Abbreviations

ASD - Anterior segment dysgenesis

CYP1B1 - cytochrome P450, family 1, subfamily B, polypeptide 1

FOXC1 – forkhead box protein C1

IOP – intra-ocular pressure

JOAG – juvenile open angle glaucoma

LTPB2 – latent transforming growth factor beta binding protein 2

MYOC - myocilin

PACG - primary angle closure glaucoma

PCG – primary congenital glaucoma

POAG - primary open-angle glaucoma

Abstract

Introduction: Primary congenital glaucoma (PCG) is a rare autosomal recessive trabeculodysgenesis. Genetic heterogeneity has been identified but mutations in the *CYP1B1* gene seem to be an important etiology in different populations.

Objectives: To clinically characterize 34 Portuguese patients with primary congenital glaucoma, to analyze the role of *CYP1B1* mutations in this cohort and to determine whether there is a correlation between mutations and disease severity in congenital glaucoma patients.

Methods: This study included 34 patients from 31 unrelated families with primary congenital glaucoma and 100 unrelated, healthy controls in Portugal. *CYP1B1* was amplified from genomic DNA, followed by direct DNA sequencing to identify disease-causing variants.

Results: Fourteen different mutations in *CYP1B1* were identified in 26 patients (76.47%). Twelve of these patients had homozygous mutant alleles and 12 had compound heterozygous mutations. We also found one homozygous with a compound allele and one heterozygous carrying only one mutated allele. No mutations were identified in eight (23,52%) probands. Thirteen mutations have been previously described as disease-causing in different populations. To the best of our knowledge,

p.L378Q was novel. The disease phenotypes of patients with *CYP1B1* mutations may be more severe compared with that of patients negative for CYP1B1 mutations.

Conclusions: Mutations in the *CYP1B1* gene are a major cause for PCG in our patients. This study describes the mutational spectrum of *CYP1B1* in a Portuguese cohort of PCG patients. Further studies are necessary to describe the genotype-phenotype correlations in patients with PCG.

Resumo

Introdução: Glaucoma congénito primário é uma trabeculodisgenesia rara de transmissão autossómica recessiva. Trata-se de uma doença geneticamente heterogénea mas mutações no gene *CYP1B1* parece desempenhar um papel importante em diferentes populações.

Objectivos: Caracterizar clinicamente 34 doentes portugueses com glaucoma congénito, analisar o papel de mutações do gene *CYP1B1* nesta população e determinar se existe correlação entre as mutações e a severidade da doença no glaucoma congénito.

Métodos: Foram incluídos neste estudo 34 doentes de 31 famílias independentes com glaucoma congénito e 100 controlos saudáveis. O gene *CYP1B1* foi amplificado a partir de ADN genómico, seguido por sequenciação directa para identificar variantes causadoras de doença.

Resultados: Foram identificadas 14 mutações em *CYP1B1* em 26 doentes (76.47%). Doze destes doentes são homozigotas e 12 são heterozigotas compostas. Foi igualmente identificado 1 doente homozigoto com um alelo composto e 1 caso de heterozigotia. Nenhuma mutação foi encontrada em oito (23,52%) doentes. Treze das mutações estão previamente descritas noutras populações. A mutação p.L378Q é nova. O fenótipo dos doentes com mutações em *CYP1B1* parecem ser mais severos quando comparados com o dos doentes sem mutações detectáveis.

Conclusões: Mutações em *CYP1B1* são uma causa major de glaucoma congénito na população portuguesa. Este estudo descreve o espectro de mutações *CYP1B1* na população portuguesa. São necessários mais estudos para descrever correlações genótipo-fenótipo em doentes com glaucoma congénito primário.

Keywords: *CYP1B1* gene, PCG, population mutational screening.

Introduction

Glaucomas are a heterogeneous group of optic neuropathies, whose common feature is progressive loss of vision, due to degeneration of the retinal ganglion cells and optic nerve damage, frequently associated with elevated intraocular pressure (IOP) (Vasiliou & Gonzalez, 2008) (Sarfarazi, 1997). They are among the most common causes of irreversible blindness worldwide, accounting for 15% of cases (Quigley, 1996). Glaucomas can be categorized based on etiology (primary versus secondary), anatomy of the anterior chamber (open-angle versus closed-angle), and time of onset (infantile, juvenile or adult-onset). The 3 major categories are: primary open-angle glaucoma (POAG), primary congenital glaucoma (PCG), and primary angle closure glaucoma (PACG). Anterior segment dysgenesis (ASD), which includes Peters anomaly, Riegers anomaly, aniridia, iris hypoplasia, and iridogoniodysgenesis have been associated with elevated IOP and glaucoma. (Vasiliou & Gonzalez, 2008). POAG is the most common form of glaucoma, affecting more than 35 million people worldwide (Quigley & Broman, 2006). Based on the age of onset, POAG can be divided into juvenile-onset POAG (JOAG, appearing between early childhood and the age of 40 years) and adult-onset POAG. (Vasiliou & Gonzalez, 2008)

PCG is a rare and severe disease seen in the neonatal or early infantile period, generally before age three. This term is reserved for cases with anatomical defects due to isolated trabeculodysgenesis which encompasses failure in the proper development of the trabecular meshwork, a structure that serves as a filter for the aqueous humor (Sarfarazi *et al.*, 2003) (Choudhary *et al.*, 2009). This leads to the obstruction of

aqueous outflow from the anterior chamber resulting in increased IOP and subsequent optic nerve damage (Ho & Walton, 2004). Details of the pathogenic pathways affecting glaucoma are not yet completely understood.

