MOLECULAR GENETIC ANALYSIS OF PRIMARY CONGENITAL GLAUCOMA IN INDIAN PATIENTS

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

To

THE DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
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Dedicated to all the PCS afflicted families
and
my beloved parents

UNIVERSITY OF HYDERABAD

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DECLARATION

The research work embodied in this thesis entitled, **"Molecular Genetic Analysis of Primary Congenital Glaucoma in Indian Patients",** has been carried out by me at the L. V. Prasad Eye Institute, Hyderabad, under the guidance of Profs. D. Balasubramanian and T. Suryanarayana. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other university.

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CERTIFICATE

This is to certify that this thesis entitled, **"Molecular Genetic Analysis of Primary Congenital Glaucoma in Indian Patients",** submitted by **Mr. A. Bindu Madhava Reddy** for the degree **of Doctor of Philosophy** to the University of Hyderabad is based on the work carried out by him at the L. V. Prasad Eye Institute, Hyderabad, under our supervision. This work has not been submitted for any diploma or degree of any other University or Institution.

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_ Bindu.

CONTENTS

ABBREVIATIONS

μl:	Microlitre
μM :	Micromolar
μg:	Microgram
a:	Adenine
aa:	Amino acid
ARA:	Axenfeld-Rieger anomaly
bp:	Basepair
c:	Cytosine
cDNA:	Complementary DNA
cM:	centimorgan
CP:	Ciliary processes
DMSO:	Dimethylsulphoxide
dNTPs:	deoxy nucleotide triphosphates
dup:	Duplication
g:	Guanine
JOAG:	Juvenile open angle glauocma
Ins:	Insertion
IOP:	Intraocular pressure
Kb:	Kilobase
kDa:	Kilodalton
ng:	Nanogram
ORF:	Open reading frame
PAGE:	Polyacrylamide gel electrophoresis
PCG:	Primary congenital glaucoma
PCR:	Polymerase chain reaction
POAG:	Primary open angle glaucoma
RFLP:	Restriction fragment length polymorphism
SC:	Schlemm's canal

SNP:	Single nucleotide polymorphism
t:	Thymine
TM:	Trabecular meshwork

AMINO ACIDS

Ala	A	alanine
Arg	R	arginine
Asn	N	asparagine
Asp	D	aspartic acid
Cys	C	cysteine
Gln	Q	glutamine
Glu	E	glutamic aid
Gly	G	glycine
His	H	histidine
He	I	isoleucine
Leu	L	leucine
Lys	K	lysine
Met	M	methionine
Phe	F	Phenylalanine
Pro	P	proline
Ser	S	serine
Thr	T	threonine
Trp	W	tryptophan
Tyr	Y	tyrosine
Val	V	valine

Chapter 1
Introduction

1.0 INTRODUCTION

Primary Congenital Glaucoma, also called Buphthalmos (Gk. bous= ox-eye) or Infantile glaucoma, is an inherited cannatal anomaly of the trabecular meshwork and anterior chamber angle which leads to obstruction of aqueous outflow, increased intraocular pressure and optic nerve damage, ultimately resulting in childhood blindness. Figure. 1.1 illustrates the normal outflow of aqueous humor.

Congenital enlargement of the eye has been recognized since the time of Hippocrates (460-377 B.C), but the association of buphthalmos (Figure. 1.2) with elevated intraocular pressure was not known until the middle of the eighteenth century (Deluise et al 1983). At that time, congenital glaucoma was grouped together with a variety of other conditions such as high myopia and anterior or staphyloma (Ritch et al 1996) but later Van Muralt (1869) described the disease as one belonging to the glaucomas. Pathological studies in 19th century by Reis et al (1999) demonstrated the maldevelopment of anterior chamber angle and the Schlemm's canal to be the primary characteristic, with inflammatory factors playing a secondary role.

A dramatic improvement in the understanding and treatment of patients with primary congenital glaucoma (PCG) occurred when Barkan (1938) introduced goniotomy as an operation for primary infantile glaucoma.

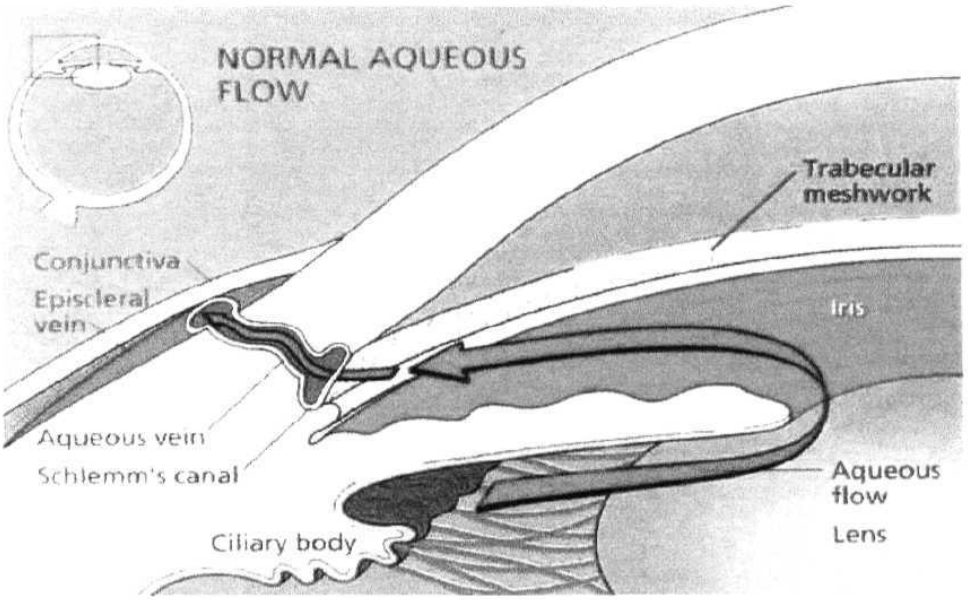


Figure 1.1. Diagrammatic cross section of the anterior segment of the normal eye, showing the aqueous outflow

Reproduced from "Basic and Clinical Science Course", Glaucoma, AAO (1998-99)

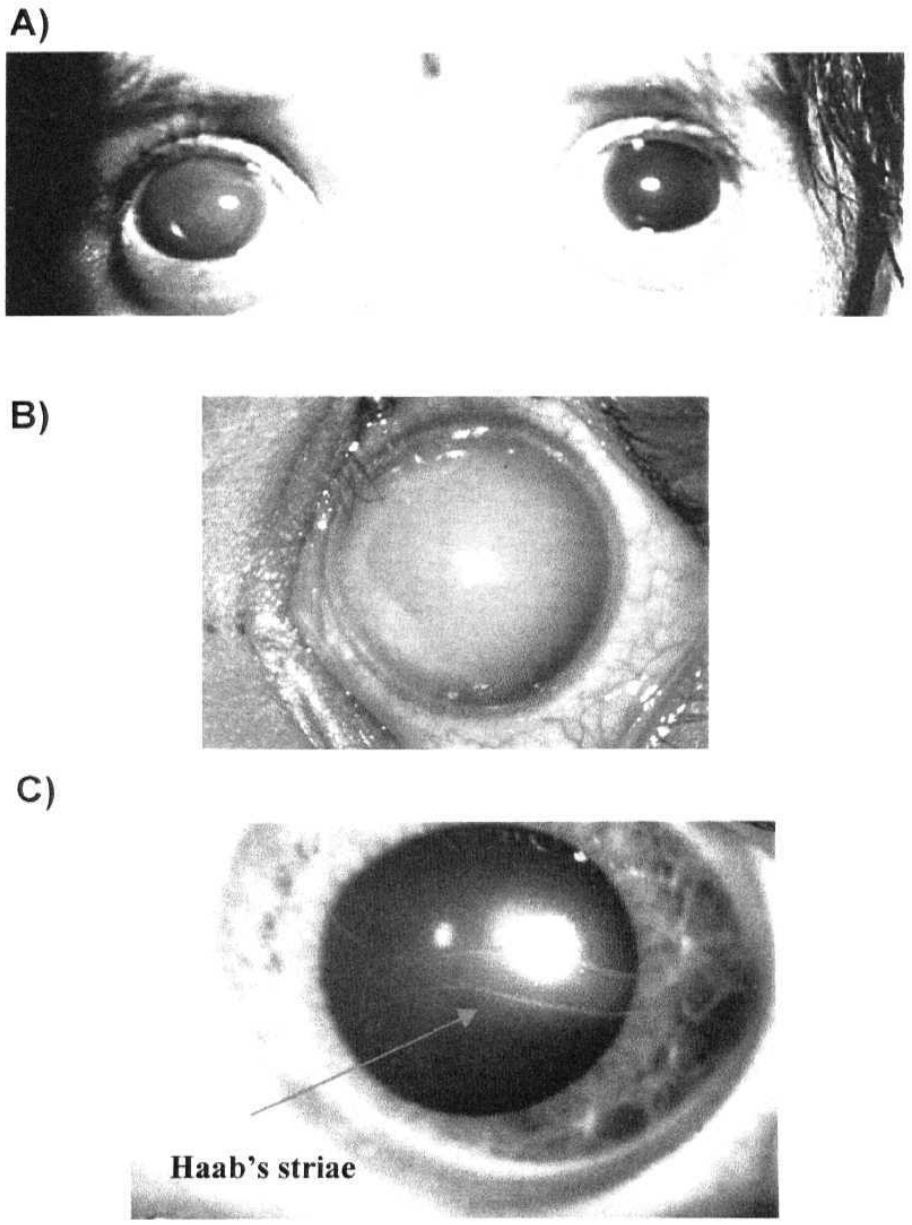


Figure 1.2.Clinical photographs from congenital glaucoma patients eye showing

A) Buphthalmos
B) Corneal edema
C) Haab's striae

Courtesy: A & B from Dr. Mandal A.K.; C from "Clinical Ophthalmology" by Kanski, J. J. (1999)

Further understanding of the pathogenesis and development of newer surgical techniques like trabeculectomy and trabeculotomy (Dannheim 1971), which is the most widely used surgical treatment for PCG/glaucoma, have shown impressive results in these patients.

Usually, glaucomas are classified based on the age of onset as congenital glaucoma / developmental glaucoma (presents during early life: 0-3 years), juvenile glaucoma (2^{nd} to 3^{rd} decade of life) and adult glaucoma (from the 4^{th} decade of life). Congenital glaucomas were further classified by Hoskin et al (1981) called as Hoskin's anatomic classification of developmental glaucoma, by Shaffer and Weiss (1970) as Shaffer-Weiss classification of congenital glaucoma (Kolker et al 1983).

Based on the population studies, some of the main risk factors for all types of glaucomas have been found, which are as shown in Table 1.1. However, Congenital glaucoma is mainly caused by genetic factors, though it does occur in occasional instances in children because of systemic infection like rubella to their mothers during pregnancy (Yanoff 1999).

Numerous developmental disorders associated with anomalies of the anterior chamber angle can lead to elevation of intraocular pressure. Based on the present knowledge, the initial event in the case of developmental

DEMOGRAPHIC	Age Gender Race
OCULAR	Intraocular Pressure Optic nerve damage Myopia Hypermetropia
SYSTEMIC	Diabetes Systemic hypertension
GENETIC	Family history
OTHERS	Cigarette smoking Alcohol intake Socioeconomic factors

Table. 1.1 Main Risk Factors for Glaucoma

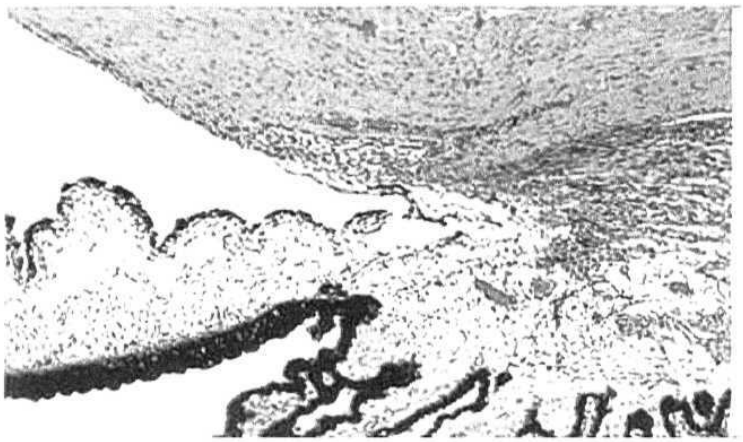
glaucomas appears to be a genetic defect, although some cases may involve an acquired intrauterine insult (Ritch et al 1999).

1.1. Normal development of the anterior chamber angle

Anderson (1981) studied the normal development of the infant angle using scanning and transmission electron microscopy and phase contrast electron microscopy and found that the anterior surface of the iris meets the corneal endothelium at five months of gestation to form the peripheral aspect of the anterior chamber. Slightly posterior to this junction are cells forming the developing trabecular meshwork, being separated by the loose connective tissue. The trabecular meshwork later becomes exposed to the anterior chamber as the angle recess deepens and moves posteriorly (Figure. 1.3A).

In the normal newborn eye, the iris and ciliary body usually have recessed to at least the level of the scleral spur and usually posterior to it. Thus, on gonioscopy of a normal newborn eye, the insertion of the iris into the angle wall is seen posterior to the scleral spur in most cases. However the posterior sliding of the uveal tissue continues during the first 6-12 months of life, which is apparent gonioscopically as formation of the angle recess (Figure. 1.3A).

A)



B)

Figure 1.3. Histological cross section of chamber angle

A) Normal eye
B) Congenital glaucoma eye

Reproduced from "Glaucoma" by Flammer, J. (2002)

Anderson (1981) also demonstrated that the iris and ciliary body fail to recede posteriorly, and thus the iris insertion and anterior ciliary body overlap the posterior portion of the trabecular meshwork in congenital glaucoma eye (Figure. 1.3B). Electron microscopic examination of trabecular meshwork sections from congenital glaucoma patients revealed thickened cords of the uveal meshwork, and compression of the meshwork with resultant reduction in trabecular spaces which are occluded by membrane (endothelium) that shows small pores that causes the obstruction of aqueous humor outflow (Figure. 1.4 and 1.5) (Kupfer et al 1971; Broughton et al 1980; Maul et al 1980; Anderson 1981).

During the early stages of life (first 3 years) the collagen fibres of the eye are softer and more elastic than in older individuals. Thus elevation of intraocular pressure causes rapid enlargement of the globe, which is especially apparent as progressive corneal and limbal enlargement. This can result in linear ruptures (Haab's striae) (Figure. 1.2B), which in turn can lead to corneal stromal and epithelial edema (Figure. 1.2C), as well as to corneal scarring. As the eye enlarges, the iris is stretched and the overlaying stroma may appear thinned. Enlargement of the scleral ring through which the optic nerve passes could lead to enlargement of the optic cup nerve fibers. In eyes with advanced disease, the optic nerve may show complete cupping resulting in irreversible blindness (Figure. 1.6). Surgical incision into the trabecular sheets by goniotomy or trabeculotomy relieves the obstruction and

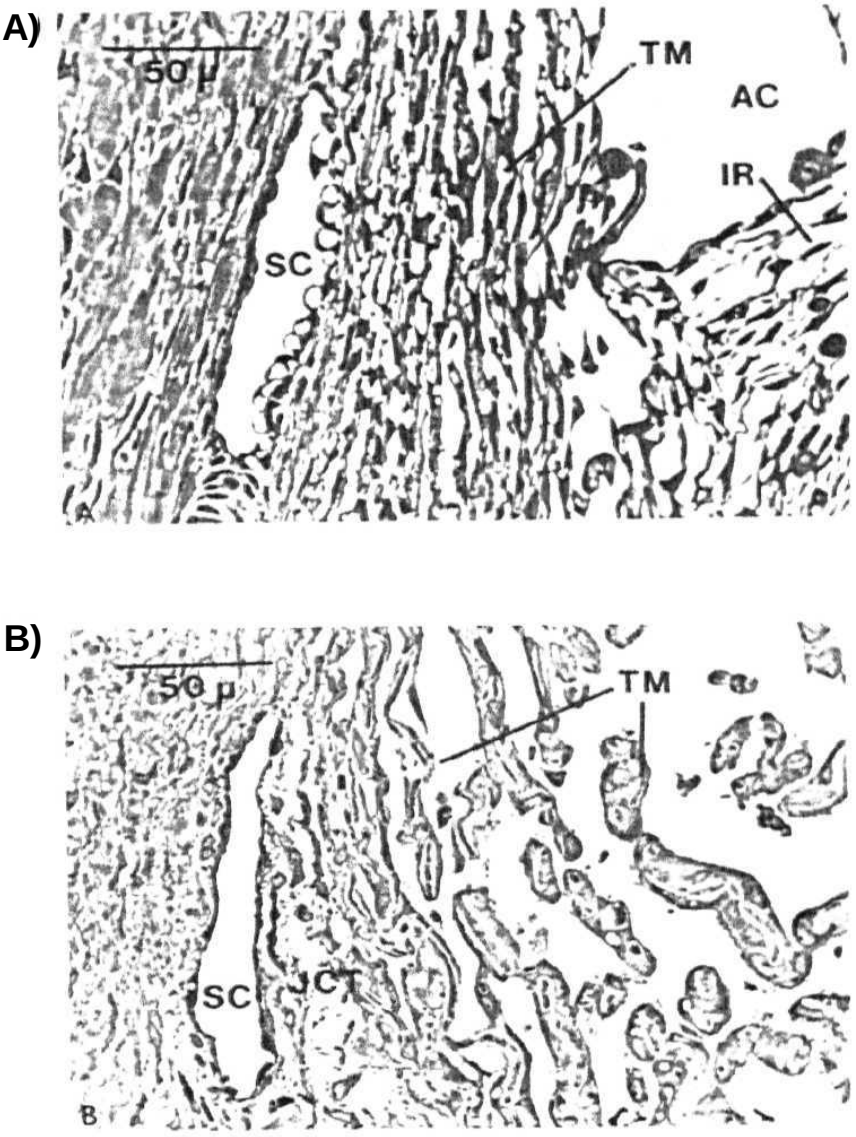
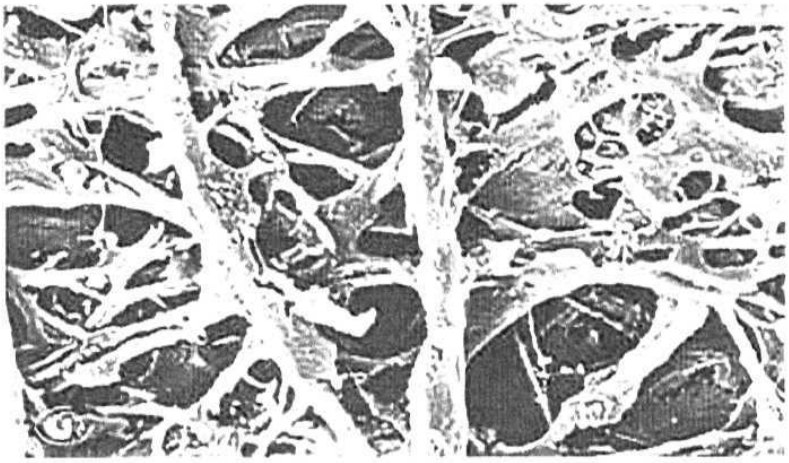


Figure 1.4. Histological cross section of angle structures showing the comparision of thickness of trabecular beams and abnormal formation of the trabecular meshwork (TM).

A) Normal eye
B) Congenital glaucoma eye

Reproduced from "Ophthalmic Pathology" by Spencer, W. H. (1996)

A)



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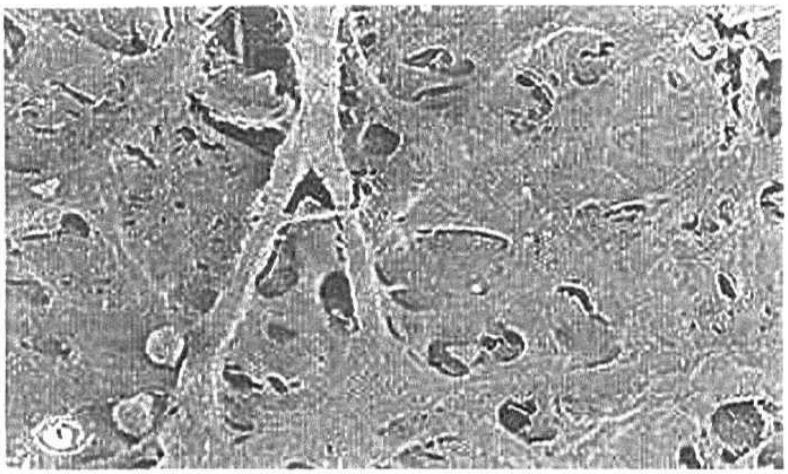


Figure 1.5. Histological cross section of the trabecular meshwork

A) Normal eye
B) Congenital glaucoma eye

Reproduced from "Glaucoma" by Flammer, J. (2002)

A)

B)

Figure 1.6. Advanced buphthalmia with deep anterior chamber elongated axial length and optic nerve cupping

A) Normal eye
B) Congenital glaucoma eye

Reproduced from "Glaucoma in Infants and Children" by Kwitko, M. L. (1973)

normalizes the intraocular pressure in most cases. Successful surgical intervention in the early stages of the disease prevents visual loss in these children (Quigley 1982).

The classical germ layer theory of the development of the human body perpetuated the idea that particular types of the tissues could be matched with specific embryonic origins. Thus it was believed originally that these were three layers in the developing embryo; the ectoderm which give rise to surface epithelia and to the nervous system, the endoderm, which formed the gut; and the mesoderm; which gave rise to all other structures that were not derived from either the ectoderm or endoderm. In accordance with this notion, experimental studies, most using chick as the animal model, have shown that a major portion of the ocular mesenchyme is derived from neural crest cells (Streeter 1951; Duke-Elder 1963; Mann 1964). The tissue forming the anterior segment of the eye are almost exclusively of neural crest origin (O'Rahilly 1974; Johnston et al 1979). Neural crest cells also contibute to numerous non-ocular structures such as craniofacial, cervical skeleton, ligaments and ganglia of craniofacial nerves (Noden 1975; Noden 1978). In case of congenital glaucoma, abnormalities are seen only in the anterior chamber angle. A number of studies show the involvement of the anterior chamber angle in the pathogenesis of primary congenital glaucoma (Ritch 1996). Ultimately, the opportunity of understanding the basic cause of the disease came from recent molecular genetic studies (Sarfarazi and Stoilov 2000).

