direct DNA sequencing for *MYOC* and *CYP1B1* has been shown. The proband is the only affected individual in the family having a unique combination of variant alleles of *MYOC* (c.1196 G>A; p.Gly399Asp) and *CYP1B1* (c.1057 G>A; p.Glu229Lys & c.1060 G>A; p.Glu230Lys) which is not present in any other family members. (B) Representative chromatograms showing the *MYOC* variant (c.1196 G>A and p.Gly399Asp) in the upper panel and two heterozygous mutations in *CYP1B1* (c.1057 G>A; p.Glu229Lys and c.1060 G>A; p.Glu230Lys). From left-to-right, the chromatograms are from individuals III-1, III-2 and III-3, respectively.

while his sister (III-3 in figure 1) was heterozygous only for p.Glu230Lys suggesting that the two changes were present in two different alleles. The p.Glu229Lys (c.1057G>A) in *CYP1B1* was reported previously from different studies as a causal mutation for PCG in compound heterozygous condition (Michels-Rautenstrauss *et al*. 2001; Panicker *et al*. 2002). This mutation was also reported in heterozygous condition in French PCG patients (Colomb *et al*. 2003), raising the possibility of a dominantly inherited mutation causing PCG, generally a recessive disease. However, the possibility of a second mutation in the other allele cannot be excluded. Recently, the p.Glu229Lys mutation has been reported in heterozygous condition causing POAG (Lopez-Garrido *et al*. 2006). Another example where the mother of a PCG patient was found to be compound heterozygous for p.Glu229Lys change and a deletion mutation (c.1064-1076del) in *CYP1B1*, but did not show any symptom of PCG (Chavarria-Soley *et al*. 2006). Since the age of that individual was not mentioned in the report, we could not completely rule out the possibility of her being affected POAG at later stage of life.

The other *CYP1B1* allele, c.1060 G>A (p.Glu230Lys) is a novel variant found in this study. The absence of this change in 170-unrelated controls and similar nature, and adjacent position of the change to p.Glu229Lys strongly argues in favour of its pathogenic role.

Screening of the *CYP1B1* in other family members revealed that the proband is the only individual in the family who inherited the unique combination of both maternal (p.Glu229Lys) and paternal (p.Glu230Lys) defective alleles (III-1 in figure 1) in *CYP1B1*. Proband's sister (III-3 in figure 1), who was homozygous for the MYOC variant (p.Gly399Asp), did not inherit the maternal (p.Glu229Lys) CYP1B1 mutant allele, but inherited p.Glu230Lys from the father.

We also conducted haplotype analysis using five SNPs (rs10012, p.Arg48Gly; rs1056827, p.Ala119Ser; rs1056836, p.Leu432Val; rs1056837, p.Asp449Asp and rs1800440, p.Asn453Ser) in the coding region of *CYP1B1*. Previously, we reported that rs10012 and rs1056827, are in complete linkage disequilibrium (Acharya *et al*. 2006), but in this case we found one exception. The unaffected sister of the proband (III-3 in figure 1) carried the most common haplotype among controls (C-G-C-C-A), while the proband was heterozygous for only rs1056827, thus carrying both C-G-C-C-A and C-T-C-C-A haplotypes. In a recent study analysing the global scenario of haplotype backgrounds of *CYP1B1*, the C-G-C-C-A was found to be the second most common haplotype among controls and patients without *CYP1B1* mutation, however, they did not report the C-T-C-C-A haplotype (Chakrabarti *et al*. 2006). Hence, the variant found in our study under the C-T-C-C-A haplotype background is probably a rare and private mutation found in the proband. It would be interesting to conduct more extensive studies in diverse Indian populations to identify the major haplotypes present in different ethnic groups that might help in identifying populations at-risk of not only glaucoma, but a lot of other common complex diseases in general. A recent nation-wide study has already provided the required framework for such analyses (Indian genome variation consortium 2008).

The *CYP1B1* represents the first example where mutations in a member of the cytochrome P450 super family result in a primary developmental defect in terms of PCG (Stoilov *et al*. 1997). It has been speculated earlier that CYP1B1 participates in the metabolism of an as-yet-unknown biologically active molecule that is a participant in eye development (Stoilov *et al*. 1997). Vincent *et al*. (2002) suggested MYOC and CYP1B1 might interact through a common pathway and that glaucoma might be multi-allelic in some cases and proposed that MYOC function might be influenced by changes in CYP1B1 (Vincent *et al*. 2002).

This study reports a unique observation where POAG is not caused by a seemingly pathologic variant in *MYOC*, irrespective of homozygosity or heterozygosity, but manifested as a phenotype only when present in combination of two defective alleles in *CYP1B1*. One can argue that the *MYOC* variant is not a mutation but a rare variant, but reported findings, involving the same codon (Vincent *et al*. 2002), makes that unlikely. There are two other possibilities to consider: (i) p.Glu229Lys in *CYP1B1* is the pathologic allele; this is unlikely because the phenotypically normal mother of the proband is also heterozygous for the change, or (ii) p.Glu229Lys and p.Glu230Lys in *CYP1B1* together cause the phenotype. We favour the second option, as this is only found in the proband. We suggest, based on the early-onset POAG case presented in this article, that both *MYOC* and *CYP1B1* should be routinely screened for variants in POAG and PCG patients.

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