PCG is the most common form of glaucoma in childhood, with more than 80% of cases diagnosed in the first year of life. It is usually a bilateral condition (80% of cases) and males are more affected than females. (Vasiliou & Gonzalez, 2008) The incidence of PCG varies substantially among populations of different ethnicities. It occurs in 1:10.000-20.000 live-births in western countries, but is notably higher in communities with high levels of consanguinity, such as Slovakian Gypsies (1 in 1250) and Saudi Arabians (1 in 2500) (Challa, 2008).

Clinically, children typically present with epiphora (excessive tearing), photophobia (hypersensivity to light), corneal opacity and edema, blepharospasm, enlargement of the globe (known as buphthalmos) and Haab's striae (breaks in the Descemet's membrane) (Challa, 2008) (Vasiliou & Gonzalez, 2008) (Sarfarazi *et al.*, 2003).

PCG is most commonly a recessive inherited disorder. Linkage studies have shown a high degree of genetic heterogeneity and three loci on chromosomes 2p21 (GLC3A) (Stoilov *et al.*, 1997), 1p36 (GLC3B) (Akarsu *et al.*, 1996) and 14q24.3 (GLC3C) (Stoilov & Sarfarazi, 2002) have been mapped. Until 2008, the only gene that had been identified was the cytochrome P4501B1 gene (*CYP1B1*), linked to GLC3A locus.

Recent studies have implicated the myocilin (*MYOC*) gene in PCG. *MYOC* is located on chromosome 1q25 at the GLC1A locus and codes for myocilin/trabecular meshwork-induced glucocorticoid response TIGR protein. (Tanwar *et al.*, 2010). *CYP1B1* has been suggested to be a modifier of *MYOC* expression. (Vincent *et al.*, 2002)

The Forkhead box protein C1 (*FOXC1)* gene, located on chromosome 6p25, was also selected for mutation analysis in PCG cases, but a limited role in the PCG pathogenesis was demonstrated. (Chakrabarti *et al.*, 2009)

Mutations in the *CYP1B1* gene are the main known cause of disease. The proportion PCG cases whose disease is due to *CYP1B1* vary widely across different populations and ranges from 20% to 100%. (Sarfarazi *et al.*, 2003) Incomplete penetrance has been observed in several families (Bejjani *et al.*, 2000).

The *CYP1B1* gene belongs to the family 1 of the *CYP450* superfamily, which contains 58 putatively functional genes in the human genome. (Vasiliou & Gonzalez, 2008). The members of the *CYP450* superfamily have a major role in clearing the body from a wide range of endogenous and exogenous (xenobiotics) chemicals and organic lipophilic drugs (Choudhary *et al.*,2009). The *CYP1B1* gene contains three exons (two of which are coding) and two introns. The three exons have 371, 1,044 and 3,707 base pairs, and the two introns have 390 and 3,032 base pairs. The coding region starts at the 5' end of the second exon and ends within the third exon (Tanwar *et al.*, 2009).

It codes for a 543 amino-acid polypeptide that contains three regions: the 53 residue membrane-bound N-terminal, a 10 residue proline-rich region called hinge, and a cytosolic globular domain with a length of 480 residues. (Vasiliou & Gonzalez, 2008)

This protein functions as a monooxygenase enzyme, catalyzing oxidative, peroxidative and reductive reactions, with roles in the metabolism of various exogenous and endogenous substrates (including xenobiotics, vitamins, melatonin and steroids) (Choudhary *et al.*, 2009). The role of CYP1B1 in congenital glaucoma is not well understood. It is thought that this gene is probably responsible for the metabolism of compounds that are critical in the trabecular meshwork development. In fact, expression of *CYP1B1* is found in higher levels in fetal eye tissues (Vasiliou & Gonzalez, 2008).

The protein is expressed in the trabecular meshwork, iris, cliliary body, and retina, in addition to 15 different non-ocular tissues (Stoilov *et al.*, 1997) (Stoilov *et al.*, 1998).

Recently, a second gene implicated in PCG has been identified, the *LTBP2* gene on chromosome 14q24.3. Is the largest member of the latent transforming growth factor (TGF)-beta blinding protein family. Defects in *LTBP2* may increase the elasticity of the ciliary body structures, causing changes in the support of the surrounding tissues. (Ali *et al.*, 2009)

Probably, all known loci/genes for glaucoma account for a minority of total cases of glaucoma, with many others genes remaining to be identified. (Tanwar *et al.*, 2010) *CYP1B1* may have a more significant role than initially expected in glaucoma pathogenesis. *CYP1B1* has been found to be a causative gene in PCG, a modifier gene in POAG, and, rarely, a causative gene in POAG and several ASD disorders (Vasiliou & Gonzalez, 2008).