1.2. Molecular Genetics of Primary Congenital Glaucoma

According to Westerlund (1947), the first allusion to an accumulation of familial cases of congenital glaucoma was made by Grelios in 1836 when he noted that the disease occurs endemically in the Jewish population in Algiers. Jungken in 1842 documented a Swedish family in which seven brothers were affected by congenital glaucoma while the parents and two sisters had normal eyes (Sarfarazi and stoilov 2000). Since then, the detailed history of PCG families has shown the classical Mendelian autosomal recessive mode of inheritance with variable penetrance. Parental consanguinity has been frequently reported. There is a high rate of concordance among monozygotic twins and discordance among dizygotic ones. Pseudodominant inheritance has also been documented in families with high rate of inbreeding and multiple consanguinity. In contrast to the prevalence of 1:10000 in the West, the prevalence of PCG is as high as 1:1250 among the Romany population of Slovakia, and 1:2500 in the Middle East, where inbreeding occurs, suggesting a genetic etiology (Francois 1961,1972; Duke-Elder 1969; Gencik 1989; Bejjani et al 1998). In the Indian State of Andhra Pradesh, the prevalence is estimated to be 1:3300, and the disease accounts for 4.2% of all childhood blindness (Dandona et al 1998; Dandona et al 2001) like as shown in the case of Irish population (Morin et al 1974).

The universal validity of the autosomal recessive model has been challenged by reports of the disease transmission in sucessive generations, unequal sex distribution among the affected individuals, and a lower than expected number of affected siblings in the familial cases. These observations have raised the possibility of PCG being a genetically heterogeneous disorder (Jay et al 1978; Demenias et al 1979; Gencik et al 1980). The first molecular evidence for this possibility came from the genetic linkage studies by Sarfarazi et al (1995), Akarsu et al (1996) and Stoilov & Sarfarazi (2002). By using a combination of candidate gene markers and general positional mapping strategies, Sarfarazi et al (1995) mapped the GLC3A locus to the short arm of chromosome 2 in a group of 17 Turkish families with primary congenital glaucoma, and later the same group mapped another two loci, GLC3B (1p36) (Akarsu et al 1996) and GLC3C (14q24.3) (Stoilov & Sarfarazi 2002), in Turkish families which were not linked to GLC3A and thus provided the evidence of genetic heterogeneity in this disorder. The identification of three separate genetic loci associated with the disease confirms that PCG is indeed genetically heterogeneous. Most of the PCG families studied around the globe have been reported to be linked to the GLC3A locus on 2p21 and the candidate gene has been identified as that for a member of the cytochrome P450 family of enzymes, namely the gene *CYP1B1* (Figure. 1.7). Recent studies estimate that the presence of mutations in *CYP1B1* varies from 20% (in Japanese patients) to 100% (in Slovakian Romany patients) (Plasilova et al 1998; Ohtake et al 2000;

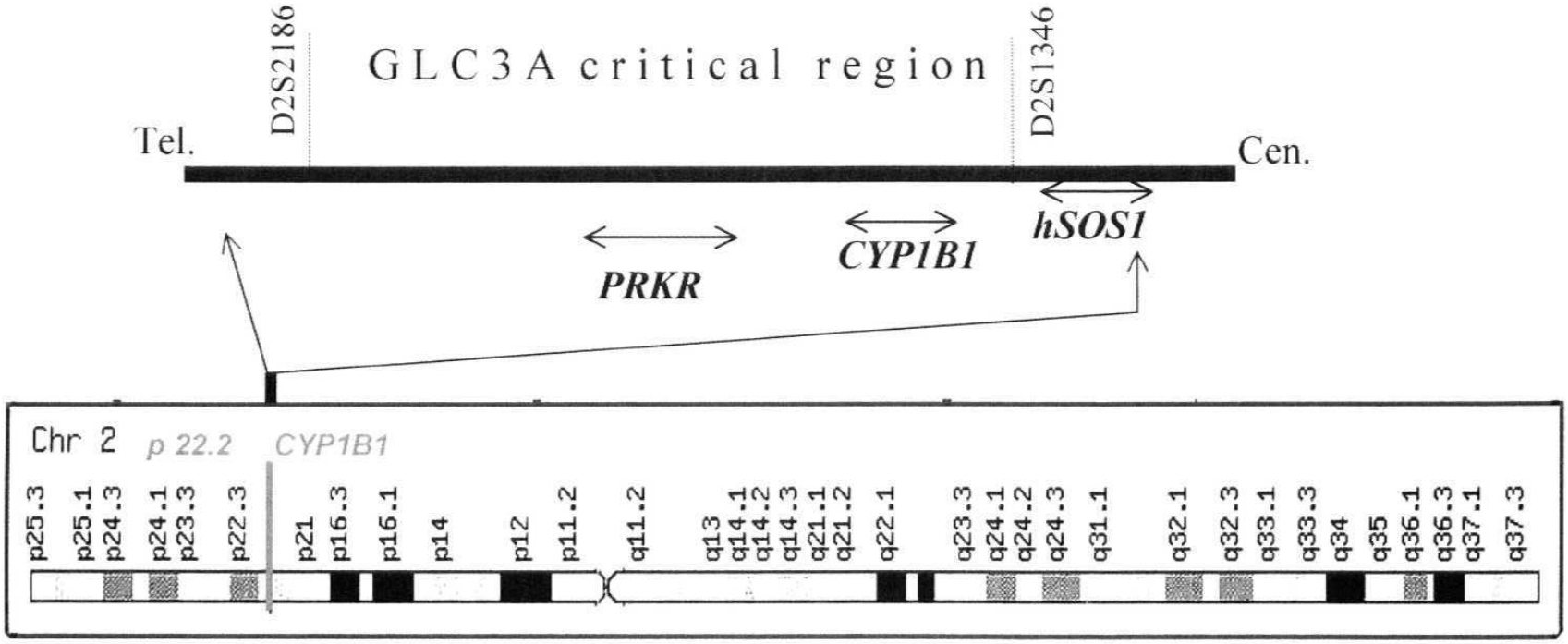


Figure 1.7. Cytogenetic map of chromosome 2 showing the location of CYP1B1 gene

Reproduced from 'GeneCards' at http://bioinfo.weizmann.ac.il/cards/

Kakiuchi-Matsumoto et al 2001; Mashima et al 2001) . However a few other families were linked to GLC3B & GLC3C on 1p36 & 14q24.3 respectively, but the genes have not been identified for these loci (Akarsu et al 1996; Stoilov & Sarfarazi 2002).

Direct sequencing of the potential candidate genes hSOS1, PRKR and *CYP1B1* in the GLC3A critical region that is flanked by markers D2S2186 and D2S1346, which is about ~2.5cM region, revealed pathogenic mutations in human *CYP1B1* gene in individuals with PCG (Figure 1.7). This initial report was followed by several studies on the role of *CYP1B1* in the etiology of PCG in various ethnic groups including Amish, Arabs, Brazil, Canada, Indonesia, Japanese, Romany, Turkey and our report from India (Stoilov et al 1997, 1998, 2002; Plasilova et al 1998; Bejjani et al 1998,2002; Martin et al 2000; Ohtake et al 2000; Kakiuchi-Matsumoto et al 2001; Mashima et al 2001; Michels-Rautenstrauss et al 2001; Panicker et al 2002; Sitorus et al 2003; Soley et al 2003; Ohtake et al 2003). These studies revealed extensive allelic heterogeneity by identifying about 42 distinct *CYP1B1* mutations segregating with the disease phenotype (Table 1.2). In large family panels from Turkey, Saudi Arabia and in Romany Slovakians, *CYP1B1* mutations were observed in 90-100% of the studied families. Reduced penetrance was reported first in the Saudi Arabian families, later on reports have come from Japan, Brazil, and India (from this study) which may be attributed to the existence of a dominant modifier locus that is located on 8p (Bejjani et al 2000). Evidence

S.No.	Nucleotide change	Predicted effect	Ethnic origin	Reference
1	409 c→t	Q19X	Brazil	Stoilov et al 2002
2	505 del 'c'	Frameshift	Japanese	Ohtake et al 2003
3	517 g→a	W 57 X	Brazil, Hispanic	Stoilov et al 2002; Stoilov et al 1999
4	528 g→a	G 61 E	Saudi Arabia, Turkish	Bejjani et al 1998; Stoilov et al 1999
5	576 t→c	L 77 P	Saudi Arabia	Bejjani et al 2000
6	622 del 'c'	Frameshift	Turkish	Soley et al 2003
7	779-789 del	Frameshift	Saudi Arabia	Bejjani et al 2000
8	847 ins 't'	Frameshift	Turkish	Stoilov et al 1999
9	921 a→t	D 192 X	Japanese	Ohtake et al 2003
10	959g→a	E 229 K	Lebanon	Rautenstrauss et al 2001
11	992 g->t	S215I	Indonesia	Sitorus et al 2003
12	1152-1160 del	Frameshift	Saudi Arabia	Bejjani et al 2000
13	1186 c→a	C 280 X	Japanese	Ohtake et al 2003
14	1187 c→t	E 281X	Turkish	Stoilov et al 1999
15	1189 g→t	E 281X	Turkish	Sitorus et al 2003
16	1209 ins 'c'	Frameshift	Turkish	Stoilov et al 1997
17	1281 del 'g'	Frameshift	Brazil	Stoilov et al 2002
18	1316-1317 ins 'at'	Frameshift	Japanese	Ohtake et al 2000, 2003
19	1334 g->t	A 330 P	Japanese	Ohtake et al 2003
20	1355 c→t	A 330 P	Japanese	Ohtake et al 2003
21	1379 c→t	L 345 F	African American	Vincent et al 2002
22	1407-1418 del	Frameshift	Turkish	Sitorus et al 2003
23	1409 c→t	R 355 X	Turkish	Rautenstrauss et al 2001
24	1410-1422 del	Frameshift	Turkish, Brazil, Japanese	Stoilov et al 1997, 2002; Martin 2000, Rautenstrauss et al 2001; Sitorus et al 2003
25	1436 g→a	V 364 M	Japanese, Indonesia	Ohtake et al 2003, Sitorus et al 2003
26	1439 g→t	G 365 W	American	Stoilov et al 1999
27	1449 g→a	R 368 H	Saudi Arabian, East Indian, Brazil	Bejjani et al 2000, Vincent et al 2002, Stoilov et al 2002
28	1466 g→a	D 374 N	Saudi Arabia	Bejjani et al 1998
29	1482 c→t	P 379 L	Turkish	Stoilov et al 1999
30	1505 g→a	E 387 K	Hispanic, Slovakia, Canada, Brazil	Stoilov et al 1999, 2002; Plasilova et al 1999; Martin et al 2000
31	1514 c→a	R 390 S	Saudi Arabia	Bejjani et al 2000
32	1515 g→a	R 390 H	American, Turkish, British	Stoilov et al 1999
33	1546-1555 dup	Frameshift	Turkish, German, Brazil, American, Costa Rica	Stoilov et al 1999, 2002; Rautenstrauss et al 2001, Vincent 2002; Soley 2003
34	1620 ins 'g'	Frameshift	Japanese	Kakiuchi et al 1999
35	1656 c→t	P 437 L	Turkish, Brazil	Stoilov et al 1999, 2002
36	1674 c→g	A 443 G	Brazil	Stoilov et al 2002
37	1677 g→a	R 444 Q	Japanese	Kakiuchi et al 2001

38	1691 del 'g'	Frameshift	Hispanic, Brazil	Stoilov et al 1999, 2002
39	1723-24 del 'ag'	Frameshift	Brazil	Stoilov et al 2002
40	1749-1775 dup	Frameshift	Turkish	Stoilov et al 1999
41	1751 c→t	R 469 W	Turkish, British	Stoilov et al 1999
42	1809 c→t	R 469 W	Saudi Arabian	Bejjani et al 1998

Table 1.2. All Known *CYP1B1* pathogenic mutations reported in the literature

***Mutations from this study is not included**

for founder effect evidence is presented for the Romany population of Slovakia and Sundanese Indonesians. All affected individuals in the Romany group identified were homozygous for the Glu 387 Lys mutation and it is the Val 364 Met mutation in the Indonesian-Sundanese, who also shared identical haplotypes for the six known intragenic single nucleotype polymorphisms markers among these individual groups.

1.3. Cytochrome P450 1B1 *{CYP1B1)*

Cytochrome P450s are a multigene superfamily of monomeric mixed function oxidases or mono oxygenases, and they function by inserting one atom of molecular oxygen into the substrate and one atom into water. These cytochrome P450s play an important role in adding a hydroxyl group in a step in xenobiotic metabolism and in catalyzing specific biochemical reactions in various metabolic pathways (Gonzalez 1989; Nelson 2001).

Dioxin induced *CYP1B1* gene is the largest known human P450 located on chromosome 2p22-21 spanning approximately 12 kb of DNA and is composed of three exons and two introns. The mRNA is 5.2 kb long, and the open reading frame begins at the 5' end of the second exon, in contrast to other P450s in all of which it begins in Exon l. The predicted protein is 543 amino acids long (Tang et al 1996).

Deletion analysis and site directed mutagenesis identified four important regions, two antisense Sp1 sites 'GGGCGG' (-84 to -89 and -68 to -73), a TATA like box 'TTAAAA' (-34 to -29) and an initiator motif 'TTGACTCT' (-5 to +3) at the 5' end for maximum promoter activity. The long 3' un-translated region contains multiple polyadenylation sites (Sutter et al 1994; Wo et al 1997). Structural alignment and molecular modeling studies of membrane bound cytochromes such as CYP1B1 show that they adopt similar molecular structures. A transmembrane domain is located at the amino terminal end followed by a proline rich 'hinge' region which permits flexibility between the membrane spanning domain and the cytoplasmic portion of the molecule. The carboxy terminal ends are highly conserved, containing a set of conserved core structures and signature sequences responsible for the heme binding ability. Between the hinge region and conserved core structure lies a less conserved substrate-binding region. Analysis of the predicted amino acid sequence indicates that there are three potential furin cleavage sites commencing at amino acid 38, which suggests that there could be post-translational N-terminal processing. (Shimizu et al 1988; Gonzalez 1989; Graham-Lorence et al 1996; Yamazaki et al 1993; Chen et al 1996; Lewis et al 1999; Werck-Reichhart et al 2000; Achary et al 2003 unpublished data).

Identification of *CYP1B1* as the gene affected in primary congenital glaucoma is the first example in which mutations in a member of the

cytochrome P450 superfamily is shown to result in a primary developmental defect. However *CYP1B1* is not the only such example, there are reports where mutations in 'drug-metabolizing' enzymes can cause aberrant development (Nebert 1991; Stoilov 2001; Stoilov et al 2001). In Drosophila, mutations affecting the *disembodied (dib) (CYP302A1)* locus prevent dorsal closure, and interfere with embryonic cuticle disposition, mid gut morphogenesis and head involution (Chavez et al 2000). In another case, the *ROTUNDIFOLIA 3 (ROT3) (CYP90C1)* locus in Arabidopsis is involved in the regulation of leaf morphogenesis (Kim et al 1999). Mutations affecting such enzymes generally produce recessive phenotypes because in heterozygotes the normal allele is capable of compensating for the mutant allele. From this point of view, several studies have inferred it as logical that a recessive phenotype like PCG should be caused by mutations in an enzyme such as CYP1B1.

The presence of *CYP1B1* mRNA in a variety of fetal tissues (kidney, lung, adrenal gland, brain) has led to the suggestion that *CYP1B1* may have an important role in normal fetal development (Murray et al 2001). Although the exact function of the CYP1B1 protein in the eye is unclear, CYP1B1 expression has been found in ocular tissues like the ciliary body and outer pigment epithelial cells of the eye at postnatal day 4 and its expression is shown to continue into adulthood (Bejjani et al 2002). These findings strongly suggest that CYP1B1 may have an important role in the normal fetal

development and terminal maturation of the anterior chamber angle of the eye. This hypothesis has received support as a result of mutations in the *CYP1B1* gene being linked with the development of primary congenital glaucoma.

Studies have shown that the expression of cytochrome P450s such as CYP26 (de-Ross 1999) and CYP2B19 (Keeney et al 1998) are relevant to the pattern of development. Similarly CYP1B1 has been shown to be expressed in the developing eye in a pattern consistent with its proposed function as regulator of the anterior chamber angle development. This cytochrome P450 may convert an endogenous substrate to a metabolite for elimination, or into a morphogen, or activate it to a teratogen or carcinogen. In the case of PCG, absence or mutation of *CYP1B1* causes maldevelopment of the trabecular meshwork in the eye. Similarly in the another instance, conversion of the metabolite retinoic acid to the less active 4-hydroxy-retinoic acid by the enzyme retinoic acid 4-hydroxylase (CYP26) enables developmental changes during embryonic development (de-Roos et al1999). Identification and characterization of such morphogens will enable us to know the role of cytochromes in the normal developmental process.

Stoilov et al (2001) have proposed two possible scenarios on how *CYP1B1* mutations may trigger pathogenic responses resulting in abnormal eye development. These are: 1) The spatial and temporal expression of the

genes controlling the anterior chamber angle development may be altered by the absence of a regulatory molecule produced by *CYP1B1.* 2) Alternately, the signs of developmental arrest may reflect the toxic effect of a metabolite that is normally eliminated by CYP1B1.

In order to understand the role of CYP1B1 in the development of PCG, Buters et al (1999) constructed a *CYP1B1* null mouse. However, surprisingly the homozygous animal did not show any evidence of glaucoma. Unfortunately, the methods used to evaluate the mouse may not be sensitive enough to detect glaucomatous changes in the mouse eye (John et al 1997). In addition, the mouse phenotype may differ from the human, since the anterior chamber angle has undergone evolutionary changes only very recently in time. For example only humans and higher apes have the typical trabecular meshwork, while in contrast, other organisms have a reticular type meshwork (Rohen 1961).

Computer based modeling studies by Achary et al (2003) for four CYP1B1 mutations namely G61E, P193L, E229K and R368H revealed that built mutant structures are associated with geometrical strain, lack of some hydrogen and ionic interactions. Molecular dynamic simulation studies on these models show some regions, including the conserved core structure and the functionally important regions to undergo significant structural changes as compared to the wild type structure.

The identification of this new member of the *CYP1B1* gene family has stimulated considerable research activity to elucidate its biological significance. Recent studies have shown that CYP1B1 is capable of metabolizing a variety of putative human carcinogens and also that it shows an increased expression in a wide range of human tumors (Spink et al 1998; McFadyen et al 1999). CYP1B1 catalyses the oxidation of 17 β-estradiol. *In vitro* studies of two mutant forms of CYP1B1, namely G61E and R469W, expressed and purified from *E. coli,* have shown reduced expression, stability and altered catalytic activity towards endogenous steroid substrate metabolism (Janson et al 2001). It has been reported that there are at least eight to ten *CYP1B1* gene polymorphisms in humans.

The studies have shown that the polymorphisms R 48 G, A 119 S and L 432 V also exhibited altered kinetics with significantly increased apparent k_m and lowered V_{max} values for both the 2-and 4- hydroxylation of estradiol (Shimada et al 1997,1999,2001; Akillu et al 2002). These *CYP1B1* polymorphisms show significant association in breast cancer, head & neck squamous cell cancer and prostate cancer patients (Inoue et al 2000; Tang et al 2000; Ko et al 2001). These studies suggest that polymorphic human variants may cause some altered catalytic specificity and may influence susceptibility of individuals towards endogenous and exogenous carcinogens.

1.4. Congenital glaucoma associated with chromosomal abnormalities and systemic abnormalities

The association of congenital glaucoma with chromosomal abnormalities has been reported earlier by several groups. PCG in these instances is accompanied by dysmorphic features, multisystem abnormalities, developmental delay so on. Keith (1966), Hopener et al (1972) and Litcher (1975) have described several cases with Trisomy 13 syndrome exhibiting bilateral congenital glaucoma. Broughton (1983) described a family with pericentric inversion of chromosome 11, where all cytogenetically affected members had congenital glaucoma that appeared clinically consistent with primary congenital glaucoma. Katsushima et al (1987) reported a patient with Trisomy 2q(q33→ q ter) and Monosomy 9p(p24→p ter), both of which were found to manifest PCG. Traboulsi et al (1988) also examined five patients with Trisomy 21 and primary congenital glaucoma. Later several studies reported chromosomal abnormalities in 1, 6, 16, and 18 chromosomes were associated with primary congenital glaucoma (Kwitko 1973). Very recently we reported a patient having down's syndrome like features with PCG having a karyotype of 22p+ variant (Mandal et al 2003). These reports emphasize the possible role of chromosomal aberrations as another factor in the heterogeneous origin of this disease.

In 1946, Guerry reported two cases with bilateral congenital glaucoma associated with rubella infection of their mothers during the first trimester. Alfano (1966) also described four PCG patients associated in a similar way with maternal rubella infection. On examination of the affected eyes, it was noted that the anterior chamber was deep and the trabeculum was partially obstructed by thin fibrous bands stretching from the anterior iris surface.

1.5. PCG: Genetic Heterogeneity

Though genetic heterogeneity has been shown for PCG, homogeneity in phenotype as well as genotype in Slovakian Gypsies (E387K), Indonesian-Sundanese (V364M) and common haplotypes (G61E, D374N and R469W) of *CYP1B1* in Saudi Arabian population has been reported. Recent studies estimate that the presence of mutations in *CYP1B1* varies from 20-100%. A similar genetic heterogeneity has also been shown in case of "early onset glaucoma", usually referred to as juvenile open angle glaucoma (JOAG). In this case, digenic inheritance with mutations in the primary candidate gene *MYOC* and also in the gene *CYP1B1* have been identified (Vincent et al 2002). Similarly, in case of Peters' anomaly, mutations where identified in *CYP1B1,* though the putative gene is *PITX2* (Vincent et al 2001). These findings might be understandable because the tissues forming the anterior segment of the eye are almost exclusively of neural crest origin. Very recently Libby et al (2003) found that mice carrying mutations in the genes encoding

FOXC1 and *CYP1B1* exhibited histopathological angle abnormalities consistent with those seen in humans with PCG. The same study propose an association between glaucoma and ocular albinism by identifying the structural abnormalities in the eyes of glaucoma with *CYP1B1* mutations were substantially more severe than in *CYP1B1* mutant mice with normal pigmentation. Even without a *CYP1B1* mutation, tyrosinase deficiency alone caused mild histopathological defect in the iridocorneal angle of the albino mice (Alward 2003). These findings not only emphasize the genetic heterogeneity and complexity behind the pathogenesis of glaucoma but also open new avenues for research.

1.6. Scope of the study

One severe form of glaucoma, which occurs at birth or early infancy (seen up to 3 years) is primary congenital glaucoma (PCG) and is mainly inherited as an autosomal recessive disorder. In the Indian state of Andhra Pradesh, the incidence is 1:3,300 and it accounts for 4.2% of all childhood blindness. The disease shows both clinical and genetic heterogeneity. Varying in severity (mild - severe) and manifestations (unilateral - bilateral) of this devastating childhood blindness have been identified in India, but the genetic etiology is yet to be clarified.

Since PCG is mainly a congenital disorder, early and reliable diagnosis of the disease is vital, so that appropriate and prompt medical and surgical interventions can be initiated in time. This could in turn prevent unwanted visual loss and hence help in saving the vision of the child. To this end, we have taken up this molecular genetic investigation in order to

1. identify the possible underlying genetic defect of this disorder;
2. screen the most predominant mutations (if any) using diagnostic methods like PCR-RFLP;
3. investigate the association of PCG with other candidate genes like *FOXC1*, *PAX6* and *MYOC* which are linked to the other anterior segment disorders like Axenfeld-Rieger anomaly (ARA), Aniridia and adult glaucoma (JOAG/POAG) respectively, and

4. establish genotype-phenotype correlations.

Several studies have shown that mutations in the *CYP1B1* linked to GLC3A locus are predominantly associated with the disease. Hence we have screened the entire coding and promoter region of *CYP1B1* in 146 clinically well characterised PCG patients and identified several pathogenic mutations and single nucleotide polymorphisms in many of these patients.

For eight of these mutations we have developed PCR based RFLPs, which, we hope, help us in screening the respective mutations in the afflicted families, carrier detection, population screening and genetic counselling.

We next screened the candidate genes of other Anterior segment disorders like Axenfeld-Rieger anomaly (*FOXC1*), Aniridia *{PAX6)* and adult glaucomas *(MYOC)* in 10 non *CYP1B1* PCG probands, in order to investigate the genetic heterogeneity of PCG. Our study could identify one missense mutation in *MYOC* gene in one PCG proband and one known polymorphism in two of the PCG probands studied. Finally we have also undertaken genotype-phenotype correlation in these instances.

Chapter 2
Materials and Methods

2.0. MATERIALS AND METHODS

2.1. Clinical Evaluation and Patient Recruitment

The study project and protocol was approved by the Hyderabad Eye Research Foundation and the L.V. Prasad Eye Institutional Review Board from both the scientific and ethical angle. After getting informed consent from volunteers in accordance with the guidelines of the Declaration of Helsinki, 139 pedigrees of 155 patients were recruited for the study from the Jasti V. Ramayanamma Childrens Eye Care Clinic of the Institute. All patients and their family members were evaluated by our collaborator and glaucoma specialist, Dr. Anil K. Mandal. Ophthalmic examination included slit lamp biomicroscopy, gonioscopy, measurement of intraocular pressure (IOP) and perimetry in some cases. Clinical manifestations included elevated IOP, enlargement of globe, edema and opacification of cornea with rupture of Descemet's membrane, thinning of anterior sclera and atrophy of the iris, anomalously deep anterior chamber, photophobia, blepharospasm, and excessive tearing. Individuals presenting with other associated ocular or systemic anomalies were excluded from this study. The patient selection was completely unbiased with respect to sex, consanguinity, and familial incidence of the disease.

Studies have shown the occurrence of PCG in 65% - 80% cases as bilateral (Duke-Elder et al 1969; Stoilov et al 2002), and in the United States

and Europe it occurs more frequently in males than females in a ratio of 3:2 (Faser 1974, Phelps et al 1974; shaffer 1967), whereas it is reversed in Japanese patients (Mashima et al 2001; Duke-Elder et al 1969) and most of the cases appeared to be sporadic (nonfamilial) (DeLuise et al 1983). Of the total families recruited in this study, 49.1% belonged to the non-consanguineous group, sporadic cases accounted for 89.6% and bilateral 88.3%. Males accounted for 56.1% among the affected individuals (Table 2.1). These figures are similar to previously reported numbers.

About 2-10ml of peripheral blood was collected from patients and their available family members. Seventy normal ethnically matched individuals, who did not have any eye problems, were included as control subjects.

2.2. Mutation Screening of *CYP1B1*

Since mutations in *CYP1B1* are the predominant cause of PCG, the entire coding region (1.6 kb organised in exons II and III) (Tang et al 1996) and the promoter region (485 bp promoter flanking region) (Wo et al 1997) were screened for variations. Though frequently seen mutations in the gene and all pathogenic mutations have been reported so far only in the coding region (exons II and III), some of the PCG patients did not show mutations in the coding region. In order to check the possibility of promoter variations, we have screened the complete coding region along with the promoter region.

Category	Total no. of patients (%)
Total no. of patients	155(100)
Male	87(56.1)
Female	68 (43.9)
Unilateral	18(11.7)
Bilateral	137(88.3)
Consanguineous	79 (50.9)
Non-consanguineous	76(49.1)
Sporadic	139(89.6)
Familial	16(10.4)

TABLE 2.1. Demographic data of PCG patients

DNA was extracted from the peripheral leukocytes of patients, family members and controls using the standard phenol-chloroform extraction method (sambrook et al 1989). Using four sets of overlapping primers, the *CYP1B1* gene was amplified from patients and controls (Table 2.2 and Figure 2.1). The primers used were: set I (1F/1R-786 bp), set II (2F/2R-648 bp), set III (3F/3R-885 bp) and set IV (PF/PR - 485 bp). All polymerase chain reactions (PCR) were done for only 30 cycles and conditions for sets I and II were as reported earlier (Stoilov et al 1998) whereas set III and set IV conditions are given in Table 2.2. All the PCRs were done using a model PTC200 machine (MJ Research, Watertown, MA) in a 25µl reaction containing the following: 50 ng genomic DNA, 1X PCR buffer with 1.5-2.0 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each primer and 1 unit *Taq* polymerase (MBI Fermentas, Lithuania) with or without 10% dimethylsulphoxide (DMSO). Primer sets I, II and IV had 10% DMSO and 1.5 mM $MgCl_2$ whereas set III had only 2.0 mM $MgCl_2$. Amplicons were sequenced directly and the sequences of the patient and control samples were compared to identify mutations. The same sets of primers were used for PCR and bi-directional sequencing. The three amplicons were purified (pre- PCR sequencing kit (USB(R) or Amicon Microcon PCR spin columns; Millipore, Bedford,MA.), Big Dye(R) terminator cycle sequencing was done and sequencing reactions were performed on ABI Prism 377 & 3100 automated DNA sequencers (Applied Biosystems, Foster City, CA). Sequencing reactions were carried out

Exon	Primers used for PCR	Amplicon size (bp)	$Mgcl_2$ Used (mM)	Annealing temp (°C)
2	1F: 5'- TCT CCA GAG AGT CAG CTC CG -3' 1R: 5'- GGG TCG TCG TGG CTG TAG -3'	786	1.5	56
2	2F: 5'- GAT GCG CAA CTT CTT CAC G -3' 2R: 5'- CTA CTC CGC CTT TTT CAG A -3'	648	1.5	56
3	3F: 5'- TCC CAG AAA TAT TAA TTT AGT CAA CTG -3' 3R: 5'- TAT GGA GCA CAC CTC AAC CTG -3'	885	2.0	60
Promoter Region	4F: 5'- AGC GGC CGG GGC AGG TTG TAC C -3' 4R: 5'- ATT GGG ATG GGG ACG GAG AA -3'	486	1.5	62

PCR conditions for all primer sets - initial denaturation at 94 "C - 3 min followed by (94 °C - 30 sec, Annealing for 30 sec , 72 °C - 45 sec) x 30 cycles with final extension at 72 "C for 10 minutes.

TABLE 2.2. *CYP1B1* primers and conditions used for PCR amplification

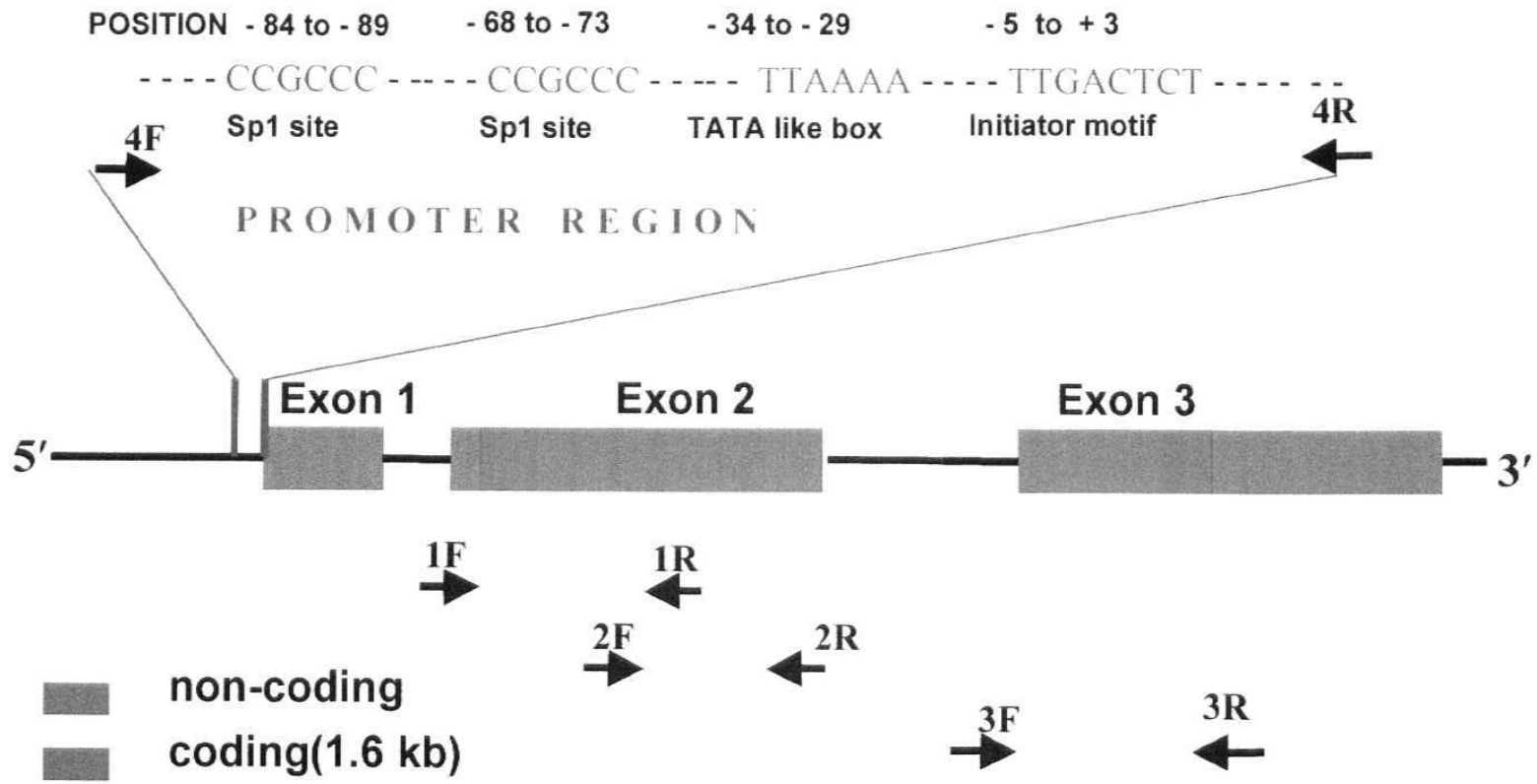


Figure 2.1. Genomic organization of *CYP1B1* gene

according to the protocol supplied by the manufacturer. The sequences seen in patient samples were compared against those in the Genebank sequence database (NCBI Accession No.U03688). Variations were confirmed as pathogenic mutations or single nucleotide polymorphisms, by multiple sequence alignment and/or by screening 70 normal controls.

In order to check the genetic heterogeneity of this disorder, which has been shown by few studies very recently (Vincent et al 2001; Libby et al 2003), we also screened the candidate genes of other anterior segment disorders namely those of Axenfeld-Rieger anomaly (*FOXC1*), Aniridia *(PAX6)* and adult glaucomas *(MYOC)* in 2 patients who showed single heterozygous mutation in *CYP1B1* PCG probands, and in 8 others who had PCG but did not show any mutations in *CYP1B1.*

2.3. Mutation Screening of *FOXC1*

Mutations in the forkhead/winged-helix transcription factor gene *FOXC1,* mapped to 6p25 (Gould 1997), have been implicated in the pathogenesis of Axenfeld-Rieger Anomaly (ARA)(Mears et al 1998; Nishimura et al 2001; Panicker et al 2002; Komatireddy et al 2003). ARA is a form of developmental glaucoma, caused by the maldevelopment of the anterior segment of the eye. Fifty to seventy percent of the ARA cases develop glaucoma in early age. Ocular features include prominent anterior

Schwalbe's line, abnormal angle tissue, hypoplastic iris, polycoria, corectopia and glaucoma. Mouse models carrying mutations in *FOXC1* show the phenotype of PCG (Libby et al 2003).

Hence, we have screened the entire coding region of 1.6kb, organised in a single exon, to check the possibility of mutations in *FOXC1*. Using four sets of primers, the entire coding region of *FOXC1* was amplified and the amplicons were sequenced using ABI Prism 377 & 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA). All the PCR reactions were performed as reported by Panicker et al 2002 (Table 2.3).

2.4. Mutation Screening of *MYOC*

Genetic linkage analysis by Sheffield et al (1993) has mapped adult glaucoma (juvenile open angle glaucoma (JOAG)/primary open angle galucoma (POAG)) to GLC1A (1q21-31) . Later Stone et al (1997) showed that mutations of the trabecular meshwork-induced glucocorticoid-responsive protein/myocilin *(TIGR/MYOC)* gene were responsible for JOAG/POAG. This gene is known previously to encode a trabecular meshwork protein postulated to have a role in the regulation of IOP (Nguyen et al 1993).

Primers for PCR amplification	Amplicon size	$Mgcl_2$ Used	Annealing temp (°C)
ARA1F: 5'- CCCGGACTCGGACTCGGC -3' **ARA1R:** 5'- AAGCGGTCCATGATGAACTGG –3'	428	1 mM	62
ARA3F: 5'- ATCAAGACCGAGAACGGTACG -3' **ARA2R:** 5'- CTGAAGCCCTGGCTATGGT -3[1]	710	1 mM	58
ARA3F: 5'- ATCAAGACCGAGAACGGTACG –3' **ARA3R:** 5'- GTGACCGGAGGCAGAGAGTA -3'	634	1 mM	58
ARA4F: 5'- TACCACTGCAACCTGCAAGC –3' **ARA4R:** 5[1]- GGGTTCGATTTAGTTCGGCT –3'	516	1. 25 mM	58

PCR conditions for all primer sets - initial denaturation at 94 ° C - 3 min followed by (94 ° C - 30 sec, Annealing for 30 sec , 72 ° C - 45 sec) x 35 cycles with final extension at 72 ″ C for 7 minutes.

TABLE 2.3. *FOXC1* primers and conditions used for PCR amplification

JOAG/POAG is generally associated with elevated IOP consequent to the abnormal resistance of aqueous outflow through trabecular meshwork a specialized tissue lining the outflow pathway of the eye (Shields 1993V

Recent studies estimate that MYOC mutations are found in S 4%-5% of sporadic cases (Alward et al 1998; Fingert et al 1999) and up to 14%-33% in familial cases of JOAG (Shimizu et al 2000; Vincent et al 2002). Studies by Vincent et al (2002) have shown mutations in *CYP1B1* (gene mapped to congenital glaucoma) to be also associated with JOAG/POAG. Some of the JOAG cases have shown the association of mutations in both *MYOC* and *CYP1B1,* showing the digenic inheritance for this phenotype (Vincent et al 2002). In order to check the possibility of *MYOC* mutations in congenital glaucoma, we have screened the entire coding region of *MYOC,* spanning exons 1-3, for mutations by direct sequencing. The primers used were as described by Alward et al (1998) (Table. 2.4).

2.5. Mutation Screening of *PAX6*

Human *PAX6* gene, a homologue of originally isolated pax6 from mouse (Walther et al 1991), located on chromosome 11p13, spans 22kb and consists of 14 exons, encoding a 422 aminoacid transcriptional regulator protein (Ton et al 1991). Mutations in *PAX6* have been identified in patients with Aniridia phenotype in humans (Dharmaraj et al 2003;

Exon	Primers used for PCR	Amplicon size (bp)	$Mgcl_2$ Used (mM)	Annealing temp (°C)
1	**1F:** 5'- GGC TGG CTC CCC AGT ATA TA -3' **1R:** 5'- ACA GCT GGC ATC TCA GGC -3'	180	1.5	58
1	2F: 5'- ACG TTG CTC CAG CTT TGG -3' 2R: 5'- GAT GAC TGA CAT GGC CTG G –3'	196	1.5	58
1	3F: 5'- AGT GGC CGA TGC CAG TAT AC -3' 3R: 5'- CTG GTC CAA GGT CAA TTG GT –3'	190	1.5	58
1	4F: 5'- AGG CCA TGT CAG TCA TCC AT -3' 4R: 5'- TCT CTG GTT TGG GTT TCC AG –3'	214	1.5	58
1	5F: 5'- TGA CCT TGG ACC AGG CTG -3' 5R: 5'- CCT GGC CAG ATT CTC ATT TT -3'	200	1.5	58
1	6F: 5'- TGG AGG AAG AGA AGA AGC GA –3' 6R: 5'- CTG CTG AAC TCA GAG TCC CC -3'	185	1.5	58
2	7F: 5'- AAC ATA GTC AAT CCT TGG GCC -3' 7R: 5'- TAA AGA CCA TGT GGG CAC AA -3'	223	1.5	58
3	8F: 5'- TTA TGG ATT AAG TGG TGC TTC G -3' 8R: ATT CTC CAC GTG GTC TCC TG –3'	177	1.5	58
3	9F: 5'- AAG CCC ACC TAC CCC TAC AC -3' 9R: 5'- AAT AGA GGC TCC CCG AGT ACA -3'	184	1.5	58
3	**10F:** 5'- ATA CTG CCT AGG CCA CTG GA -3' **10R:** 5'- CAA TGT CCG TGT AGC CAC C –3'	192	1.5	58
3	**11F:** 5'- TGG CTA CCA CGG ACA GTT C -3' **11R:** 5'- CAT TGG CGA CTG ACT GCT TA -3'	197	1.5	58
3	**12F:** 5'- GAA CTC GAA CAA ACC TGG GA -3' 12R: 5'- CAT GCT GCT GTA CTT ATA GCG G -3'	195	1.5	58
3	13F: 5'- AGC AAG ACC CTG ACC ATC C -3' **13R:** 5'- AGC ATC TCC TTC TGC CAT TG -3'	179	1.5	58

PCR conditions for all primer sets - initial denaturation at 94 °C - 3 min followed by (94 °C – 30 sec, Annealing for 30 sec , 72 °C – 45 sec) x 35 cycles with final extension at 72 °C for 5 minutes.

TABLE 2.4. *MYOC* primers and conditions used for PCR amplification

http://www.hgu.mrc.ac.uk/Softdata/PAX6/). The gene has been studied in a number of organisms, including *Drosophila,* and plays a central role in eye development. It has been conserved throughout the animal kingdom (Quiring et al 1994; Loosli et al 1996). Variations in PAX6 show phenotypes other than Aniridia, including Peter's anomaly (Hanson et al 1994), ocular anterior segment anomalies (Azuma et al 1998), isolated foveal hypoplasia (Azuma et al 1996), autosomal dominant keratitis (Mirzayans et al 1995) and congenital cataracts (Glaser et al 1994).

Since PCG is also a developmental disorder, we decided to check the possibility of the association of PAX6 mutations with PCG, and screened the entire coding region using 14 sets of primers as described by Gronskov et al (1999) and Dharmaraj et al (2003) (Table 2.5).

2.6. PCR-RFLP Analysis

Some of the mutations resulted in either loss or gain of recognition sites. For determining the co-segregation of mutant alleles with disease phenotype in the family and also population screening, the respective fragment harbouring the mutation was amplified from all family members and normal controls. An aliquot of amplicons was digested with the corresponding restriction enzymes (Table 2.6). Restriction enzymes were procured either from MBI Fermentas, Lithuania, or New England Biolabs, Inc. Beverly, MA., and all the assays were done following the manufacturer's instructions. The

fragments were separated on 8% polyacrylamide gel, stained with ethidium bromide and visualized to distinguish the wild type and the mutant alleles.