The visual outcome of a child with PCG is closely related to the age at which the diagnosis is made. Thus, early and reliable diagnosis and adequate treatment is vital to improve the child's visual prognosis. (Tanwar *et al.*, 2009)

In the present study, we screened all coding exons of CYP1B1 gene in 34 unrelated PCG patients, to determine whether there is a correlation between *CYP1B1* mutations and disease severity in PCG.

Population and Methods

This study adhered to the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of the University Hospital of Coimbra. A total of 34 individuals with PCG were included in this study, all patients have been clinically assessed at the Centre for Hereditary Eye Diseases of the Department of Ophthalmology, University Hospital of Coimbra. Nineteen patients were female and 15 patients were male. They belonged to 31 unrelated families residing in different regions of Portugal. Control DNAs (n=100) were obtained from randomly selected healthy adults.

All individuals included in the study were informed about its objectives and volunteered to participate. Informed consent was obtained from all subjects.

The diagnosis involved clinical, ocular and systemic evaluation. Ophthalmological assessment included best corrected visual acuity (BCVA), biomicroscopy, gonioscopy, and IOP measurement, and optic nerve examination (whenever possible). Inclusion criteria are: increased corneal diameter (>12.0mm), raised IOP (>21mHg) with or without Haab's striae, and optic disc changes (whenever examination was possible). Individuals presenting with other ocular or systemic anomalies were excluded.

CYP1B1 mutation screening and sequence analysis: Genomic DNA was isolated from peripheral blood lymphocytes using an automated DNA extractor (BioRobot EZ1, Qiagen, Hilden, Germany). The entire coding region, including exon-intron boundaries, was amplified from DNA using polymerase chain reaction (PCR), using previously described primers and conditions (Stoilov *et al.*, 1998). To detect sequence changes, all

CYP1B1 amplicons were purified with QIA-quick Gel Extraction Kit (Qiagen). Sequencing reactions were performed using the 4-dye terminator cycle sequencing ready reaction kit (BigDye DNA Sequencing Kit, Applied Biosystems, Foster City, CA). Sequence products were resolved in an ABI Prism 3130 (Applied Biosystems).

Mutation nomenclature: Mutations and polymorphisms were named based on genomic DNA sequences U56438 and NT_004487 of *CYP1B1*.

Results

Clinical and molecular findings

Thirty four probands with PCG belonging to 31 independent families were enrolled in the study. Fifteen patients (44,12%) were male, and 19 patients (55,88%) were female. In two families, more than one individual were affected (6,45%). All patients included in the present study had an aggressive form of PCG and had undergone multiple surgeries for IOP control.

All cases had typical symptoms and signs of PCG such as megalocornea, tearing and photophobia. Most cases had corneal opacity and ruptures in Descemet's Membrane. Severe phenotypes were observed in at least one eye of the patients. No significant differences in the clinical findings were found between the patients with or without *CYP1B1* mutation. The clinical findings are summarized in **Table I**.

We identified 14 different *CYP1B1* mutations (in a total of 39 mutations found) in 26 (76.47%) of the 34 index cases: three point mutations (1 G>A, 1 C>T and 1 T>A), six deletions (c.881delG, c.1410del13bp, c.1063del1076, c.1064_1076del, c.1691delG and c.880delG), three duplications (c.1556dup10bp, c.1200_1209dupTCATGCCACC and c.1776dup27bp) and one in-frame insertion (c.1198_1207dup). The point mutations predicted three missense (p.E387K, p.P437L, and p.L378Q) amino acid substitutions. Deletions and duplications result in nine frameshift mutations. We identified one nonsense mutation leading to a premature stop (R444X). All mutations are summarized in **Table II** and **Table III** and a schematic representation of the location of these

mutations is provided in **Fig.1**. **Fig.2** shows a representative pedigree in this study (family 4).

Thirteen of these mutations have been previously described as disease-causing in different populations. To the best of our knowledge, p.L378Q was novel. This mutation was present on a single case (family 9), associated with a milder phenotype. This change was not present in either the tested unaffected family members or in the 100 chromosomes from healthy Portuguese controls. This novel T>A transition at nucleotide 1133 – L378Q - replaces an amino acid residue for another of different nature – leucine (nonpolar, neutral, hydropathy index (HI) of 3.8) for glutamine (polar, neutral and HI of -3.5)

The detected mutations were present in the probands as follow: 12 (35,29%) were homozygous (3 for c.1776dup27bp, three for p.E387L, two for c.881delG, two for c.1410del13bp, one for c.1556dup10bp and one for c.1064_1076del13). Twelve (35,29%) were compound heterozygous. One (2,94%) was homozygous with a compound allele (homozygous c.880delG with heterozygous c.1691delG). One (2,94%) was heterozygous carrying only one mutated allele (c.1063_1076del). No mutation was found in eight (23,52%) probands.

In this study, c.881delG was found to be the most frequent change, followed by p.E387K and c.1410del13bp. This three mutations together were present in 57,69% of the studied alleles.