Exon	Primers used for PCR	Amplicon size (bp)	$Mgcl_2$ Used (mM)	Annealing temp (°C)
1	**1F:** 5'- CTC ATT TCC CGC TCT GGT TC –3' **1R:** 5'- AAG AGT GTG GGT GAG GGA AGT –3'	197	2.5	61.5
2	2F: 5'- TTA TCT CTC ACT CTC CAG CC –3' 2R: 5'- AAG CGA GAA GAA AGA AGC GG –3'	276	2.5	60
3	3F: 5'- TCA GAG AGC CCA TCG ACG TAT -3' 3R: 5'- CTG TTT GTG GGT TTT GAG CC -3'	193	2.5	60
4	4F: 5'- TTG GGA GTT CAG GCC TAC CT -3' 4R: 5'- GAA GTC CCA GAA AGA CCA G -3'	153	2.5	58
5	5F: 5'- CCT CTT CAC TCT GCT CTC TT -3' 5R: 5'- CAT AAT TAG CAT CGT TTA CAG TAA -3'	284	1.75	60
5A	5AF: 5'- TGA AAG TAT CAT CAT ATT TGT AG -3' 5AR: 5'- GGG AAG TGG ACA GAA AAC CA -3'	237	2.0	58
6	6F: 5'- GTG GTT TTC TGT CCA CTT CC -3' 6R: 5'- AGG AGA GAG CAT TGG GCT TA -3'	299	2.0	60
7	7F: 5'- CAG GAG ACA CTA CCA TTT GG –3' 7R: 5'- ATG CAC ATA TGG AGA GCT GC -3'	252	1.5	60
8	8F: 5'- GGG AAT GTT TTG GTG AGG CT -3' 8R: 5'- CAA AGG GCC CTG GCT AAA TT -3'	371	1.5	60
9	9F: 5'- GTA GTT CTG GCA CAA TAT GG -3' 9R: 5'- GTA CTC TGT ACA AGC ACC TC -3'	206	2.0	58
10	**10F**: 5'- GTA GAC ACA GTG CTA ACC TG –3' **10R**: 5'- CCC GGA GCA AAC AGG TTT AA -3'	243	1.75	60
11	**11F**: 5'- TTA AAC CTG TTT GCT CCG GG -3' **11R**: 5'- TTA TGC AGG CCA CCA CCA GC –3'	208	3.0	60
12	**12F**: 5'- GCT GTG TGA TGT GTT CCT CA -3' 12R: 5'- TGC AGC CTG CAG AAA CAG TG –3'	228	1.75	60
13	**13F**: 5'- CAT GTC TGT TTC TCA AAG GGA -3' **13R**: 5'- CAC CAA AAT GAA TAA AAG TTT G -3'	393	3.0	55

PCR conditions for all primer sets - initial denaturation at 94 °C - 3 min followed by (94 °C - 30 sec, Annealing for 30 sec , 72 °C - 45 sec) x 35 cycles with final extension at 72 °C for 7 minutes.

TABLE 2.5. *PAX6* primers and conditions used for PCR amplification

S.No.	Name of the candidate Gene	Mutation position/ codon change	Mutation type	Restriction enzyme for RFLP	Primer sets used for PCR
1	*CYP1B1*	376 ins A/ Ter@223	Frameshift	-*Eco* 130I	CYP1B1 set 1F/ 1R
2	*CYP1B1*	del 446 to 468 bp/ Ter@52	Frameshift	-*Bbv* C I	CYP1B1 set 1F/ 1R
3	*CYP1B1*	G 528 A/G61E	Missense	+ *Taq* I	CYP1B1 set 1F/ 1R
4	*CYP1B1*	G 689 C / A115P	Missense	-*Bsg* I	CYP1B1 set 1F/ 1R
5	*CYP1B1*	T 741G / M132R	Missense	-*Nla* III	CYP1B1 set 1F/ 1R
6	*CYP1B1*	A 777C / Q144P	Missense	-*Msp* A1I	CYP1B1 set 1F/ 1R
7	*CYP1B1*	C 923 T / P193L	Missense	+*Eco* 81I	CYP1B1 set 2F/ 2R
8	*CYP1B1*	G 959 A / E229K	Missense	-*Earn* 11o4I	CYP1B1 set 2F/ 2R
9	*CYP1B1*	del 1409 & 1410 /Ter@ 373	Frameshift	-Tag I	CYP1B1 set 3F/ 3R
10	*CYP1B1*	G 1449 A/ R368H	Missense	-*Taa* I	CYP1B1 set 3F/ 3R
11	*CYP1B1*	C 1514T/ R390C	Missense	-*Hin* 6I	CYP1B1 set 3F/ 3R
12	*MYOC*	C 1109 T/ P370L	Missense	+*Alw* NI	MYOC 10F/10R

+ : Gain of Restriction Enzyme site , - : Loss of Restriction Enzyme site.

TABLE 2.6. PCR-RFLPs used to screen for various *CYP1B1* & *MYOC* mutations

Chapter 3
Results and Discussion

3.0 RESULTS AND DISCUSSION

3.1. Mutational analysis of *CYP1B1*

3.1.1 Identification of pathogenic mutations in *CYP1B1* by direct sequencing

Direct sequencing and sequence analysis of the complete coding region of *CYP1B1* gene in 106 patients from 100 pedigrees revealed 14 pathogenic mutations in 53 patients belonging to 45 pedigrees (Table 3.1). Of the 14 mutations, nine pathogenic mutations were found in exon II and five in exon III. These include frameshift, missense and compound heterozygous mutations. Most of these amino acids which are mutated in the patients screened are highly conserved across species among cytochrome P450s (Table 3.2). We also found that none of these mutations were seen in the normal controls.

The mutations identified in this study are schematically shown in Figure 3.1 to 3.17, along with the relevant electropherograms and PCR-RFLP data. Figure 3.14 provides a schematic view of the domain arrangement in human CYP1B1. We discuss the various mutations below

S.NO.	Pedigree	Consan / non-consan	Bilateral / Unilateral	Sex Male/ Female	Sporadic/ Familial	Mutation	SNP	Promoter region variation
1	001A	C	B	F	f	P193L* & E229K*	1,2,3,5, 6	
2	001b	C	B	F	f	P193L*	1,5,6,8	
3	002A	C	B	M	S	R368H	1	
4	003A	C	B	M	S		2,3,5,6	
5	004A	C	B	M	f	Ins A 376*	2,3,5,6	
6	004D	C	B	F	f	Ins A 376*	2,3,5,6	
7	005A	C	B	F	f	R368H & R390C	5,6	
8	005c	C	B	M	f	R390C*		
9	006A	C	B	F	f	R368H	4	
10	006D	C	B	F	f	R368H	4	
11	007A	C	B	M	S		2,3,5,6	
12	008A	C	B	F	S	A115P*		
13	009A	C	B	M	S	G466D*		
14	010A	C	U	M	S		2,3,5,6	
15	011A	C	B	F	S	G61E		
16	012A	C	B	F	f	R390C*		
17	012D	C	B	M	f	R390C*		
18	013A	C	B	M	S		2,3,5,6	
19	014A	C	B	M	S			
20	015A	NC	B	F	S		5,6,7	
21	016A	C	B	M	f		2,3,5,6	
22	016c	C	B	M	f		2,3,5,6	
23	017A	NC	B	M	S	R368H		
24	018A	C	B	F	S	R390C*		
25	019A	NC	B	F	S		2,3,5,6, 7	
26	020A	C	B	F	S		2,3,5,6	c 3078 t
27	021A	C	B	M	S	S239R*	5,6	
28	022A	C	B	F	S	R368H		
29	023A	C	B	M	S		2,3,5,6	
30	024A	C	B	M	S	E229K	2,3,5,6	
31	025A	NC	B	M	S	R368H	4,5	
32	026A	NC	B	F	S		5,6	
33	027A	C	B	F	f		5,6	
34	027D	C	B	M	f		5,6	
35	028A	C	B	F	S		5,6	
36	029A	NC	B	M	S		2,3	
37	030A	C	B	M	S		5,6	
38	031A	NC	B	M	f		2,3	
39	031D	NC	B	F	f		2,3	
40	032A	NC	U	M	S		2,3,5,6	
41	033A	C	B	F	S			c 3078 t
42	034A	C	B	M	S		5,6	
43	035A	C	B	F	f	R368H	5,6	
44	035b	C	B	F	f	R368H		
45	036A	C	B	M	S		2,3	
46	037A	C	B	F	S	E229K'	5,6	
47	038A	C	U	F	S			
48	039A	NC	B	M	S	R368H	5,6	
49	040A	C	B	M	S	R368H		
50	041A	C	B	M	S		2,3,5,6	
51	043A	C	B	F	S		5,6	
52	044A	NC	B	M	S		2,3,5,6	
53	045A	C	B	F	S		2,3,5,6	
54	046A	NC	U	M	S		5,6	
55	047A	NC	B	F	S		2,3,5,6	
56	048A	NC	B	F	S		2,3,5,6	

57	049A	NC	B	F	S		2,3,5,6	
58	050A	C	B	F	S	Del 446 – 468*		
59	051A	NC	B	M	S	R368H		
60	052A	NC	U	F	S			
61	053A	NC	B	F	S			
62	055A	C	B	M	S			
63	056A	C	B	F	S			
64	057A	C	B	M	S	*E229K'*	*2,3*	
65	058A	C	B	F	S	G61E		
66	059A	NC	B	M	S			
67	059A	NC	B	F	S			
68	060A	NC	B	M	S			
69	061A	C	U	M	S			
70	062A	NC	B	M	S			
71	063A	NC	U	F	S			
72	064A	NC	B	M	S			
73	066A	NC	U	M	S			
74	067A	C	B	M	S	R368H		
75	069A	NC	B	M	S	*P193L'*		
76	070A	NC	B	M	S		*5,6*	
77	071A	NC	B	F	f	R368H		
78	071D	NC	B	F	f	R368H		
79	072A	NC	B	F	S			
80	073A	NC	B	M	S			
81	075A	NC	B	F	S	*R368H*		
82	076A	C	B	F	S	R368H		
83	077A	C	B	F	S			
84	078A	C	B	M	S			
85	079A	NC	B	M	S	R368H	*5,6*	
86	081A	C	B	M	S	Del 1409 - 1410'	2,3,5,6	
87	083A	NC	U	F	S		5,6	*c 3078 t*
88	084A	NC	U	F	S		2,3,5,6	
89	085A	NC	U	F	S		2,3,5,6	
90	086A	NC	B	M	S	*Q144P**	2,3,5,6	
91	087A	NC	B	M	S		*5,6*	
92	088A	NC	U	M	S			
93	089A	C	B	F	S		*2,3*	
94	090A	NC	B	M	S		2,3	
95	091A	NC	B	M	S		2,3,5,6	
96	092A	NC	B	F	S	R390C*	5,6	
97	093A	C	B	M	f	G61E		
98	093D	C	B	F	f	G61E		
99	094A	C	B	M	S	P437L	2,3,5,6	
100	094D	C	B	M	S	P437L	2,3,5,6	
101	095A	NC	B	M	S	*R368H*		
102	096A	C	B	M	S		2,3	
103	097A	NC	B	M	S		5,6	
104	098A	C	B	M	S			
105	099A	NC	U	M	S		*2,3,5,6*	
106	100A	C	B	M	S	*R368H*	*5,6*	
107	101A	C	B	M	S			
108	102A	NC	B	F	S			
109	103A	NC	B	M	S			
110	104A	NC	B	F	S			
111	105A	C	B	M	S			
112	107A	C	B	M	f			
113	107D	C	B	F	f			
114	108A	C	B	M	S			
115	109A	C	B	M	S			
116	110A	C	B	M	S			
117	112A	C	U	M	S			
118	114A	NC	U	F	S			
119	115A	C	B	M	S			
120	116A	C	B	F	S	*E229K'*		

121	117A	NC	B	F	S			
122	118A	NC	B	F	S			
123	121A	NC	B	M	S			
124	122A	NC	B	M	f	M132R*	5,6,7	
125	122D	NC	B	M	f	M132R*	5,6,7	
126	123A	NC	B	M	S			
127	124A	NC	U	M	S			
128	125A	NC	B	F	S	*E229K'*		
129	126A	NC	B	M	S			
130	128A	NC	B	M	S			
131	129A	NC	B	F	S			
132	130A	NC	B	M	S	R368H		
133	132A	C	B	F	S			
134	133A	C	B	F	S			
135	134A	NC	U	F	S			
136	135A	NC	B	F	S			
137	136A	NC	B	M	S	R368H		
138	137A	NC	B	F	S	R368H		
139	140A	C	B	F	S			
140	142A	C	B	M	S			
141	143A	NC	B	F	S	R390C*		
142	144A	NC	B	M	S	R368H		
143	145A	C	B	F	S			
144	146A	NC	U	F	S			
145	147A	C	B	F	S			
146	148A	NC	B	M	S			
147	150A	NC	B	M	S	R368H		
148	151A	NC	B	M	S			
149	152A	NC	B	M	S			
150	153A	C	U	M	S			
151	155A	C	B	F	S			
152	156A	C	B	F	S			
153	157A	NC	B	M	S			
154	165A	NC	B	M	F	*P370L**	2,3,5,6,8	
155	165c	NC	B	M	F	*P370L**	2,3,5,6	

A:	proband	b:	mother	c:	father
D:	probands sibling	C:	consanguineous	NC:	non-consanguineous
U:	unilateral	B:	Bilateral	M:	male
F:	female	S:	sporadic	f:	familial
Italics:	heterozygous	*:	*MYOC* mutation and the rest are *CYP1B1* mutations		
1:	t-13c variation	2:	c488g	3:	g701t
4:	g896a	5:	g1640c	6:	t1693c
7:	a1704g	8:	ins1339ggc in *FOXC1*		
*:	Novel mutations				

Table 3.1. PCG patients showing the detailed demographic data with mutations and SNPs identified in this study

376 ins 'a' (Frameshift mutation): A homozygous insertion of the nucleotide adenine (A) at cDNA position 376 in two patients from PCG004 resulted in a frameshift that truncated the open reading frame (ORF) by creating a premature stop codon (TGA). A truncated 222 amino acid (aa) protein lacking 321 aa from the C-terminus was generated. Only the first 10 aas at the N-terminus were identical in the mutant and the wild type protein, and the frameshift eliminated all CYP1B1 domains, resulting in a functional null allele. This also abolished the restriction site for *Eco* 130I in exon II (Figure. 3.1).

Del 446 to 468 (Frameshift mutation): A homozygous deletion of 23 base pairs (bp) at cDNA position 446 to 468 in a PCG050 proband resulted in a frameshift that truncated the ORF by creating a premature stop codon (TGA). This resulted in a truncated 51 aa long protein, with 492 aa missing from the C-terminus. This major deletion happens to fall at the end of the membrane domain, causing the elimination of all important domains in CYP1B1 protein. This deletion abolished a restriction site for *Bbv* CI in Exon II (Figure. 3.2).

g528a / G61E (Missense mutation): A homozygous substitution of guanine at cDNA position 528 by adenine, causing a change of glycine 61 to glutamic acid, was observed in 4 patients from 3 pedigrees (PCG011, PCG058, PCG093). The glycine residue at this position is highly conserved

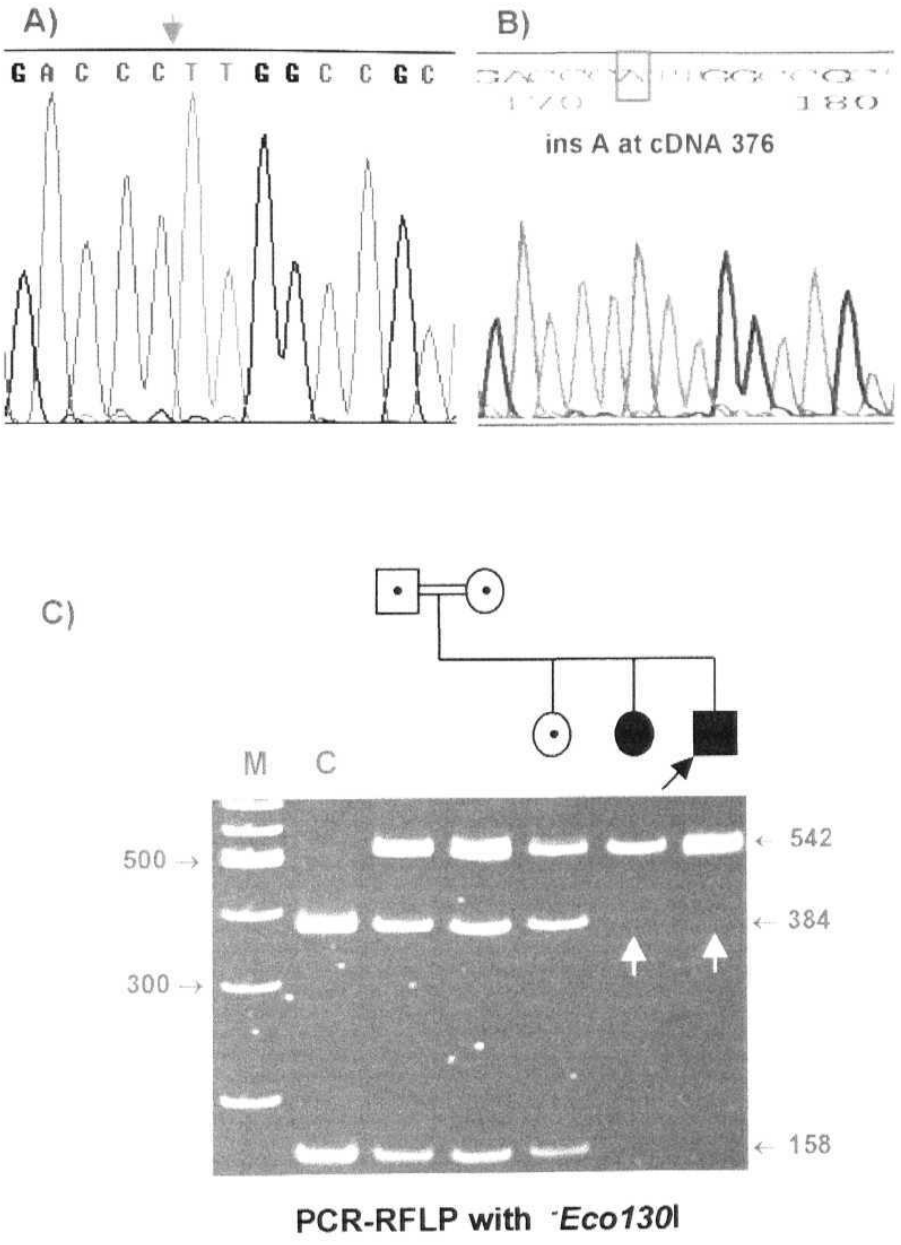
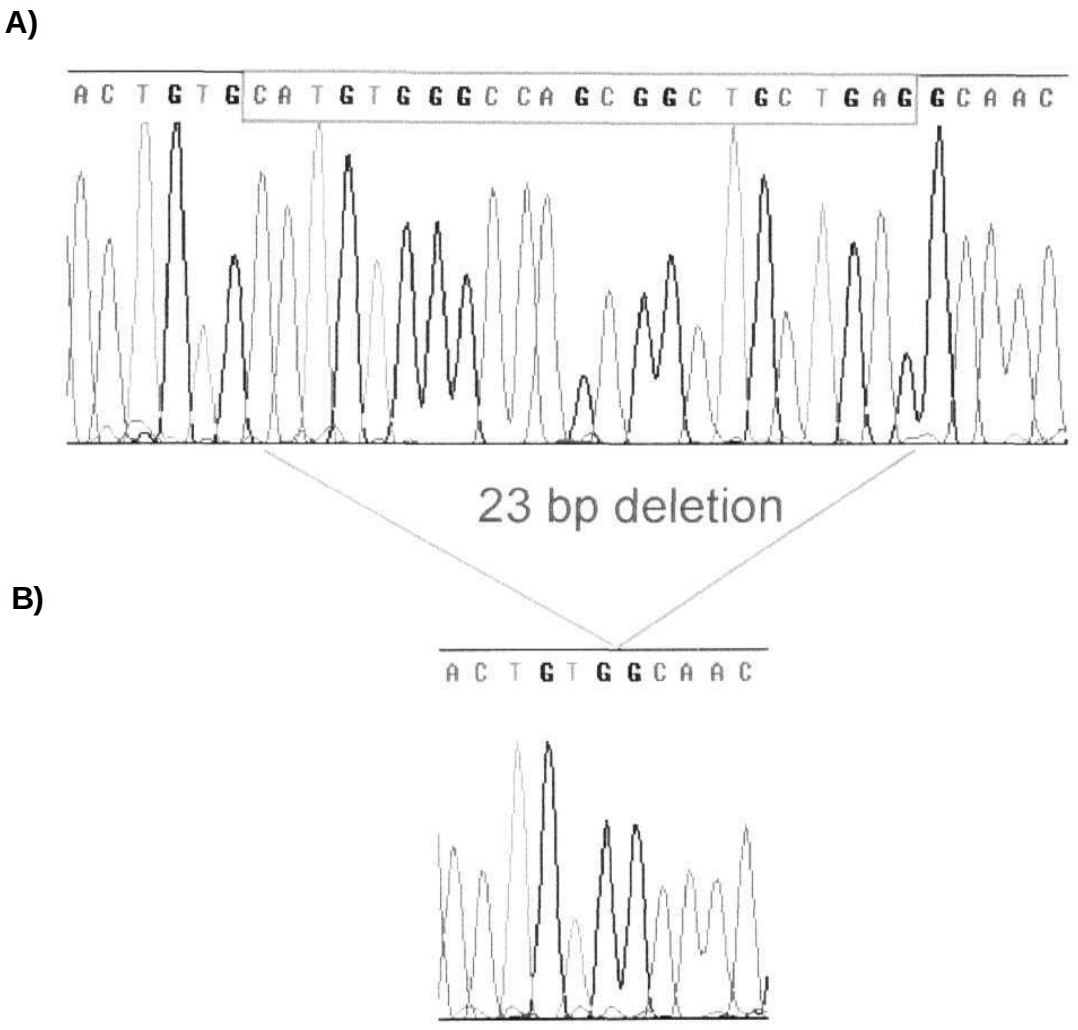