Table I: Clinical details in study patients with PCG

Family number	Current age	Gender	Clinic	Visual Acuity OD OS	C/D ratio	Visual Fields
1	32y	F	Megalocornea OU, iridectomies, shallow AC, ant polar cataract OS; nystagmus	0,2 0,4	NE NE	General reduced sensitivity OU
2	18y	M	OD megalocornea, iridectomies, corectopia, deep AC, nystagmus; OS phthisis bulbi	<0,1 NLP	0,9 -	Severely constricted
3	3y	F	Megalocornea (OU), nystagmus	0.2 (binocular testing)	0.4 0.5	NE
4	25y	F	Megalocornea, flat blebs	2/10 8/10	NE NE	constr/scot
	21y	F	Megalocornea, central opacification, Haab	<0.1 <0.1	1.0 1.0	NE
	10y	M	Megalocornea OU, Haab striae	<0.1 <0.1	0.9 0.9	Constricted
5	9y	M	Megalocornea OU, Haab striae, iridectomies OU, manifest latent nystagmus	0,6 0,5	0,6 0,6	Reduced sensitivity OU
6	8y	M	Megalocornea (OU); corectopia; iridectomies Haab striae; nystagmus	<1/10 <1/10	1.0/1.0	Constricted
7	20y	M	OD phthisis bulbi; OS megalocornea,	NLP 0,6	- 0,6	Constricted , reduced sensitivity
8	34y	F	OD megalocornea, deep AC, PC IOL, chronic conjunctivitis, valve, manifest nystagmus; OS prosthesis	NLP NLP	NE NE	Zero
9	14y	F	Megalocornea, Haab striae	5/10 <1/10	0.9/0.9	Constricted
10	33y	F	Megalocornea, Haab striae,	3/10 4/10	0,7 0,7	Constricted
11	24y	M	Megalocornea, Haab striae, iridectomies, flat conjunctival blebs	5/10 6/10	0,9 0,9	Constricted
12	30y	F	Megalocornea, complete opacification	NLP NLP	NE NE	0
13	32y	F	Megalocornea, anterior staphyloma, complete opacificication	NLP NLP	NE NE	0
14	33y	M	Complete opacification, anterior staphyloma, megalocornea	NLP NLP	NE NE	0
15	31y	F	Complete opacification, megalocornea,	NLP NLP	NE NE	0
16	4y	M	Megalocornea unilateral	<1/10 8/10	1.0/0.3	NE
17	10y	M	Megalocornea (OU), Haab striae	4/10 1/10	0.8/0.9	Constricted
18	45y	F	Megalocornea OU, Haab striae	3/10 4/10	0.8 0.8	Constricted

19	34 y	F	OD prosthesis; OS megalocornea, miosis, nystagmus	NLP LP	- 0,9	Severely constricted
20	39 y	F	OD prosthesis; OS megalocornea, opacified cornea, Haab striae	NLP 0,1	- 0,9	Severely constricted, reduced sensitivity
21	26 y	M	OD Phthisis bulbi; OS megalocornea, iridectomies, nystagmus	NLP 3/10	NE 0,6	Severely constrict
22	6y	F	Megalocornea, valves OU, trabeculectomies OU, retinal detachment OS	NLP LP	0,9 0,9	NE
23	4 y	M	Megalocornea, Haab striae, engorged conjunctival vessels, nystagmus	<1/10 <1/10	0,7 0,9	NE
24	9y	M	OD megalocornea, iridectomies, deep AC, PC IOL, flat bleb; OS no changes in anterior segment	3/10 10/10	0,7 0,3	NE; no changes LE VF
25	48y	F	OD prosthesis; OS megalocornea, iridectomies, nuclear, PSC cataract	0 <0,1	- 0,9	Severely constricted
	40y	F	Megalocornea OU, centrally opacified corneas, deep AC, iridectomies	<0,1 <0,1	1,0 1,0	Severely constricted
26	30y	F	Megalocornea OU, corneal opacification, deep AC, iridectomies, flat superior blebs	0,1 <0,1	0,9 0,9	Very constricted
27	1y	M	Megalocornea OU, Haab striae	CSM CSM	0.3 0.4	NE
28	1y	F	Megalocornea OU, Haab striae	CSM CSM	0.5 0.5	NE
29	1y	M	Megalocornea OU, Haab striae	CSM CSM	0.4 0.4	NE
30	35y	F	RE prosthesis LE complete opacification, megalocornea, anterior staphyloma	0 LP	NE NE	Navigational vision (NE)
31	37y	M	RE central graft transparent, deep AC, conjunctival changes LE prosthesis	0.03 0	NE NE	NE

OD – right eye; OS – left eye; OU – both eyes; F – female; M – male; y – year ; CSM – central, steady, maintained; NE: not evaluable; AC anterior chamber; PSC: posterior sub-capsular; PC IOL: posterior chamber intraocular lens; LP: light perception; NLP: no light perception

Table II: *CYP1B1* mutations detected in 31 families with PCG

Family Number	Number of affected individuals	Mutations	
		Allele 1	**Allele 2**
1	1	Glu387Lys	Glu387Lys
2	1	c881delG	c881delG
3	1	Glu387Lys	Pro437Leu
4	3	c1776dup27bp	c1776dup27bp
5	1	Glu387Lys	Glu387Lys
6	1	c881delG	Glu387Lys
7	1	Arg444STOP	c881delG
8	1	c881delG	c1410del13bp
9	1	c881delG	Leu378Gln (Novel)
10	1	c881delG	c1556dup10bp
11	1	c881delG	c1410del13
12	1	Glu387Lys	c1198_1207dup
13	1	c1063del1076	-
14	1	c1064_1076del13	c1064_1076del13
15	1	c1064 1076del13	c1200_1209dupTCATGCCACC
16	1	-	-
17	1	-	-
18	1	-	-
19	1	c881delG	c881delG
20	1	-	-
21	1	Glu387Lys	c1556dup10bp
22	1	Glu387Lys	Glu387Lys
23	1	c1410del13bp	c1410del13bp
24	1	-	-
25	2	c881delG	c1410del13bp
26	1	c1691delG	c880delG
27	1	-	-
28	1	-	-
29	1	-	-
30	1	c1410del13bp	c1410del13bp
31	1	c1556dup10bp	c1556dup10bp