Figure 3.1 PCG 004 proband showing cDNA 376 ins A

A) Electropherogram of control

B) Electropherogram of PCG 004 Proband

C) PCR-RFLP showing the segregation of mutant alleles ' ↑ ' in the PCG 004 pedigree



cDNA 446 to 468 del; ter @ 52aa

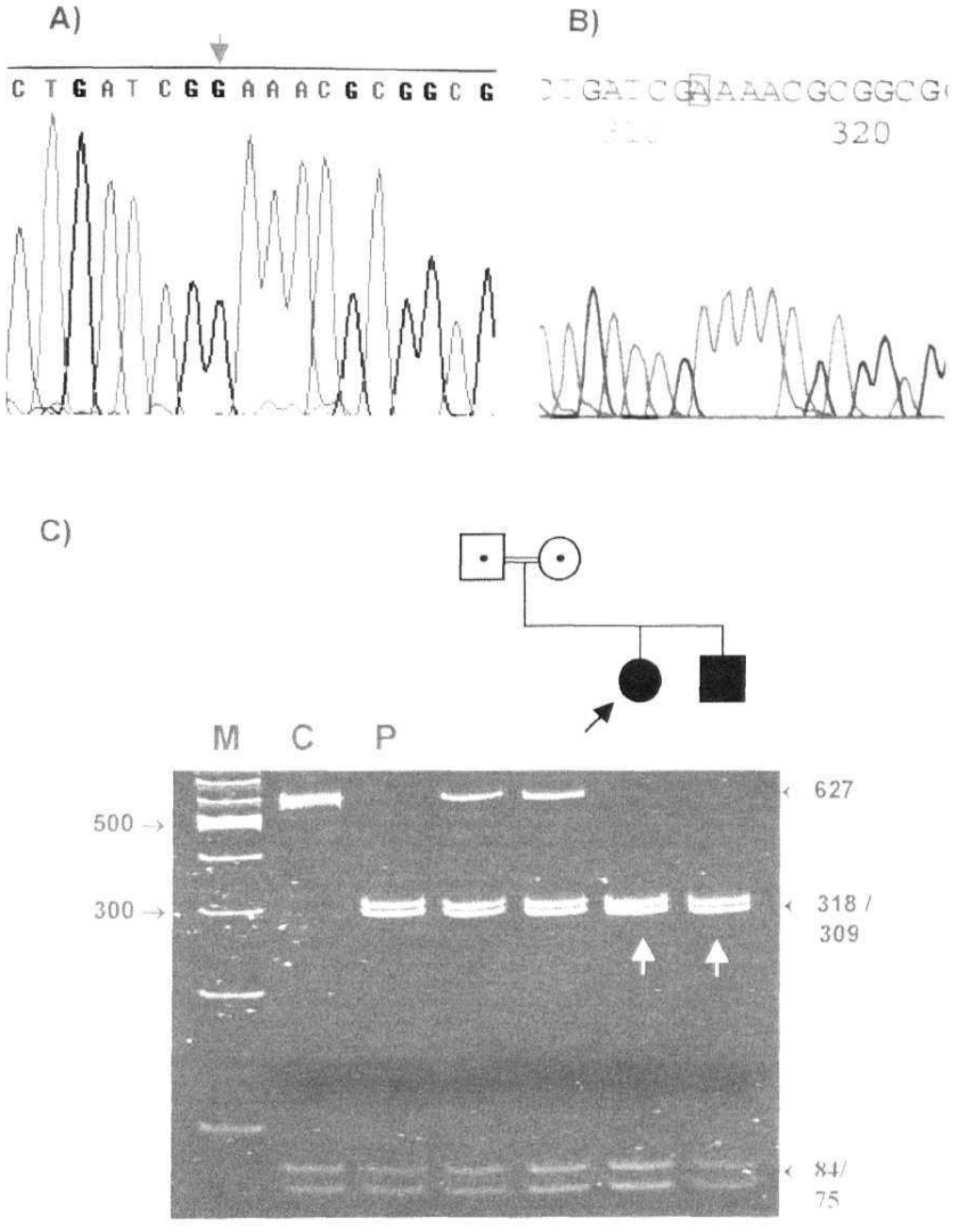
Figure 3.2. PCG 050 proband showing cDNA 446 to 468 deletion

A) Electropherogram of Normal

B) Electropherogram of PCG 050 proband

across cytochrome P450 species (Table 3.2). This mutation occurs in the C-terminal proline rich region which connects the membrane bound region and the cytosolic C-terminal domain of the protein (Figure 3.14), thereby thought to act as a hinge. Stoilov et al (1998) described the effect of substitution of G61E in restricting the flexibility of the hinge. Achary et al (unpublished data) hypothesize that the catalytic domain of CYP1B1 probably acts as a scavenger of toxic molecules and therefore the flexibility at the hinge is required in order to move the catalytic domain freely around the cytosol so as to capture these molecules. Restriction in the movement of the catalytic domain may impair its binding efficiency and the electron transfer process. This variant was also reported elsewhere (Bejjani et al 1998; Stoilov et al 1998; Bejjani et al 2000), and causes the gain of restriction site for *Taq* I in exon II (Figure 3.3).

g689c / A115P (Missense mutation): A homozygous substitution of guanine at cDNA position 689 by cytosine which causes a change of alanine 115 to proline was observed in the proband of the family PCG008. This mutation occurs in the N-terminal region of the cytosolic domain, between the B helix (100aa -107aa) and C helix (141aa - 151aa)(Figure 3.14). Though this residue is not conserved across species, it is absent in the normal population and segregates with the disease phenotype. The study by Yamazaki et al (1993) has shown the importance of proline residues in the proline rich region of microsomal cytochrome P450s. In their study, wild type



PCR-RFLP with Tag I [g528a(G61E)]

Figure 3.3. PCG 093 proband showing 528g→a (G61E)

A) Electropherogram of control

B) Electropherogram of PCG 093 Proband

C) PCR-RFLP showing the segregation of mutant alleles ' ↑ ' in the PCG 093 pedigree

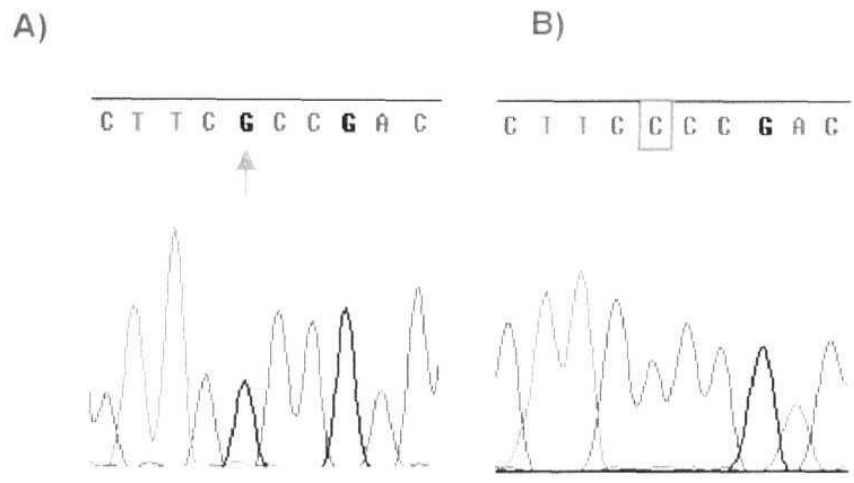


Figure 3.4. PCG 008 proband showing g689c (A115P)

A) Electropherogram of control

B) Electropherogram of PCG 008 Proband

P450 showed the typical carbon monoxide difference spectrum, whereas the mutant protein (substitution of proline by alanine) did not show the characteristic P450 spectrum. Taking this into consideration, we suspect that the change of alanine to proline might also cause a final conformational change of the protein due to the extra proline residue. This mutation also causes the loss of restriction site for *Bsg* I in exon II (Figure 3.4).

t741g / M132 R (Missense mutation): A homozygous substitution of thymine at cDNA position 741 by guanine causes the chage of methionine 132 to arginine. This mutation was observed in the proband of PCG122, and is located in the region between B & C helices of the CYP1B1 protein. Comparative modeling of CYP1B1 using CYP2C5 as a template has shown the possibility of hydrogen bond formation with main chain amide by this residue (Achary et al). This mutation could disrupt this hydrogen bond formation and may affect the final conformation of the protein. Though this is also not conserved across various cytochrome P450 species, the variation is absent in normal controls and it segregates with the disease phenotype. This mutation causes loss of the restriction site for *Nla* III in exon II (Figure 3.5).

a777c / Q144P (Missense mutation): A heterozygous substitution of alanine at cDNA position 777 by cytosine, which causes a change of glutamine 144 to proline, was observed in PCG086 proband. This mutation lies in the C helix (141aa-151aa) region of the protein (Figure 3.14). Insertion

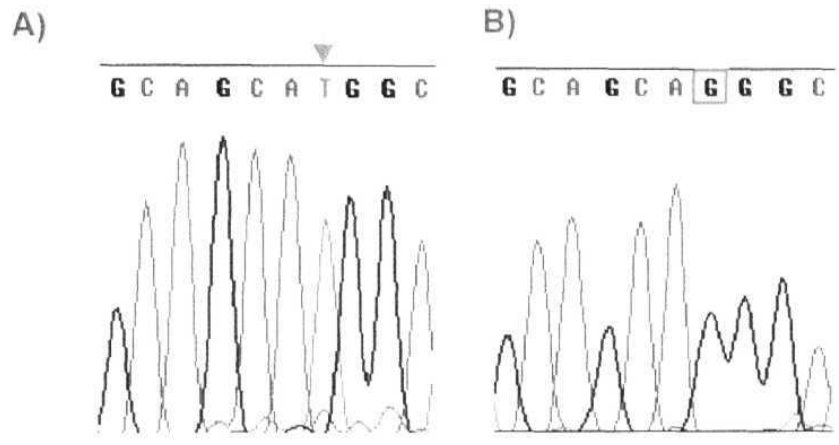


Figure 3.5. PCG 122 proband showing t741g (M132R)

A) Electropherogram of control

B) Electropherogram of PCG 008 Proband

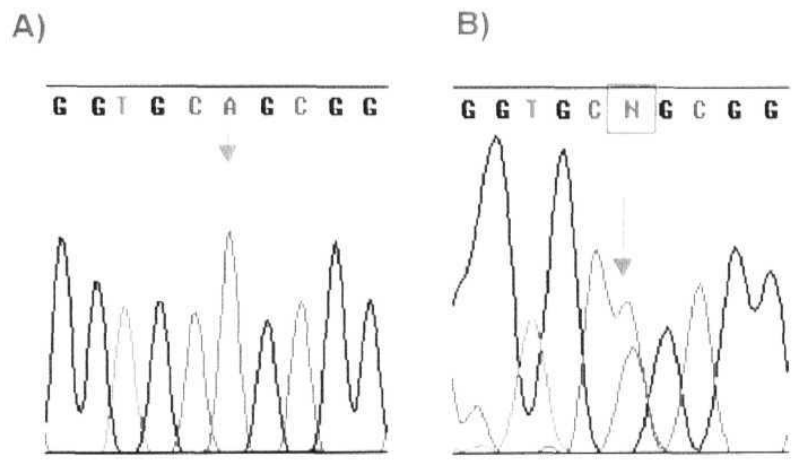


Figure 3.6. PCG 086 proband showing a777c (Q144P)

A) Electropherogram of control

B) Electropherogram of PCG 086 Proband

of proline in the middle of the C helix could induce a turn in the helix, which might affect the final conformation of protein. Though this residue is not conserved across the various cytochrome P450s, the variation was not observed in normal controls. This mutation causes loss of restriction site for *Msp* A1I in exon II (Figure 3.6).

c923t / P193L (Missense mutation): A heterozygous substitution of cytosine at cDNA position 923 by thymine causes the change of proline 193 to leucine, was observed in 3 patients from 2 pedigrees (PCG001, PCG069). Proline at this position is completely conserved in the cytochrome P450 across species (Table 3.2). This mutation occurs at the N cap position of E helix (aa193 - aa 209)(Figure 3.14). The wild type P193 is involved in hydrogen bond O(P193)..OGI(T197) formation. This mutation would cause a loss of this hydrogen bond formation and could also generate other interactions such as hydrophobic contacts and salt bridges. Molecular simulation studies by Achary et al, show the helical (cis conformation) conformation of P193 to change to β-conformation because of leucine at this position. This mutation also causes gain of restriction site for *Eco* 81I in exon II (Figure 3.8B).

g959a / E229K (Missense mutation): A heterozygous substitution of guanine at cDNA position 959 by adenine, causing a change of glutamic acid 229 to lysine, was observed in probands of 4 pedigrees. This residue is

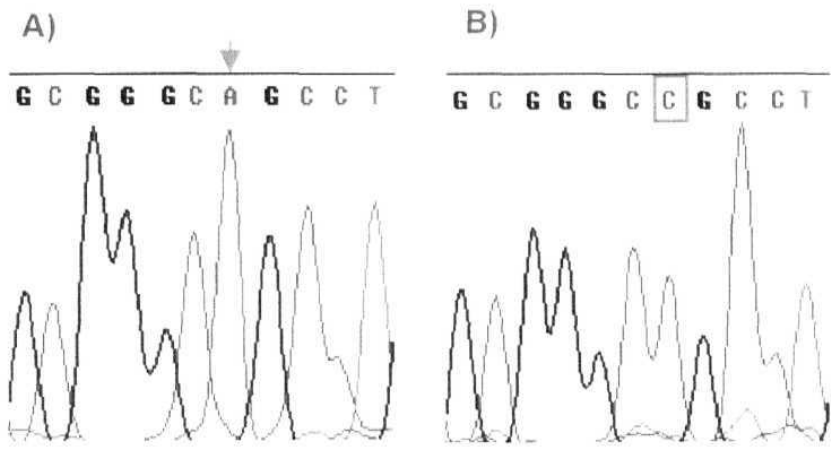


Figure 3.7. PCG 021 proband showing a1061c (S239R)

A) Electropherogram of control

B) Electropherogram of PCG 021 Proband

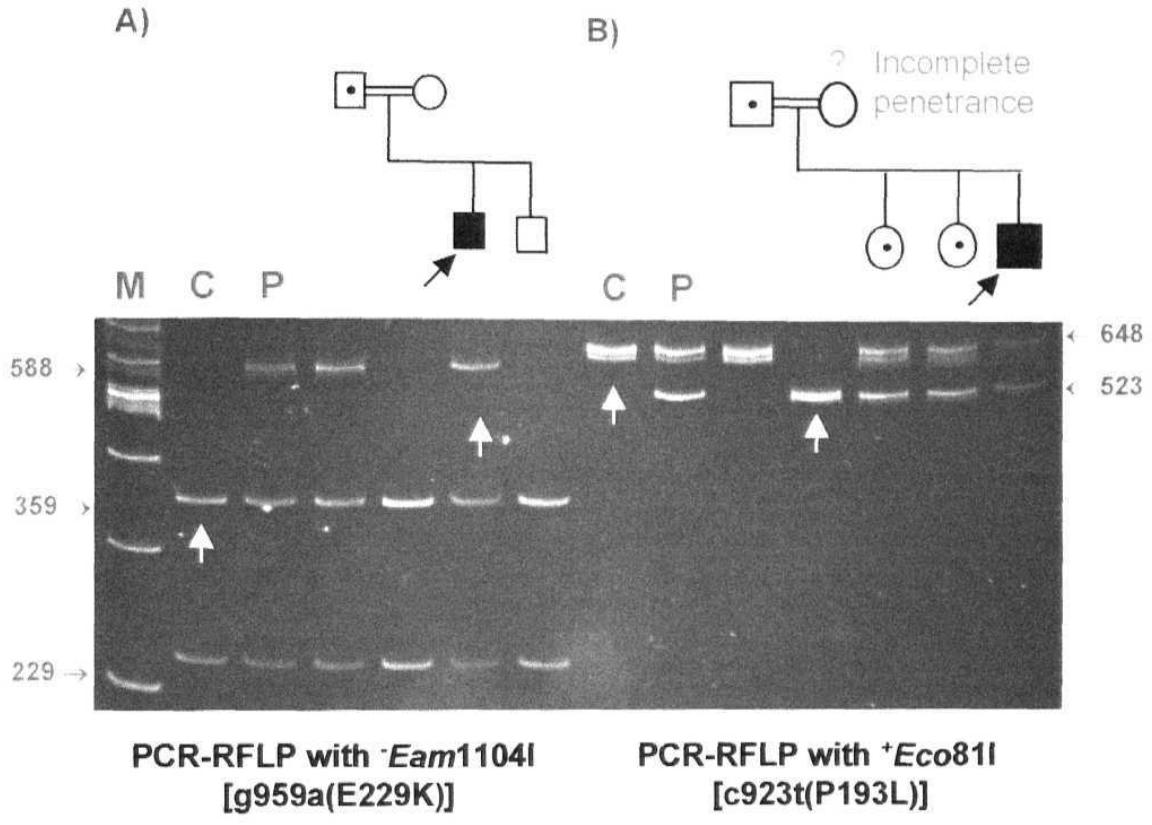


Figure 3.8. PCG 057 (A) and PCG 069 (B) pedigrees showing the segregation of mutant alleles '↑'

A) PCR-RFLP for E229K mutation in PCG 057 pedigree

B) PCR-RFLP for P193L mutation in PCG 069 pedigree showing the incomplete penetrance for the PCG phenotype

conserved in three known CYP1B1 species, and lies in the C-terminal region of the F-helix (219aa - 234aa)(Figure 3.14). About 12.8% of the normal population was also found to be heterozygous for this variation raising the doubt whether it is pathogenic. Further investigations by homozygosity mapping for this single sequence change from the same ethnic background might explain the nature of this variation. But this variation was found to be segregating with the disease phenotype and has also been reported earlier to be a pathogenic mutation (Michels-Rautenstrauss et al 2001). It also causes the loss of restriction site for *Earn* 1104I in exon II (Figure 3.8A).

a1061c / S239R (Missense mutation): A homozygous substitution of adenine at cDNA position 1061 by cytosine causes a change of serine 239 to arginine, was observed in the proband of family PCG021 (Figure 3.7). This residue is conserved in all the three CYP1B1 species (Table 3.2), and occurs between the F (219aa - 234aa) and G (259aa - 281 aa) helices of CYP1B1 protein. This variation mutation also has shown to be segregating with the disease phenotype.

Del 1409 & 1410, 'cg' (Frameshift mutation): A homozygous deletion of two nucleotides 'cg' at cDNA position 1409 & 1410 in PCG081 proband resulted in a frameshift that truncated the ORF by creating a premature stop codon (TGA) (Figure 3.9). This resulted in a truncated 373aa long protein, missing 170 aa from the C-terminus. This deletion lies in the J helix (350aa -

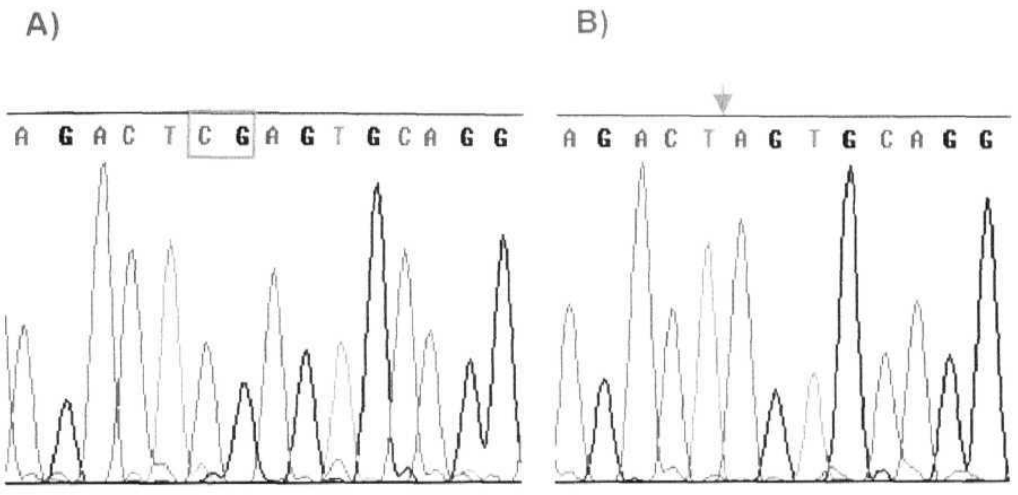


Figure 3.9. PCG 081 proband showing 2bp deletion at cDNA 1409 & 1410 nucleotides

A) Electropherogram of control

B) Electropherogram of PCG 081 Proband

363aa) and terminates before the K helix (378aa - 392aa) of the CYP1B1 protein (Figure 3.14). The resultant protein therefore lacks helices K, L and the heme binding region. This mutation causes the loss of restriction site for *Taq* I in exon III.

g1449a / R368H (Missense mutation): A homozygous substitution of guanine at cDNA position 1449 by adenine, that causes a change of arginine 368 to histidine, was observed in 20 PCG patients from 17 pedigrees (Table 3.1). This residue is completely conserved in the protein across various species (Table 3.2). This variation was absent in normal population and segregates with the disease phenotype. It falls in the meander region of the protein, connecting the two helices J (350aa - 363 aa) and K (378aa - 392aa) of the CYP1B1 protein (Figure 3.14). This mutation also has been reported elsewhere (Bejjani et al 2000; Vincent et al 2002). This mutation causes the loss of restriction site for *Taa* 1 in exon III (Figure 3.10).

c1514t / R390C (Missense mutation): A homozygous substitution of cytosine at cDNA position 1514 by thymine, causing a change of arginine 390 to cysteine was observed in 6 PCG patients from 4 pedigrees (PCG005, PCG012, PCG018, PCG092). This residue in CYP1B1 is highly conserved across species (Table 3.2), absent in normal population, and segregates with the disease phenotype. This mutation occurs at the C-terminal region of helix K (378aa - 392aa) of CYP1B1 protein (Figure 3.14). Mutations in this residue