Table III: Details of CYP1B1 mutations found in this study

Nucleotid Change	**Aminoacid change**	**Allelic frequence**	**Type of mutation**	**Exon**
c.1505G>A	*E387K*	*19,23%*	Missense	III
c.881delG	Fs	23,08%	Frameshift	II
c.1656C>T	p.P437L	1,92%	Missense	III
	p.L378Q	1,92%	Missense	III
	p.R444X	1,92%	Nonsense	III
c.1410del13bp	Fs	15,38%	Frameshift	III
c.1556dup10bp	Fs	7,69%	Frameshift	III
c.1198_1207dup	Fs	1,92%	Frameshift	III
c.1063del1076	p.R355fs	1,92%	Frameshift	III
c.1064_1076del	p.Arg355HisfsX69	5,77%	Frameshift	III
c.1200_1209dupTCATGCCACC	p. Thr404SerfsX30	1,92%	Frameshift	III
c.1691delG	Fs	1,92%	Frameshift	III
c.880delG	Fs	3,85%	Frameshift	II
c.1776dup27bp	In-frame insertion	11,54%	In-frame insertion	III

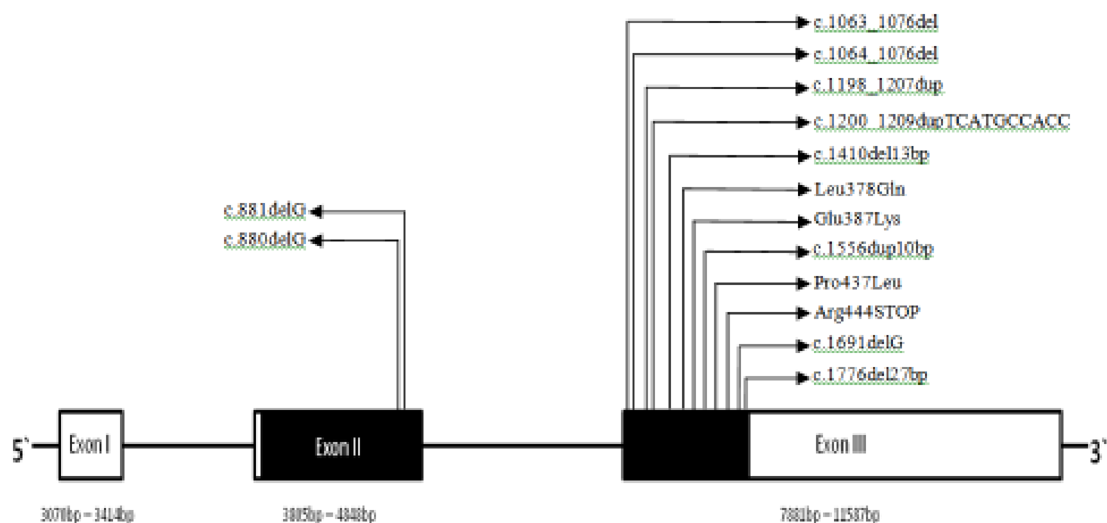

Figure 1: Schematic representation of the *CYP1B1* mutations in the coding region. *Arrows:* locations of the mutations within the *CYP1B1* gene. *Shaded regions:* coding exons 2 and 3 of the *CYP1B1* gene.

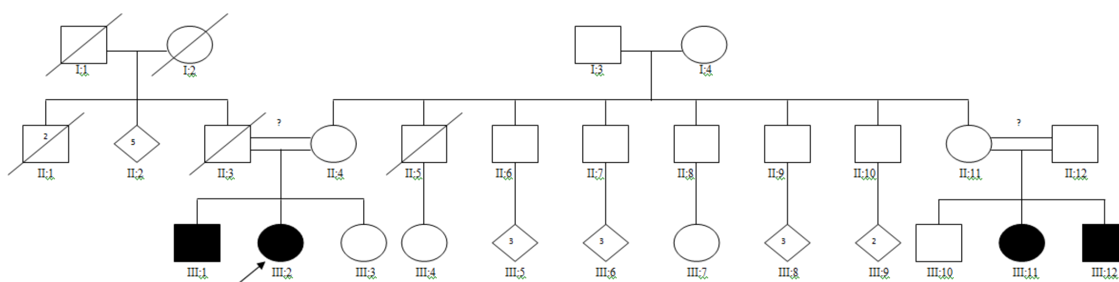

Figure 2: Pedigree of family 4. Affected individuals are homozygous for the *CYP1B1* (c1776dup27bp) mutation. Blackened symbols denote affected status for PCG. Unblackened symbols denote unaffected status. Arrow indicates the proband.

Discussion

Mutations in the *CYP1B1* gene have been associated with PCG with varying frequencies across different ethnic communities and geographic boundaries. *CYP1B1* mutations are structured by geographic backgrounds. (Chakrabarti *et al.*, 2006)

In present study, 26 of 34 patients with PCG (76.47%) had *CYP1B1* mutations, underscoring the clinical importance of this gene. This percentage is lower than the 90% to 100% reported in Arab patients with PCG (Bejjani, *et al.*, 2000) and in Romanian gypsy patients with PCG (Plásilova *et al.*, 1999). However, our percentage is higher than the 50% reported in Brazilian patients with PCG (Stoilov, 2002), 30% reported in India (Reddy *et al.*, 2004) and Morocco (Belmouden *et al.*, 2002), 20% to 30% of cases in Japan and in ethnically mixed populations (Kakiuchi-Matsumoto *et al.*, 2001) (Mashima *et al.*, 2001) and 12% of cases in native americans from Ecuador (Curry *et al.*, 2004). Estimated frequency of *CYP1B1* mutations is around 10%-15% in simplex cases. (Mashima *et al.*, 2001) (Stoilov, 2002) Direct comparison between studies may be difficult due to differences in sample size, sample composition (familial/sporadic, unilateral/bilateral) and the screening method used.

PCG is generally inherited in an autosomal recessive pattern. Mutations are generally homozygous or compound heterozygous in affected individuals and with phenotypically normal heterozygotes, whereas in this study, 1 patient (family 13) was heterozygous of *CYP1B1* (only one mutant allele was detected). Heterozygous mutations of *CYP1B1* in patients with PCG have been previously reported. (Reddy *et al.*, 2004) (Alfadhli *et al.*,

2006) It was reasonable to assume that these individuals may have mutations affecting the promoter region of the gene, (Mashima *et al.*, 2001) or affecting genes linked to other loci like, GLC3B and GLC3C. Mutations in other genes such as *MYOC* and *FOXC1*, resulting in digenic inheritance, remain a previously described possibility.

In this study, 8 (23,52%) probands didn´t carry any *CYP1B1* mutations. These results suggest the possibility of other candidate genes, or loci, not yet identified, that may cause PCG as it has been suggested by previous studies. (Ali *et al.*, 2009) (Chakrabarti *et al.*, 2009) (Narooie-Nejad *et al.*, 2009)

A wide range of mutations in the coding regions of *CYP1B1* were detected in these patients. The different mutation spectrum in our study patients may be attributed to genetic heterogeneity.

We identified 14 different mutations: three point mutations (2 transitions and 1 transversion predicting three missense amino acid substitutions), six deletions and three duplications (predicting nine frameshift mutations), one in-frame insertion and one non-sense amino acid substitution. It was previously described that missense mutations affected highly conserved amino acid residues located predominantly either in the hinge region or the CCS part of the CYP1B1 molecule. This may interfere with fundamental properties of the cytochrome P450 molecule. (Sarfarazi & Stoilov, 2000)

The most frequent missense mutation found was p.E387K (6 times in twenty six probands with *CYP1B1* mutation). Three probands were homozygous and three were compound heterozygous. This mutation substitutes the highly conserved amino acid glutamic acid for lysine, in exon 3. Glu387 is located in helix K, which is one of the highly conserved core structural elements. These two residues form the sequence GluXXArg, which is conserved among all members of the cytochrome P450 superfamily, supporting its importance for the normal function of the P450 molecule

(Plásilova *et al.*, 1999) (Sivadorai *et al.*, 2008). Homozygosity for the E387K mutation is always associated with severe disease, and such was also the case in our population.

Frameshifts introduce premature stop codons in the CYP1B1 reading frame and eliminate at least the haem-binding region from the C-terminus of the molecule. It is expected that these mutations result in functional null alleles. (Sarfarazi & Stoilov, 2000) The two most frequent frameshifts in this study were c.881delG and c.1410del13bp. The translation of one nucleotide long deletion c.881delG was found in ten probands (two were homozygous and eight were compound heterozygous). This change will lead to a premature stop and is likely to induce non-sense mediated decay with complete loss of function. Thus, the resulting phenotype is severe. A 13-bp deletion in the *CYP1B1* gene (c.1410del13bp) was found in six probands (two were homozygous and four were compound heterozygous). The deletion removed nucleotides 1410 to 1422 from exon 3 and resulted in a frameshift that truncates the open reading frame by creating a premature stop codon (TGA) 203 bp downstream of the deletion. This truncation eliminates the carboxy-terminus end, which includes the essential haem domain. (Stoilov *et al.,*1997)

The frequency with which these three mutations arose suggested that position 390 and nucleotides 881 and 1410 emerged as a hot spot for mutations in the *CYP1B1* gene.

One novel mutation [Leu378Gln (p.L378Q)] was found in one compound heterozygous proband. This changes a conserved residue throughout the phyla and is likely to disrupt the secondary structure of CYP1B1, as a result of the change of a neutral for a charged amino acid. Functional studies will be needed to fully understand the consequences of this mutation.

CYP1B1 mutations associated with PCG have now been reported in diverse ethnic groups. However, limited information is available on genotype-phenotype correlations.