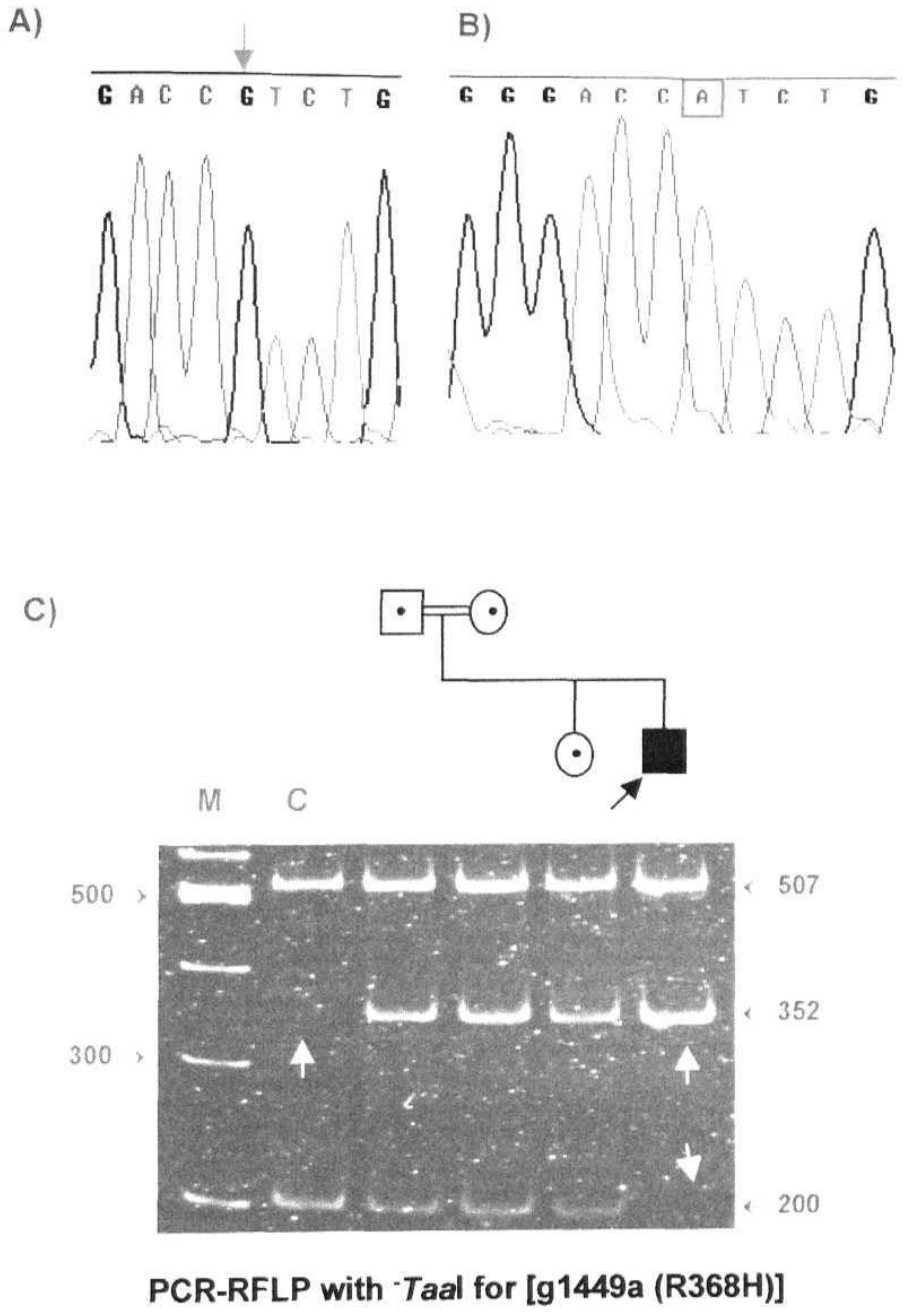


Figure 3.10. PCG 002 proband showing 1449g→a (R368H)

A) Electropherogram of control

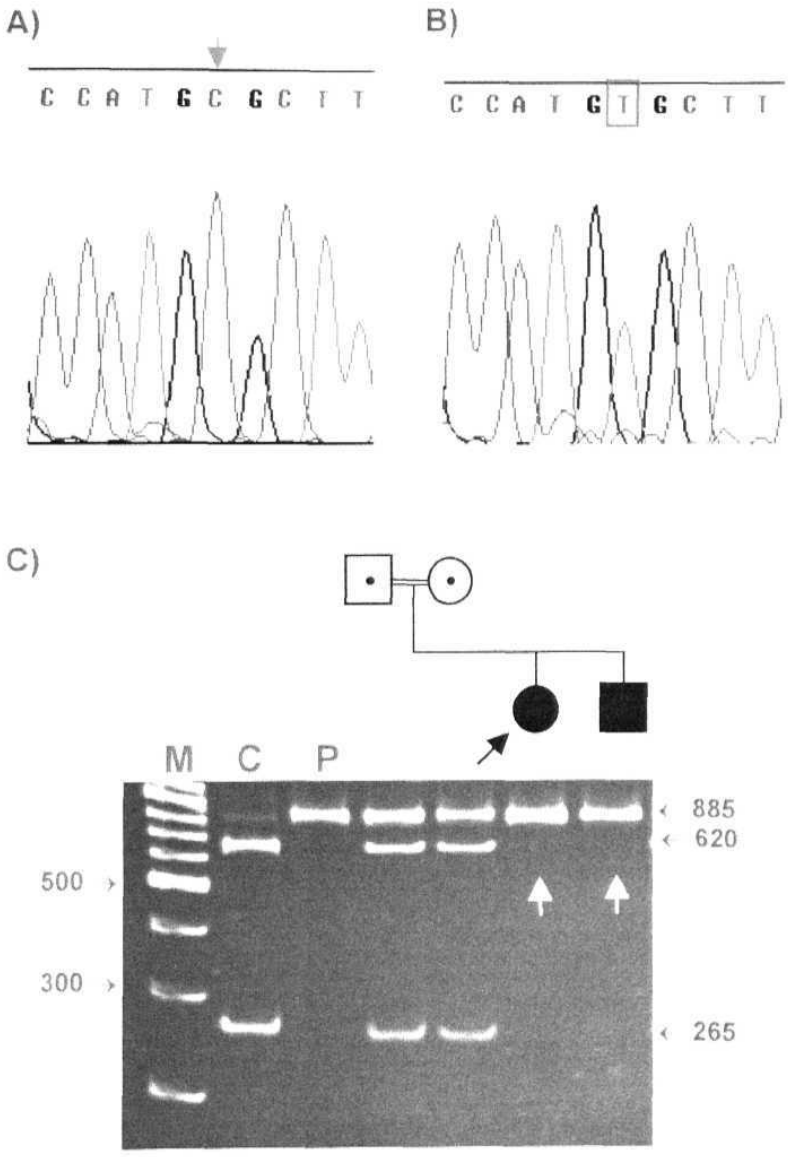
B) Electropherogram of PCG 002 Proband

C) PCR-RFLP showing the segregation of mutant alleles '↑↓' in the PCG 002 pedigree

have been reported earlier where arginine is changed to either histidine or serine (Stoilov et al1998; Plasilova et al 1999). Here we observed the change of arginine to cysteine at the same codon position. It also causes the loss of restriction site for *Hin* 6I in exon III (Figure 3.11).

c1656t / P437L (Missense mutation): A homozygous substitution of cytosine at cDNA position 1656 by thymine that causes a change of proline 437 to leucine was observed in the PCG094 proband (Figure 3.12). This residue is highly conserved across the species (Table 3.2), absent in the normal population and found to be segregating with the disease phenotype. This mutation has also been reported elsewhere (Bejjani et al 1998; Stoilov et al 2002), and lies between the K helix (378aa - 392aa) and L helix (473aa to 487aa)of CYP1B1 protein.

g1742a / G 466 D (Missense mutation): A homozygous substitution of guanine at cDNA position 1742 by adenine, causing a change of glycine 466 to aspartic acid was observed in the PCG009 proband. This residue is highly conserved across the species (Table 3.2) and is part of the 'signature sequence' (NH_2- FXXGXXXCXG -COOH). The signature sequence is present in all heme binding cytochromes at the C-terminus of the protein and the cysteine residue in this sequence acts as the fifth ligand to heme (Figure 3.13). The other conserved residues and hydrophobic amino acids like phenylalanine and glycine next to the axial ligand (cysteine) are very



PCR-RFLP with *HhaI* [c1514a(R390C)]

Figure 3.11. PCG 012 proband showing c1514a (R390C)

A) Electropherogram of control

B) Electropherogram of PCG 012 Proband

C) PCR-RFLP showing the segregation of mutant alleles '↑' in the PCG 012 pedigree

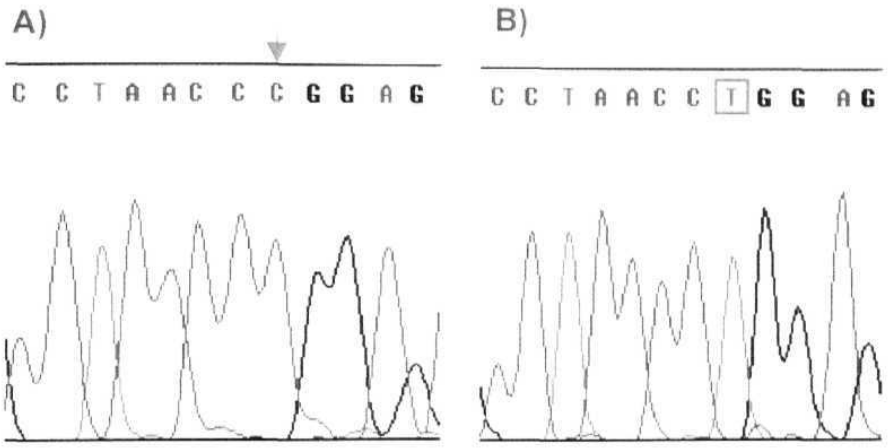


Figure 3.12. PCG 094 proband showing c1656t (P437L)

A) Electropherogram of control

B) Electropherogram of PCG 094 Proband

important for the apoprotein to hold and/or incorporate the heme plane at the active site of P450. Site directed mutagenesis of glycine to glutamic acid at the same position in rat liver cytochrome $P450_d$ showed lower absorption intensities of CO-bound forms of the mutant protein compared to the normal protein (Shimizu et al 1988).

In PCG001, proband mutations P193L / E229K and in PCG005 proband missense mutations R368H / R390C were observed as compound heterozygous mutations. Both these pedigrees are showing parent to child transmission of phenotype (pseudo-dominant pattern of inheritance). Segregation of two independent mutant alleles and consanguinity seen in these pedigrees might increase the probability of occurrence of phenotype in these pedigrees.

3.1.2. Identification of single nucleotide polymorphism in *CYP1B1*

In addition to the earlier mentioned pathogenic mutations, we identified seven single nucleotide polymorphisms (SNPs) (Table 3.1). Among these polymorphisms, c488g (R48G), g701t (A119S) in exon II and g1640c (V432L), t1693c (D449D) in exon III were seen predominantly in PCG patients (Table 3.3). These polymorphisms have also been reported widely in all ethnic populations studied. The two polymorphisms of exon II (c488g and g701t) and two polymorphisms of exon III (g1640c and t1693c) were found to

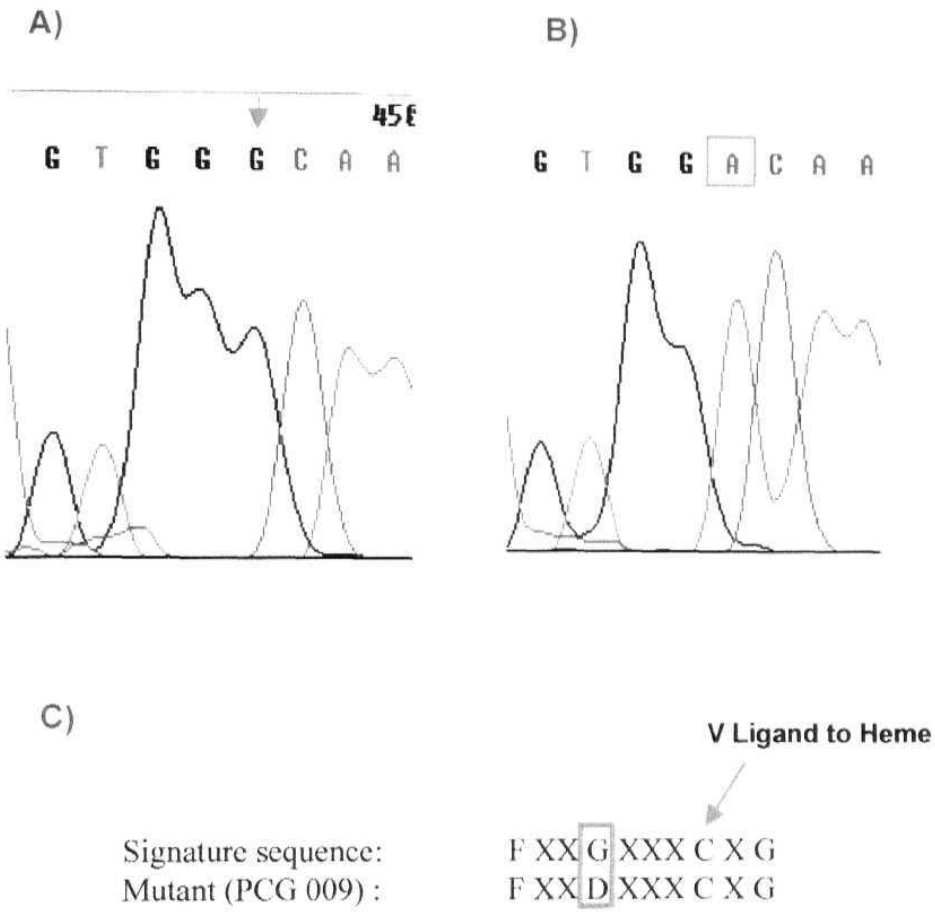


Figure 3.13. PCG 009 proband showing 1742g→a (G466D)

A) Electropherogram of control

B) Electropherogram of PCG 009 Proband

C) Signature sequence showing the mutated residue

Name of the Helix	Region in CYP1B1
A- Helix	74aa - 80aa
B-Helix	100aa-107aa
C-Helix	141aa - 151aa
D-Helix	166aa-183aa
E-Helix	193aa-209aa
F-Helix	219aa-234aa
G-Helix	259aa - 264aa
H-Helix	272aa – 281aa
I-Helix	292aa - 298aa
J-Helix	319aa – 363aa
K-Helix	379aa - 392aa
L-Helix	473aa-487aa

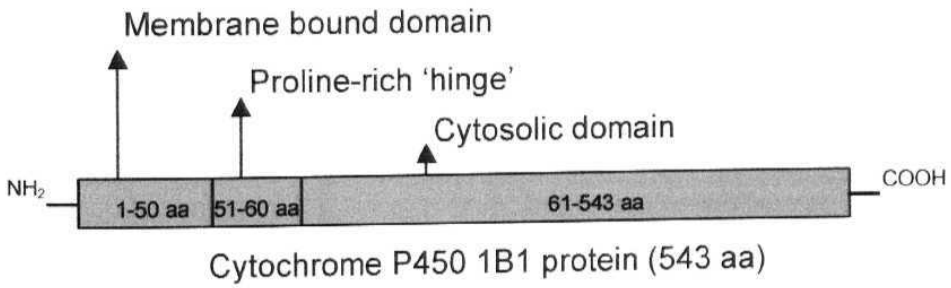


Figure 3.14. A schematic view of domain arrangement in human CYP1B1 sequence

CYP1A1 guanine pig	NH_2--G---	-M--	---R---	---P---	---N---	---N---	---R---	---R---	---P---	---G----COOH
CYP1A1 human	NH_2--G---	-M--	---R---	---P---	---N---	---N---	---R---	---R---	---P---	---G----COOH
CYP1A1 mouse	NH_2--G---	-M--	---R---	---P---	---N---	---Y---	---R---	---R---	---P---	---G----COOH
CYP1A1 trout	NH_2--G---	-L--	---R---	---P---	---D---	---N---	---R---	---R---	---P---	---G----COOH
CYP1A2 human	NH_2--G---	-L--	---R---	---P---	---H---	---N---	---R---	---R---	---P---	---G----COOH
CYP1A2 trout	NH_2--G---	-L--	---R---	---P---	---D---	---N---	---R---	---R---	---P---	---G----COOH
CYP1B1 human	NH_2--G---	-M--	---Q---	---P---	---E---	---S---	---R---	---R---	---P---	---G----COOH
CYP1B1 mouse	NH_2--G---	-L--	---Q---	---P---	---E---	---S---	---R---	---R---	---P---	---G----COOH
CYP1B1 rat	NH_2--G---	-L--	---R---	---P---	---E---	---S---	---R---	---R---	---P---	---G----COOH
CYP2A7 human	NH_2--G---	-V--	---L---	---P---	---L---	---E---	---R---	---R---	---P---	---G----COOH
	61G→S	**132M→R**	**144Q→S**	**193P→L**	**229E→K**	**239S→R**	**368R→H**	**390R→C**	**437P→L**	**466G→D**

Table 3.2. Multiple sequence alignment showing the conservation of aa showing the missense mutation residues identified in this study

Allele type	c488g / R 48 G	g701t / A119S	g1640c / V 432 L	a1704g/ D 449 D
Wilt type allele	42.6%	42.6%	49.1%	49.1%
Heterozygous mutant	32.7%	32.7%	31.1%	31.1%
Homozygous mutant	24.5%	24.5%	19.6%	19.6%

Table 3.3. Frequency of predominant polymorphism seen in this study patients

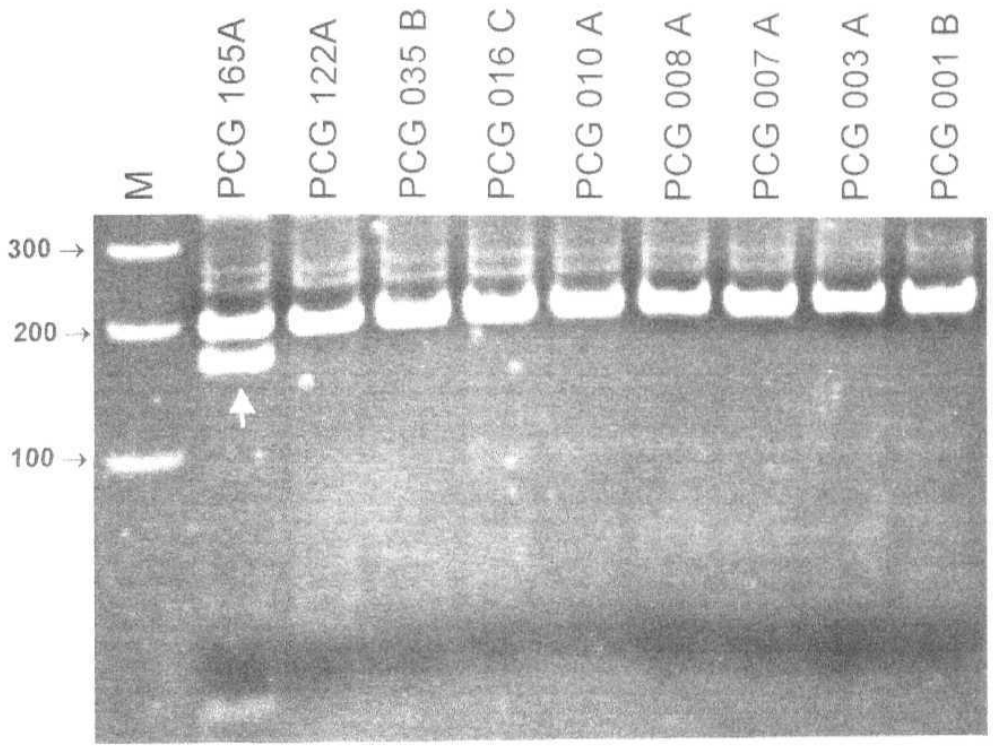
segregate together in all the PCG cases studied, but exon II polymorphisms and exon III polymorphisms were seen to be segregating independently.

3.2. Direct sequencing of *MYOC*

Direct sequencing of coding region of *MYOC* in two unrelated PCG patients who showed single heterozygous mutation in *CYP1B1* (PCG 01b & PCG035b) and 8 others who had PCG but did not show any mutation in the coding region of *CYP1B1* (probands of families from PCG 003, 007, 008, 010, 013, 014, 016, 165) revealed one heterozygous missense mutation in PCG165 proband. It involved the substitution of cytosine 1109 by thymine causing a change of proline 370 to leucine. This mutation has also been associated with an Indian primary open angle glaucoma patient (Mukhopadhay et al 2002). This mutation lies in exon III and results in loss of restriction site for *Alw* NI (Figure 3.15). PCR-RFLP analysis for this mutation revealed the presence of same mutation in affected father.

3.3. Direct sequencing of *FOXC1*

We next did direct sequencing of the complete coding region of *FOXC1* in two patients who showed single heterozygous mutation in *CYP1B1* and 8 others who had PCG but did not show any mutation in the coding region of *CYP1B1*. The sequence showed no pathogenic mutation. However,



PCR-RFLP with $^{+}$*Alw* NI for c1109t (P 370 L)

Figure 3.15. PCR-RFLP with *Alw* NI for *MYOC* mutation c1109t (P 370 L) with different PCG patient samples

Lane 1 sample showing positive for P 370 L mutation

one known polymorphism was found in PCG001b and PCG165A, which was an insertion of the trinucleotide repeat CCG at ORF position 1339 causing the addition of one glycine in a stretch of 10 glycines in the C-terminal end of the protein. This has been widely reported as a polymorphism and is seen in normal controls (Mears et al 1998).