(Stoilov, 2002) (Hasnain & Balasubramanian, 2002) (Ohtake *et al.*, 2003) (Panicker *et al.*, 2004) PCG is known to have variable penetrance and expressivity. In our study this heterogeneity is observed even in patients within the same family. In family 4, one of the probands has better visual acuity, less significant optic disc damage than the other two relatives. This confirms the presence of significant phenotypical intrafamilial variability.

In the present study, comparisons between the probands with *CYP1B1* mutation and no mutation revealed significant differences in sex preponderance. Compared to the no mutation probands, the proportion of female patients was significantly higher in the probands with mutation. Interestingly, previous reports had estimated that male cases account for approximately 65% of PCG cases overall. (Vasiliou & Gonzalez, 2008) Also patients with PCG and *CYP1B1* mutations tend to have a more severe phenotype than those without mutations.

Individuals who are compound heterozygote for *CYP1B1* mutations may exhibit complex phenotypes, which could account for the phenotypic heterogeneity of PCG. For example, families 8 and 11 had the same heterozygous mutations (c.881delG and c.1410del13bp), but family 8 had a worst phenotype. The phenotypic variability of *CYP1B1* would require comparative analysis of the clinical presentation, phenotype and genotype of the relevant *CYP1B1* mutations.

Of all mutations studied, frameshift mutations tend to cause a more severe phenotype than missense mutations. This fact was described in previous studies. (Panicker *et al.*, 2002). In this study, some patients with both frameshift and missense mutations had severe phenotypes. This may be due to: (1) Centre for Hereditary Eye Diseases of the Department of Ophthalmology is a referral centre and patients with milder phenotypes may not have been referred to us. (2) Our population includes mostly adult and quality

of treatment was less efective decades ago. (3) A delay in health care and treatment options related to social circumstances.

In this study, six probands were married: proband of the family 12 with proband of the family 13, proband of the family 14 with proband of the family 15 and proband of the family 34 with proband of the family 35. This fact emphasizes the importance of prenatal diagnosis. Genetic testing of *CYP1B1* mutations may help predict new cases and their prognoses.

In summary, the present study describes the spectrum of *CYP1B1* mutation associated with PCG in a Portuguese population and their phenotype. Mutations of the *CYP1B1* gene are less prevalent in Portuguese populations with PCG compared with Arabic or Romany populations. Extend the *CYP1B1* screening to other populations around the world is necessary for a better evaluation of its role in the pathogenesis of PCG. Further studies involving larger number of families from many ethnic backgrounds would be required in establishing appropriate genotype-phenotype correlations. This may help in predicting disease prognosis, guiding therapy and patient counseling.

References

Alfadhli, S., Behbehani, A., Elshafey, A. *et al.* (2006). Molecular and clinical evaluation of primary congenital glaucoma in Kuwait. *Am J Ophthalmol. ,* 141(3):512-516.

Ali, M., McKibbin, M., Booth, A. *et al.* (2009, May 15). Null Mutations in LTBP2 Cause Primary Congenital Glaucoma. *The American Journal of Human Genetics,* 84:664-671.

Akarsu A. N., Turacli M. E., Aktan S. G. et al. (1996) A second locus (GLC3B) for primary congenital glaucoma (Buphtalmos) maps to 1p36. Hum Mol Genet 5:1199-1203

Bejjani, B. A., Stockton, D. W., Lewis, R. A., Tomey, K. F., *et al.* (2000). Multiple CYP1B1 mutations and incomplete penetrance in an inbred population segregating primary congenital glaucoma suggest de novo events and a dominant modifier locus. *Human Molecular Genetics ,* 9(3):367-374.

Belmouden, A., Melki, R., Hamdani, M., *et al.*, (2002). A novel frameshift founder mutation in the cytichrome P450 1B1 (CYP1B1) gene is associated with primary congenital glaucoma in Morocco. *Clin Genet ,* 62:334-9.

Chakrabarti, S., Kaur, K., Kaur, I., *et al*. (2006, January). Globally, CYP1B1 Mutations in Primary Congenital Glaucoma Are Strongly Structured by Geographic and Haplotype Backgrouns. *Invest Ophthalmol Visl Sci ,* 47(1):43-47.

Chakrabarti, S., Kaur, K., Rao, K. N., *et al.* (2009). The Transcription Factor Gene FOXC1 Exhiits a Limited Role in Primary Congenital Glaucoma. *Invest Ophthalmol Vis Sci ,* 50:75-83.

Challa, P. (2008). Glaucoma genetics. *Int Ophthalmol Clin ,* 48(4):73-94.

Choudhary, D., Jansson I., Schenkman J. B. (2009) CYP1B1, a developmental gene with a potential role in glaucoma therapy. *Xenobiotica*, 39(8):606-615

Curry, S. M., Daou, A. G., Hermanns, P., *et al.* (2004). Cytochrome P4501B1 mutations cause only part of primary congenital glaucoma in Ecuador. *Ophthalmic Genet ,* 25:3-9.

Hasnain, S. E., Balasubramanian, D. (2002). Identification of novel mutations causing familial primary congenital glaucoma in Indian pedigrees. *Invest Ophthalmol Vis Sci. ,* 43:1358-1366.

Ho, C. L., Walton, D. S. (2004). Primary congenital glaucoma. *J Pediatr Ophthalmol Strabismus ,* 41:271-288.

Kakiuchi-Matsumoto, T., Isashiki, Y., Ohba, N., *et al.* (2001). Cytochrome P450 1B1 gene mutations in Japanese patients with primary congenital glaucoma. *Am J Ophthalmol ,* 131(3):345-50.

Mashima, Y., Suzuki, Y., Sergeev, Y., *et al.* (2001). Novel cytochrome P4501B1 (CYP1B1) gene mutations in Japanese patients with primary congenital glaucoma. *Invest Ophthalmol Vis Sci. ,* 42(10):2211-2216.

Narooie-Nejad, M., Paylakhi, S. H., Shojaee, S., *et al.* (2009). Loss of function mutations in the gene encoding latent transforming growth factor beta binding protein 2, LTBP2, cause primary congenital glaucoma. *Human Molecular Genetics ,* 18(20):3969-3977.

Ohtake, Y., Tanino, T., Suzuki, Y., *et al.* (2003). Phenotype of cytochrome P4501B1 gene (CYP1B1) mutations in Japanese patients with primary congenital glaucoma. *Br J Ophthalmol ,* 87:302-304.

Panicker, S.G., Reddy, A., Mandal, A., *et al.* (2002). Identification of novel mutations causing familial primary congenital glaucoma in Indian Pedigrees. *Invest Ophthalmol Vis Sci.*, 43:135-66

Panicker, S. G., Mandal, A. K., Aramati, B. M., *et al.* (2004). Correlations of Genotype with Phenotype in Indian. *Invest Ophthalmol Vis Sci. ,* 45:1149-1156.

Plásilova, M., Stoilov, I., Sarfarazi, M., *et al.* (1999). Identification of a single ancestral CYP1B1 mutation in Slovak Gypsies (Roms) affected with primary congenital glaucoma. J Med Genet. , 36(4):290-294.

Quigley, H. A. (1996). Number of people with glaucoma worldwide. Br J Ophthalmol, 80:389-393

Quigley, H.A., Broman, A.T. (2006). The number of people with glaucoma worldwide in 2010 and 2020. Br J Ophthalmol, 90:262-267

Reddy, A. B., Kaur, K., Mandal, A. K., *et al.* (2004). Mutation spectrum of the CYP1B1 gene in Indian primary congenital glaucoma patients. Mol Vis. , 10:696-702.

Sarfarazi, M., et al. (1997). Recent advances in molecular genetics of glaucoma. Hum Mol Genet, 6:1667-1677

Sarfarazi, M., Stoilov, I. (2000). Molecular genetics of primary congenital glaucoma. *E*ye , 14:422-428.

Sarfarazi, M., Stoilov, I., Schenkman, J. B. (2003). Genetics and biochemistry of Primary Congenital Glaucoma. Ophthalmol Clin North Am, 16:543-54

Sivadorai, P., Cherninkova, S., Bouwer, S., *et al.* (2008, July). Genetic heterogeneity and minor CYP1B1 involvement in the molecular basis of primary congenital glaucoma in Gypsies. *Clin Genet. ,* 74(1):82-87.

Stoilov, I., Akarsu, A. N., Sarfarazi, M. (1997, April). Identification of three different truncating mutations in cytochrome P4501B1 (CYP1B1) as the principal cause of primary congenital glaucoma (Buphthalmos) in families linked to the GLC3A locus on chromosome 2p21. *Hum Mol Genet. ,* 6(4):641-7.

Stoilov, I., Akarsu, A. N., Alozie, I. et al. (1998). Sequence analysis and homology suggest that primary congenital glaucoma on 2p21 results from mutations disrupting either the hinge region or the conserved core structures of cytochrome P4501B1. Am J Hum Genet 62:573-584

Stoilov, I.R., Costa, V. P., Vasconcellos, J. P., *et al.* (2002). Molecular genetics of primary congenital glaucoma in Brazil. *Invest Ophthalmol Vis Sci. ,* 43(6):1820-1827.

Stoilov, I. R., Sarfarazi, M. (2002). The third genetic locus (GLC3C) for primary congenital glaucoma (PCG) maps to chromosome 14q24.3. *Invest. Ophthalmol. Vis. Sci. ,* 43:3015.

Tanwar, M., Dada, T., Sihota, R., *et al.* (2009). Mutation spectrum of CYP1B1 in North Indian Congenital Glaucoma patients. *Molecular Vision*, 15:1200-1209

Tanwar, M., Dada, T., Sihota, R. (2010). Mitochondrial DNA analysis in primary congenital glaucoma. *Molecular Vision ,* 16:518-533.

Tanwar, M., Kumar, M., Sihota, R., et al. (2010). MYOC and FOXC1 gene analysis in Primary Congenital Glaucoma. Molecular Vision, 16:1996-2006

Vasiliou, V., Gonzalez, F. J. (2008). Role of CYP1B1 in Glaucoma. *Annu. Rev. Pharmacol. Toxicol. ,* 48:333-58

Vincent, A. R., Billingsley, G., Buys, Y., *et al.* (2002). Digenic Inheritance of early-onset glaucomas: CYP1B1, a Potencial Modifier Gene. *Am J Human Genet*, 70:448-460

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