3.4. Direct sequencing of *PAX6*

Direct sequencing of the complete coding region of *PAX6* in two patients who showed single heterozygous mutation in *CYP1B1* and 8 others who had PCG but did not show any mutation in the coding region of *CYP1B1,* did not show either pathogenic mutation or SNPs.

3.5. PCR-RFLP analysis of *CYP1B1 & MYOC*

Among the 15 mutations identified in this study in both *CYP1B1* and *MYOC* (14 In *CYP1B1* and 1 in *MYOC),* 12 mutations resulted in either loss or gain of restriction site for distinct restriction enzymes (11 in *CYP1B1* and I in *MYOC*). In order to screen for these mutations in the corresponding pedigrees to determine the segregation of mutant alleles, to identify carriers, to screen other PCG patients for respective mutations, and to screen normal controls, we conducted PCR-RFLP analysis in 49 patients belonging to 46

pedigrees from PCG101 to PCG165. This resulted in identification of any one of the 11 *CYP1B1* mutations in 8 PCG patients from this cohort (Table 3.1).

In sum, direct sequencing and PCR-RFLP screening in 155 patients from 139 pedigrees resulted in identification of 14 distinct *CYP1B1* and 1 *MYOC* mutation in 57 patients from 45 pedigrees. This corresponds to 35.4% of PCG patients showing *CYP1B1* mutations of which 25.4% patients showed single heterozygous mutation.

Interestingly 16% of the PCG patients studied showed R368H mutation in this population. This mutation has been reported only in a very few PCG families from Saudi Arabia and Brazil, and at a very low frequency (Bejjani et al 2000; Stoilov et al 2002; Reddy et al 2003). But in the present study based on mutation screening, we find it to be a predominant allele associated with PCG in India. This is the highest reported frequency for this mutation from all ethnic backgrounds studied so far, indicating that the frequency of this mutation could vary based on the ethnic origin as well as geographical location.

Segregation analysis of *CYP1B1* positive pedigrees for the respective mutations using these PCR-RFLP methods was performed. In the pedigree PCG0017 (Figure 3.16), the father (I.1) and a second unaffected sibling (II.3) carry both wild type alleles for R368H. The mother (I.2) and the other

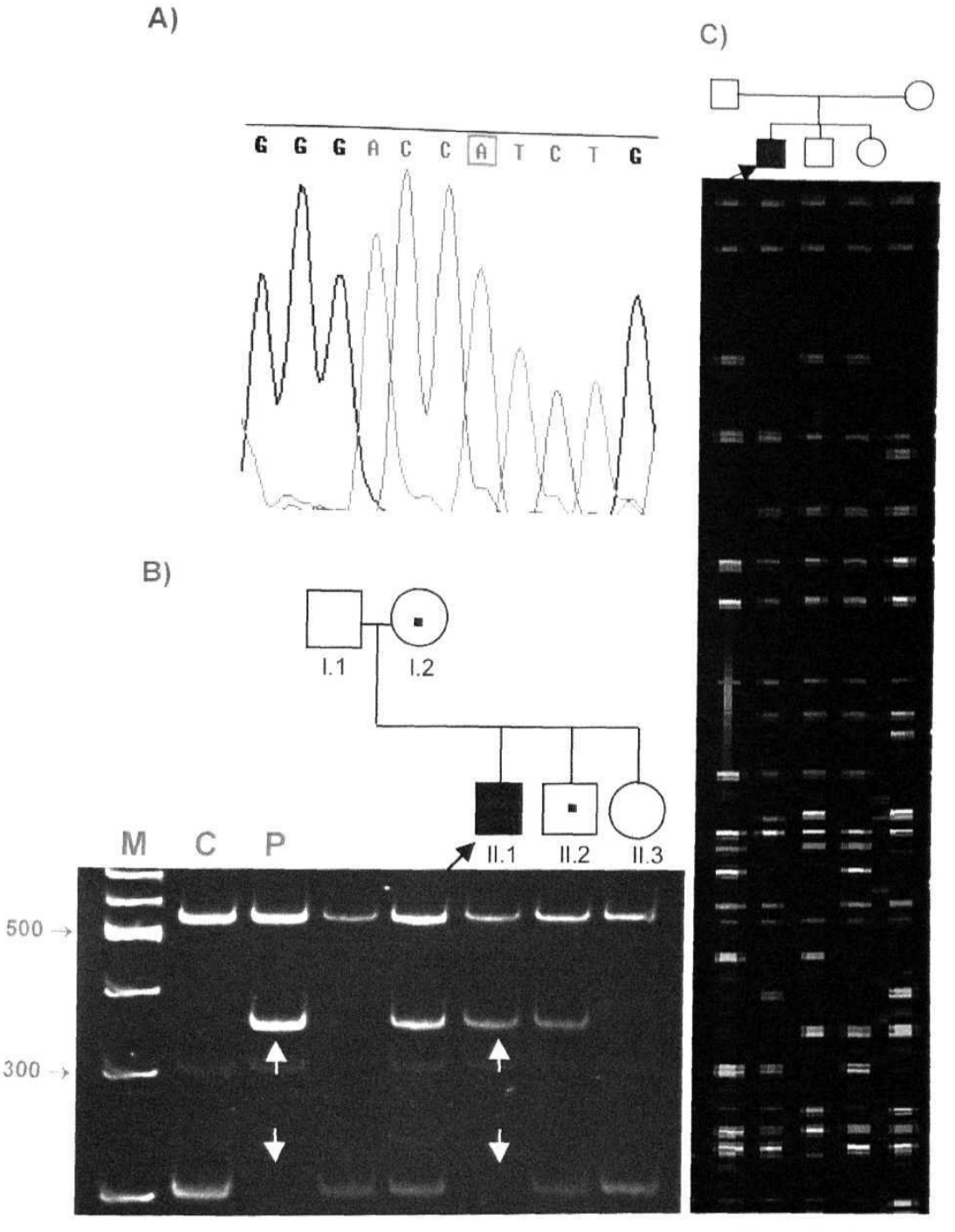


Figure 3.16. PCG 017 proband showing 1449g→a (R368H)

A) Electropherogram of PCG 093 Proband

B) PCR-RFLP showing the segregation of mutant alleles (arrow) in the PCG 093 pedigree

C) Gel picture showing the STR analysis in PCG 017 pedigree

unaffected sibling (II.2) are carriers for allele R368H, whereas the proband has both mutant alleles for R368H. We have ruled out the possibility of non-paternity in this pedigree by performing the short tandem repeat (STR) analysis with the help of Dr. Hasnain, CDFD. STR analysis from the X- and Y-chromosomes revealed that for the Y-linked markers, father (I.1) and proband (II.1) shared identical haplotype. Because the DNA sample used for analysis was extracted from peripheral blood, it is poaaible that this de *novo* mutation might be an example of a germinal mosaicism. Stoilov et al (1998) have also reported similar findings in one American pedigree.

In another pedigree, PCG069, we identified an incomplete penetrance of the phenotype. In this pedigree as shown in figure 3.8B, though the mother (I.2) has both mutant alleles for P193L, she did not show the phenotype. Whereas the proband (II.3) and other unaffected members (I.1, II.1, II.2) are heterozygous for this allele (P193L), only the proband showed the phenotype. Molecular simulation studies by Achary et al (2003) show that the proline at this position is involved in hydrogen bond formation, change of residue proline to leucine changes to the helical conformation to β-conformation. Hence proline at this position might be having a functional significance of CYP1B1 protein. In this pedigree, though mother is carrying a homozygous mutation, she does not show the phenotype, this could be due to the presence of a dominant modifier locus which suppresses the effect of the mutation in the case of the mother (I.2) (Figure 3.8B). Similar findings were also reported by

Bejjani et al (2000) in a Saudi Arabian pedigree. Repeated sequencing of both coding and promoter region of *CYP1B1* in case of this proband did not show any other heterozygous variation.

3.6. Promoter analysis of *CYP1B1*

Around 64 % of the 155 patients studied by both direct sequencing and RFLP methods did not show mutations in *CYP1B1* coding region and also 25% were heterozygous for the single mutations. To check the possibility of the promoter variation in these non-*CYP1B1* cases and in patients who showed single heterozygous mutation in the coding region of *CYP1B1,* we screened a 320 bp flanking region of all the four important elements required for maximal promoter activity (Wo et al 1997). In the PCG pedigrees PCG020, PCG033, PCG083, the probands showed a heterozygous variation - substitution of guanine at genomic DNA position 3078 by thymine (Figure 3.17). This variation lies 24 nucleotides next to the initiator motif region (-5 to +3) (Wo et al 1997), This initiator motif is one of the four known important regions for maximal promoter activity. But the variation seen here is not falling in any of these four known important regions. We failed to screen for this variation in the controls. All three probands did not show any variations in coding region of *CYP1B1*. The effect of this variation for the pathogenesis of this phenotype is highly speculative.

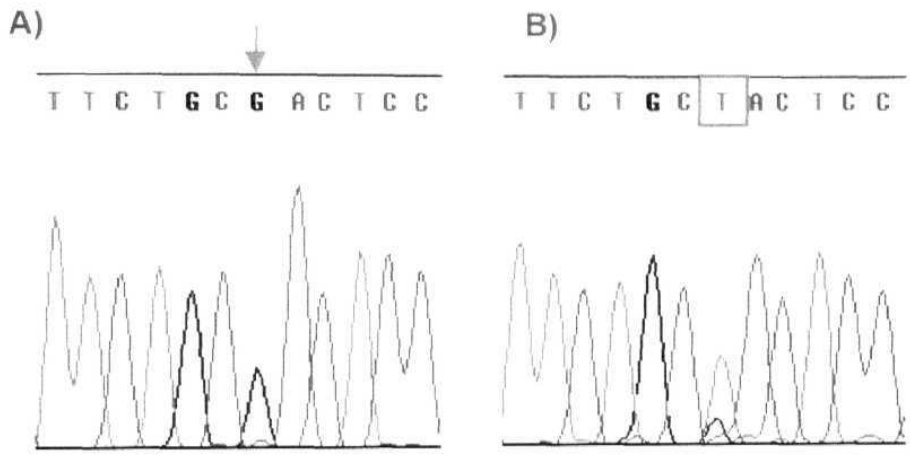


Figure 3.17. PCG 020 proband showing *3078g→t

A) Electropherogram of control

B) Electropherogram of PCG 020 Proband

* genomic DNA position

3.7. Genotype-Phenotype correlation

We attempted to study the genotype-phenotype correlation in order to correlate the effect of genotype (mutation) on the severity of the phenotype. Because of varying age of onset, diagnosis and time of presentation to the medical treatment with each patient, it is very difficult to correlate the phenotype-genotype and prognosis with medical / surgical treatment accurately. Based on the available clinical history (Table 3.4), and the help of our collaborator and glaucoma specialist Dr. Anil K. Mandal, we graded the severity of the phenotype as normal, mild, moderate and severe as shown in Table 3.5. Based on this grading we compared the clinical findings of all the PCG patients who showed mutation in either *CYP1B1* or *MYOC*. Based on this assessment, it was found that depending on the combination of genotype, the phenotype varies. Among all these mutations, 376 ins A, signature sequence mutation (G466D) and all the patients with R390C homozygous mutation showed most severe phenotypes (Table 3.6).

Clinical parameters	Range
Age of onset	By birth - 3 years
Age of diagnosis	By birth - 30 yrs
IOP (mm Hg)	24-55
Cup to disc ration of the optic nerve	0.3:1 (total cupping)
Corneal diameter (mm)	11-17
Last recorded vision	6/6 - NPL (normal – blind)
Corneal changes	Corneal scar, haab's striae, edema, buphthalmos, megalocornea
Treatment	Medical - Surgical (1-3)

Table 3.4. Clinical data of PCG patients for genotype-phenotype correlation

Clinical parameters used for grading	Normal	Mild	Moderate	Severe / Very Severe
Corneal diameter (mm)	< 10.5	>10.5-12	>12 - 13	>13
IOP (mm Hg)	<16	>16-20	>20 -30	>30
C / D ratio	0.3-0.4	>0.4-0.6	>0.6 -0.8	>0.8
Last recorded vision	20/20	<20/20 - 20/60	<20/60 - 20/200	<20/200 - 20/400, < 20/400 - NPL*
Corneal clarity	No edema	Mild edema	Severe edema	Severe edema & Haab's striae

*: Very severe

Table. 3.5. Severity index used for grading PCG phenotype

Pedigree	Age of Intervention	Mutations Identified	Severity Eye wise	Prognoses Eye wise
004p	5 mo#	Ter @ 223	Very severe OU	Very poor OU*
004s	3 mo#	Ter @ 223	Very severe OU	Very poor OU*
093p	1 mo	G61E	Severe OU	Poor OU
093s	2 mo	G61E	Severe OD	Poor OD
			Very severe OS	Poor OD
011p	2 wks	G61E	Mild OU	Good OU
058p	1 wk	P193L	Severe OU	Poor OU
001p	ND	P193L(h)	Mild OU	Good OU
		E229K (h)		
001m	ND	P193L(h)	Normal OD	Good OD
			Very severe OS	Very poor OS*
069p	1.6 yrs	P193L(h)	Very severe OU	Very poor OU
024p	1 mo	E229K (h)	Very severe OU	Very poor OU
037p	5 yrs	E229K (h)	Very severe OU	Very poor OU
047p	10 yrs#	E229K (h)	Very severe OU	Very poor OU
125p	3 mo	E229K(h)	Severe OU	Poor OU
002p	35 days#	R368H	Mild OD	Good OD
			Severe OS	Poor OS

Table 3.6. Contd..

006p	8 mo	R368H	Mild OD	Good OD
			Severe OS	Poor OS
017p	9 yrs	R368H	Very severe OD	Very poor OD*
			Severe OS	Poor OS
040p	5 mo	R368H	Very severe OD	Very poor OD
			Severe OS	Poor OS
076p	1 yr	R368H	Severe OD	Poor OD
			Moderate OS	Fair OS
079p	3 mo	R368H	Very severe OD	Very poor OD
			Severe OS	Poor OS
130p	3 yrs	R368H	Severe OD	Poor OD
			Very severe OS	Very poor OS
137p	1 mo	R368H	Severe OD	Poor OD
			Moderate OS	Fair OS
144p	1 mo	R368H	Severe OD	Poor OD
			Moderate OS	Fair OS
006s	4 mo	R368H	Severe OU	Poor OU
022p	6 mo#	R368H	Very severe OU	Very poor OU
035s	28 days	R368H	Very severe OU	Very poor OU
051p	5 mo	R368H	Very severe OU	Very poor OU
071p	2 wks	R368H	Severe OU	Poor OU
071s	2 wks	R368H	Severe OU	Poor OU
075p	1 wk	R368H	Very severe OU	Very poor OU
150p	1 mo	R368H	Moderate OU	Fair OU
136p	2 mo	R368H	Moderate OU	Fair OU
067p	2 mo	R368H	Severe OU	Poor OU
025p	10 yrs	R368H (h)	Very severe OU	Very poor OU

Table 3.6. contd..

035p	8 yrs	R368H (h)	Very severe OU	Very poor OU
095p	1.2 yrs	R368H (h)	Very severe OU	Very poor OU
100p	1.3 yrs #	R368H (h)	Moderate OD	Fair OD
			Severe OS	Poor OS
039p	21 days#	R368H (h)	Moderate OU	Fair OU
005p	**29 days**	R368H (h)	Moderate OU	Fair OU
		R390C (h)		
005f	**2** mo	R390C	Very severe OU	Very Poor OU*
012p	**4** mo	R390C	Very severe OU	Very Poor OU
012s	2 mo	R390C	Very severe OU	Very poor OU
018p	**1** wk	R390C	Very severe OU	Very poor OU
092p	29 days	R390C	Very severe OU	Very poor OU
008p	2 wk	A115P	Moderate OU	Fair OU
086p	1mo	Q114P (h)	Moderate OU	Fair OU
122p	14yrs	M132R	Severe OU	Very poor OU
122s	3mo	M132R	Moderate OU	Fair OU
009p	3mo	G466D	Very severe OU	Very poor OU
057p	8yrs	E229K (h)	Severe OU	Poor OU
021p	4yrs	S239R	Severe OU	Poor OU
051p	7yrs	del 446 - 468	Severe OU	Poor OU
165p	11 yrs	P370L *(MYOC)*	Moderate OU	Fair OU

p-proband; s-sibling; m-mother; **f-father;** #-multiple surgeries done; (h)- heterozygous mutation; OD-right eye; OS-left eye; ND- not done

Table 3.6. Genotype -Phenotype correlation in PCG with various mutations identified in this study

Summary

Chapter 1 gives a general introduction and review of literature explaining the pathogenesis of primary congenital glaucoma (PCG) with respect to the embryological development of the eye and its anterior angle chamber, the histopathological abnormality of the disease and its clinical features. This chapter describes extensively the available genetic evidence for PCG, with a complete review of literature on the genetic cause of the disease and the molecular biology of the primary candidate gene *CYP1B1.* This chapter also explains the genetic and clinical heterogeneity of PCG, and the association of *CYP1B1* with other anterior segment anomalies.

Chapter 2 describes the materials and methods used in this study. It also contains the method of patient selection, the use of PCR and RFLP methods relevant to the candidate genes for anterior segment anomalies like *CYP1B1*, *MYOC, FOXC1* and *PAX6,* which are also screened in this study.

Chapter 3 describes the salient findings and a detailed discussion on these findings.

The salient features of this study are;

- First study describing the genetic cause of PCG in the Indian population.
- Largest number of cases of PCG studied in the world from the same ethnic background.
- Identified 14 pathogenic mutations and 7 single nucleotide polymorphisms in *CYP1B1.*
- Identified one pathogenic mutation in *MYOC* in one PCG patient, which provides evidence for the possible genetic heterogeneity of PCG.
- Identified one known polymorphism in the *FOXC1* gene in two PCG patients.
- PCR-RFLPs described in this study will help in faster screening for the respective mutations and genetic counseling in afflicted families.
- First study describing the genotype-phenotype correlation in PCG.

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1. Panicker, S. G., Reddy, A. B. M., Mandal, A. K., Ahmed, N., Nagarajaram, H. A., Hasnain, S. E. and Balasubramanian, D. Identification of Novel Mutations Causing Familial Primary Congenital Glaucoma in Indian Pedigrees. *Invest Ophthalmol Vis Sci.* 2002;43: 1358-66

2. Reddy, A. B. M., Panicker, S. G., Mandal. A. K., Hasnain, S. E. and Balasubramanian, D. Identification of R368H as a predominant *CYP1B1* allele causing Primary Congenital Glaucoma in Indian patients. *Invest Ophthalmol Vis Sci.* 2003; (In Press).

3. Panicker, S. G., Sampath, S., Mandal, A. K., Reddy, A. B. M., Ahmed, N., Hasnain, S. E. and Balasubramanian, D. Novel Mutation in *FOXC1* Wing region causing Axenfeld-Rieger Anomaly. *Invest Ophthalmol Vis Sci.* 2003; 43: 3613-6.

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Identification of Novel Mutations Causing Familial Primary Congenital Glaucoma in Indian Pedigrees

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PURPOSE. TO determine the possible molecular genetic defect underlying primary congenital glaucoma (PCG) in India and to identify the pathogenic mutations causing this childhood blindness.

METHODS. Twenty-two members of five clinically well-characterized consanguineous families were studied. The primary candidate gene *CYP1B1* was amplified from genomic DNA, sequenced, and analyzed in control subjects and patients to identify the disease-causing mutations.

RESULTS. Five distinct mutations were identified in the coding region of *CYP1B1* in eight patients of five PCG-affected families, of which three mutations are novel. These include a novel homozygous frameshift, compound heterozygous missense, and other known mutations. One family showed pseudodominance, whereas others were autosomal recessive with full penetrance. In contrast to all known *CYPIBI* mutations, the newly identified frameshift is of special significance, because all functional motifs are missing. This, therefore, represents a rare example of a natural functional *CYP1B1* knockout, resulting in a null allele (both patients are blind).

CONCLUSIONS. The molecular mechanism leading to the development of PCG is unknown, Because *CYP1B1* knockout mice did not show a glaucoma phenotype, the functional knockout identified in this study has important implications in elucidating the pathogenesis of PCG. Further understanding of how this molecular defect leads to PCCi could influence the development of specific therapies. This is the first study to describe the molecular basis of PCG from the Indian subcontinent and has profound and multiple clinical implications in diagnosis, genetic counseling, genotype-phenotype correlations and prognosis. Hence, it is a step forward in preventing this devastating childhood blindness. (*Invest Ophthalmol Vis Sd.* 2002;43:1358-1366)

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The glaucomas, a heterogeneous group of optic neuropathies, if untreated, lead to optic nerve atrophy and permanent loss of vision. Glaucoma accounts for 15% of blindness worldwide.[1] One severe form of glaucoma, which occurs at birth or in early infancy (up to 3 years of age), is primary congenital glaucoma (PCG), which is mainly inherited as an autosomal recessive disorder. In contrast to a prevalence of 1:10,000 in the West," prevalence is as high as 1:1250 among the Romany population of Slovakia,[3] and 1:2500 in the Middle East,[4] where inbreeding occurs, suggesting a genetic etiology. In the Indian state of Andhra Pradesh, the prevalence is 1:3300, and the disease accounts for 4.2% of all childhood blindness.[5,6] However, the genetic defect of this disorder was unknown, and this prompted us to undertake the investigation.

Genetic linkage studies by Sarfarazi et al.[7] and Akarsu et al.[8] mapped PCX; to two different loci, GLC3A (at 2p21)[7] and GLC3B (at 1p36),[8] in which mutations within the *CYPIBI* gene (encoding the cytochrome P450 enzyme at GLC3A) were associated with the disease.[9] Several *CYP1B1* mutations in various ethnic backgrounds have been implicated in the pathogenesis.[10-20] To determine the possible genetic defect underlying PCG in India, molecular analyses of five families were undertaken, and the *CYP1B1* coding region was screened for mutations. Herein, we describe the pathogenic mutations (some of which are novel), including a natural *CYP1B1* functional knockout, their genotype-phenotype correlations, structure-function relationship, and the simple diagnostic methods developed for identifying these mutations.

METHODS

Clinical Evaluation and Patient Selection

The study protocol adhered to the tenets of the Declaration of Helsinki. After providing informed consent, five consanguineous PCG families were recruited for the study. These families were selected because all family members were available for the investigation, Patients and family members were evaluated by a glaucoma specialist (AKM) and were followed up for 10 years. The clinical data of the patients are described in Table 1. Ophthalmic examinations included slit lamp biomicroscopy, gonioscopy, measurement of intraocular pressure OOP), and perimetry in some cases. Clinical manifestations included elevated IOP, enlargement of the globe, edema, opacification of the cornea with rupture of the Descemet's membrane, thinning of anterior sclera and atrophy of the iris, anomalously deep anterior chamber, photophobia, blepharospasm, and excessive tearing.

Mutation Screening and Sequence Analyses

Because mutations in *CYPIBI* are the predominant cause of PCG, the entire coding region (1,6 kb organized in exons II and III)[21] was screened for mutations, Only these two exons were screened, because both contain the mutational hot spots of the gene and all pathogenic mutations reported so far are harbored in exons II and III. DNA was extracted from the peripheral leukocytes of patients, family members and control subjects. Using three sets of overlapping primers, the *CYP1B1* gene was amplified from patients and control subjects (Table

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TABLE 1. Clinical Data of Subjects with Primary Congenital Glaucoma

Pedigree	Age of Onset	Age of Diagnosis	Presence of Haab's Striae	Corneal Diameter (mm) and Clarity at Diagnosis (OD; OS)	IOP at Diagnosis (mm Hg OD; OS)	Last C/D Ratio (OD; OS)	Last Visual Acuity (OD; OS)	Treatments (OD; OS)
PCG 4								
Proband	By birth	2 wk	Present in OU	12; 12.5 Buphthalmos OU; hazy cornea and edema	36; 38	0.9; NA	NPL OU	Medical and 1× Trab/Trab OU; 1× PK * OD
Affected sibling	By birth	3 mo	NA OU	NA; Buphthalmos OU; hazy cornea and atrophic	NA OU	NA OU	NPL OU	Medical and 1× Trab/Trab treatments at 3 mo
PCG 11, Proband	By birth	2 wk	Absent OU	12; 12.5 Corneal edema OU	30 OU	NA OU	Fixing and following light OU	Medical and 1× Trab/Trab OU; 2× Trab/Trab OS
PCG 1								
Proband	By birth	~5 y	Absent OU	NA; clear OU	24 OU	0.8; 0.6	20/25 OU	Medical treatment OU
Affected mother	Late onset in OD; >3 years	30 y	Absent OD; present OS	NA; Clear OD; hazy OS	34; 50	0.8; 0.9	20/20; NPL	Medical treatment OD
PCG 2, Proband	By birth	2 wk	Present OU	13 OU Buphthalmos OU; hazy cornea OU	NA OU	0.9 OU	20/30; PL	3× Trab/Trab OU; retinal reattachment surgery OS†; medical treatment OD
PCG 6								
Proband	By birth	9 mo	Absent OU	13; 12.5 Corneal edema OU	26; 30	0.3 OU	20/40; 20/200	1× Trab/Trab OU
Affected sibling	By birth	3 mo	Absent OU	15 OU Corneal edema and scarring OU	32 OU	NA OU	PL; HM	Medical and 1× Trab/Trab OU

IOP, intraocular pressure; OD, right eye; OS, left eye; OU, both eyes; C/D, cupdisc ratio of the optic nerve; NPL, no perception of light, PL, perception of light, HM, hand motion; NA, not available; X, Times; Trab/Trab, combined trabeculotomy and trabeculectomy; PK*, penetrating keratoplasty performed but resulted in graft failure; OS† left eye became atrophic.

2). Amplicons were sequenced directly, and the patient and control sequences were compared to identify all mutations. The primers used were as follows: set 1 (1 forward [F]/1 reverse [R], 786 bp),[12] set II (2F/2R, 648 bp),[22] and set III (3F/3R, 885 bp).[12] All PCRs were performed for only 30 cycles, and conditions for sets 1 and 11 were as reported earlier[12]; conditions for set III are given in Table 2. Twenty-five- to 50-μL polymerase chain reactions (PCR) were performed with the following: 50 to 100 ng genomic DNA, 1× PCR buffer with 1.5 to 2.0 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer, and 1 U *Taq* polymerase (Bangalore Genei, bangalore, India), with or without 10% dimethyl sulfoxide (DMSO). Primer sets I and 11 had 10% DMSO and 1.5 mM $MgCl_2$, whereas set III had only 2.0 mM $MgCl_2$. The same sets of primers were used for PCR and bidirectional sequencing. The three amplicons were purified (pre-PCR sequencing kit; USB, Cleveland, OH), terminator cycle sequencing was performed (BigDye kit; PE-Applied Biosystems, Foster City, CA), and sequencing reactions were performed on an automated DNA sequencer (ABI model 377; PE-Applied Biosystems).

PCR-Restriction Fragment Length Polymorphism Analyses and Cosegregation of Mutant Alleles with Disease Phenotype

In all cases, mutations resulted in either loss or gain of recognition sites (Table 2). For determining the cosegregation of mutant alleles with disease phenotype in the family, the respective fragment harboring the mutation was amplified from all family members, and an aliquot of amplicons was digested with the corresponding restriction enzymes (Table 2; MB1 Fermentas, Vilnius, Lithuania) The fragments were separated on 8% polyacrylamide gel, stained with ethidium bromide and visualized to distinguish the wild type and mutant alleles. Seventy volunteer donors without history of eye disorders served as control subjects.

RESULTS

Identification of Pathogenic Mutations

Novel Frameshift Mutation and Functional Null Allele. In family PCG4 (an uncle-to-niece marriage) two patients showed a homozygous insertion (Figs. 1A, 1B; Table 2) of a nucleotide A at cDNA position 376 (376insA). This novel mutation, not previously reported,[10-20] resulted in a frameshift that truncated the open reading frame (ORF) by creating a premature stop codon (TGA), 636 bp downstream from this insertion. Consequently, a truncated 222-amino-acid (aa) protein missing 321 aa from the C terminus was generated (Fig. 1C). This also abolished the restriction site *Eco* 130I in exon II. Both the wild-type and the mutant proteins contained just 10 aa at the N terminus, which is similar in both, and the frameshift eliminated all CYP1B1 domains, resulting in a functional null allele. All unaffected members in family PCG4 were heterozygous for this mutation (Fig. 2A).

Novel Compound Heterozygous Mutations and Pseudodominance. In another family (PCG1; marriage between first cousins), parent-to-child transmission of the disease was noticed. This is an interesting pedigree in which the daughter (proband) and mother were affected with bilateral PCG and the father was a normal carrier (Fig. 3A). Two affected generations showed varying severity and manifestations. The mother showed asymmetric manifestation (left eye blind, right eye mildly affected), whereas the proband displayed a uniform milder manifestation in both eyes. The proband had a novel compound heterozygous missense mutation (Table 2) within exon II. The first mutation (Fig. 3B) was a C→T substitution at 923 bp, resulting in a proline-to-leucine change at aa 193 (P193L) and a gain of the restriction site *Eco*81I. The second

TABLE 2. Mutations Causing PCG Phenotype

Pedigree	Exon	Mutation Position in cDNA (bp)	Hetero-/homozygous	Codon Change	Mutation Type	Restriction Site Change	Diagnostic Method Developed	Primers Used for Amplification (5′–3′)	Novel or Reported
PCG4	II	376insA	Homozygous	Ter@223	Frameshift	$^{-}$*Eco*130I	PCR followed by *Eco*130II digestion	1F-tctccagagagtcagctccg (3676–3695) 2FR-agtagtggccgaaagccat (4199–4217)	Novel*
PCG11	II	528G→A	Homozygous	G61E	Missense	$^{+}$*Taq*I	PCR followed by *Taq*I digestion	IF-tctccagagagtcagctccg (3676–3695) IR-gggtcgtcgtggctgtag (4461–4444)	Refs. 10, 12, 15
PCG1	II	923C→T	Heterozygous	P193L	Missense	$^{+}$*Eco*81I	PCR followed by *Eco*81I digestion	2F-gatgcgaacttcttcacg (4258–4276) 2R-ctactccgccttttcaga (4905–4887)	Novel*
	II	959G→A	Heterozygous	E229K	Missense	$^{-}$*Eam*1104I	PCR followed by *Eam*1104I digestion	2F-gatgcgcaacttcttcacg (4258–4276) 2R-ctactccgccttttcaga (4905–4887)	Novel*
PCG2 and 6	III	1449G→A	Homozygous	R368H	Missense	$^{-}$*Taa*I	PCR followed by *Taa*I digestion	†3F-tcccagaaatattaatttagtcaactg (7740–7765) 3R-tatggagcacacctcaacctg (8624–8605)	Novel* diagnostic method

Gain and loss of restriction sites are indicated by + or − signs, respectively. Nucleotide numbering is based on sequence reported by Tang et al.[21]

* Reported for the first time in this study.

† PCR conditions for set III primers are initial denaturation of 94°C for 3 min followed by (94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min) × 30 cycles. Final extension was at 72°C for 10 minutes.

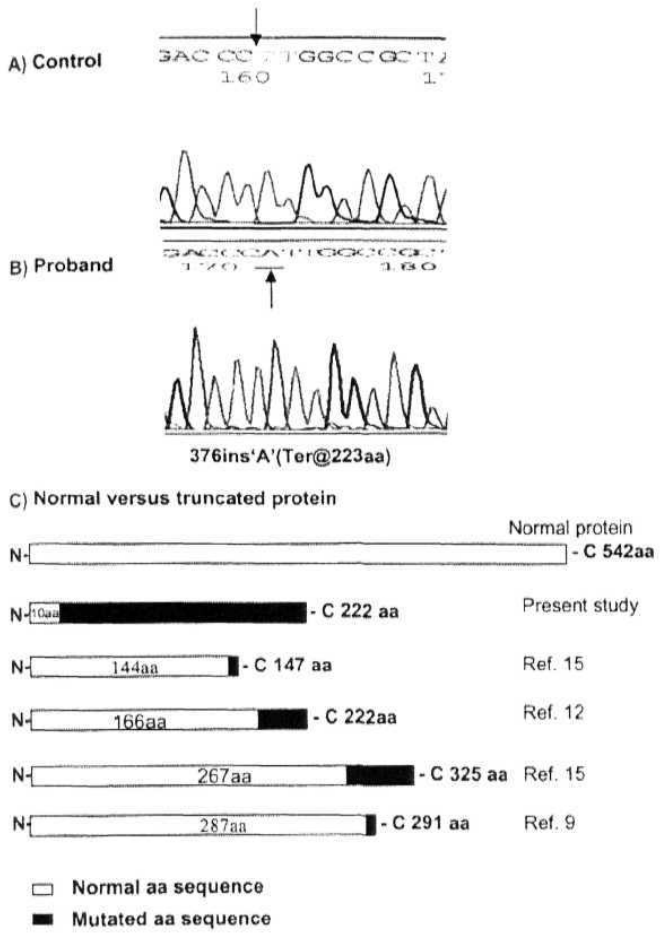

FIGURE 1. Electropherogram of the sense strand of genomic DNA from the proband in family PCG4, showing a novel homozygous frameshift mutation. Note the homozygous insertion of a nucleotide A (376insA) in the mutant allele of the proband (B), which is absent in the control (A). The mutation, which is *underlined* and shown by an *arrow*, results in premature termination at aa 223. The comparison of normal versus all truncated protein known in exon II is also shown (C). (□) Sequences that are common between normal and wild-type protein; (•) mutant protein. The length of truncated protein and its references are shown on the *right.*

mutation (Fig. 3B) was a G→A substitution at 959 bp, resulting in replacement of glutamic acid with lysine at aa 229 (E229K) and loss of restriction site *Eam*1104I (Fig. 2C).

The maternal grandfather (I.1), mother (II. 1), and proband (I1I.1) in PCG1 were heterozygous for 923C→T, whereas the father (II.2), proband (III.1), and unaffected sibling (III.2) were heterozygous for 959G→A (Figs. 3A, 3B). Consistent with the recessive mode of inheritance, the proband had inherited two heterozygous mutant alleles (III.l), one from each parent. Sequence analysis of the affected mother (II. 1) revealed only one mutant allele (Fig. 3A). The presumed second mutant allele has yet to be identified. For recessive disease to develop, the patient should have two mutant alleles either in the heterozygous or homozygous state. The unaffected sibling (III.2) had inherited one paternal heterozygous mutant allele, and it may be that she also inherited the unknown mutant allele from the mother. Repeated sequencing of the *CYP1B1* coding region in the mother (II. 1) and unaffected sibling (III.2) failed to identify the other heterozygous mutation, suggesting its possible presence either in the promoter or in some other regulatory region. Although we presume that this unaffected sibling (III.2) had inherited two mutant alleles, at 8 years of age she had not yet shown any symptoms of glaucoma and hence she could be considered as a glaucoma suspect. The first mutation (P193L) maps to a region highly conserved among various types of cytochromes, whereas the E229K mutation is conserved only among the *CYP1B1* types (Fig. 4). Screening of 70 control subjects by PCR-restriction fragment length polymorphism (RFLP) not only confirmed the absence of this compound heterozygous mutation in the normal population, but also supports that it is likely to be pathogenic. However, a few control subjects (12.8%) were heterozygous for the 923C→T (E229K) mutation, but none for the 959G→A (P193L) mutation.

Homozygous Missense Mutations. Three families were identified with two known homozygous missense mutations[10,15,23]: two with the R368H homozygous mutation and one with the G61E homozygous mutation (Table 2). Both are highly conserved across various members of the cytochrome P450 superfamily (Fig. 4). These mutations were found to segregate with four patients (families PCG2 and PCG11, one patient each; PCG6, two patients) in three unrelated consanguineous families (PCG2 and PCG6, first-cousin marriage; PCG11, uncle-to-niece marriage). Consistent with recessive inheritance, mutant alleles segregated with disease phenotypes in all families.

Patients in families PCG2 and PCG6 shewed the same homozygous mutation: G→A substitution at 1449 bp. This resulted in a arginine-to-histidine change at aa 368 (R368H) in CYP1B1 and a loss of restriction site *Taa*I in exon III (Table 2). In PCG11, substitution of a nucleotide G→A at cDNA position 528 resulted in a glycine-to-glutamic acid replacement at aa 61 (G61E) of CYP1B1 and a gain of the restriction site *Taq*\ in exon II (Table 2).

Nonpathogenic *CYP1B1* Single Nucleotide Polymorphisms

In addition to pathogenic mutations five other single nucleotide polymorphisms (SNPs; Table 3) were identified in the less conserved region of CYP1B1. Because PCG6 had two different homozygous missense mutations, the highly conserved residue (R368H, reported earlier)[15] was considered to be a pathogenic mutation, whereas the less conserved one (G184S) was taken to be a novel polymorphism (Fig. 4).

Structural Implications of Mutant Proteins

It is interesting to note that, of the four amino acid mutations (excluding insertion mutation), three occur in the less-conserved N-terminal domain of the protein. An alignment of the amino acid sequence with a homologue of known three-dimensional structure (Protein Data Bank [PDB] code: 1DT6) revealed that all the mutation sites are away from the heme-binding pocket and therefore probably do not affect directly the binding of the heme. However, these sites seem to be important in maintaining the structural integrity of the protein. The conserved glycine residue at position aa 61 is in a left-hand helical conformation and is in a very unique position where the peptide chain takes a sharp turn. Position aa 193 forms the N-capping region of the helix (aa 173-210) and is most suited for proline, which is also highly conserved. Any amino acid change at this position may disrupt the helical structure. The same is probably true for the position E229, which is in the middle of the helix (aa 218-234). R368 is probably less important structurally, because the site is in the loop region, which is on the surface of the protein and is probably necessary for protein-protein interactions.

Our examination of the translated product of the frameshift mutation (376insA) revealed that the amino acid sequence of

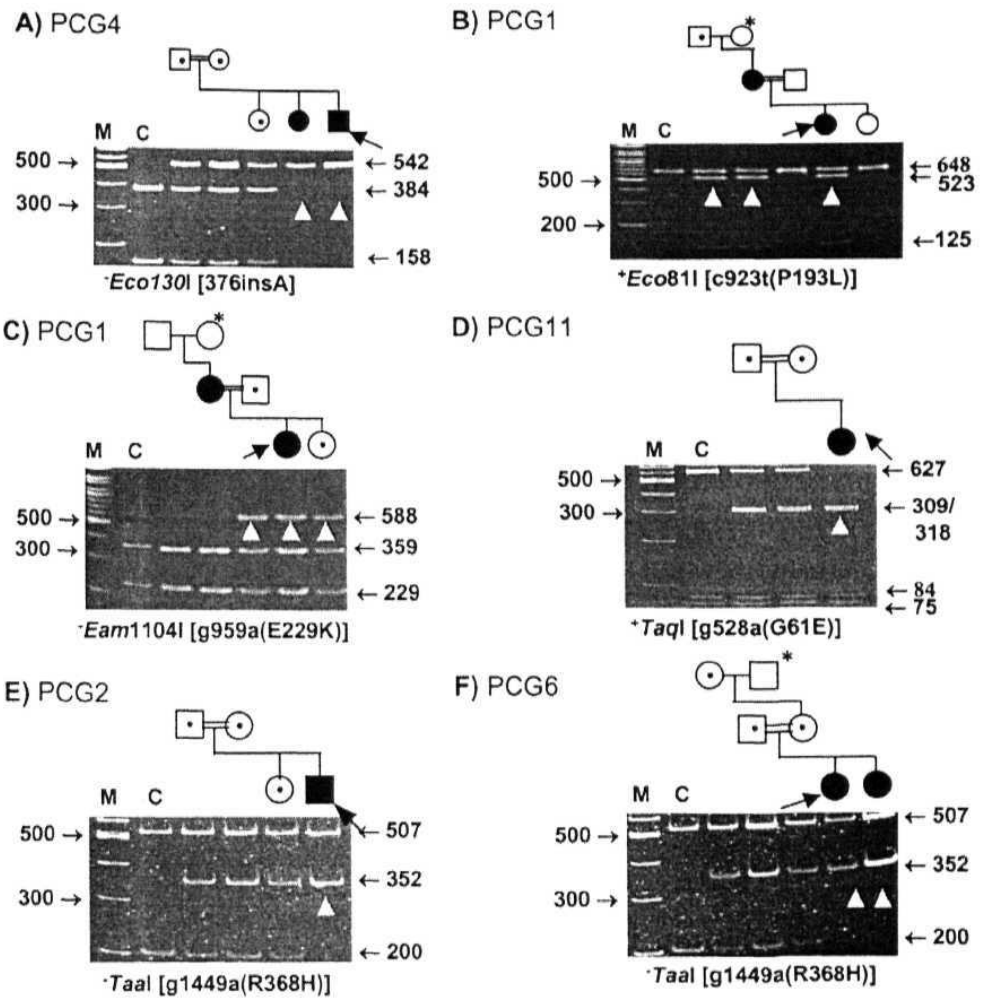

FIGURE 2. PCR-RFLP analyses of the cosegregation of different mutations with disease phenotypes. *Pilled squares* and *circles:* Affected individuals; *arrow:* probands; *dot* in *open symbol:* carriers; *double line:* consanguinity. DNA molecular weight marker *(lane M)* in base pairs *(lefty,* allele sizes *(right);* control *(lane C);* mutant allelc(s) *(arrowheads).* Restriction site changes and mutations (nucleotide as well as amino acid changes) are shown at the *bottom* of each panel. (*) Sample for analysis unavailable, (A) Wild-type allele amplification and restriction digestion of amplicon from control DNA generated 384- and 158-bp fragments *(lane C).* Mutation abolishes the *Eco*130I site. In heterozygous individuals (carriers) in addition to the wild-type allele, a mutant allele of 542 bp was present. In the disease phenotype (homozygous) only a mutant allele of 542 bp was evident. (B) C→T substitution in PCG1 results in a gain of an *Eco*811 site, which is evident from the cleavage of the 648-bp fragment *(lane C)* into 523-bp and 125-bp fragments. In carriers, in addition to the wild-type allele a mutant allele of 648 bp was present. (C) Restriction digestion of the wild-type allele in the control generated 359- and 229-bp *(lane C)* fragments and abolished the *Eam*1104I site. In carriers, in addition to the wild-type allele 588, mutant alleles of 359 and 229 bp were present. (D) Restriction digestion of the wild-type allele in the control showing undigested fragment of 627 bp *(lane C).* Mutation creates a *Taq*\ site. In carriers, in addition to the wild type allele, mutant alleles of 318 and 309 bp were present. In the disease phenotype (homozygous) only mutant alleles of 318 and 309 bp were present, (E, *V)* Restriction digestion of wild type allele in the control generated 507- and 200-bp fragments *(lane C).* Mutation creates a *Taa*\ site. In carriers, in addition to the wild-type allele, a mutant allele of 352 bp was present. In the disease phenotype (homozygous) only mutant alleles of 507 and 352 bp were present.

the new ORF does not show an appreciable match with any of the known protein sequences in the PDB. A secondary structure prediction of the sequence showed that the translated product is mostly made of coiled regions.

Genotype–Phenotype Correlations

Correlation between genotype and phenotype based on this study was evident from a comparison of the different mutations associated with varying manifestations and prognoses of the disease (Table 4). The PCG phenotypes associated with various mutations showed varying severity and manifestations. In some cases, there was asymmetric manifestation between eyes of the patients (mother in family PCG1), whereas the same mutation (R368H) exhibited interfamily (families PCG2 and -6) as well as intrafamily (family PCG6) variability (Tables 1,4).

DISCUSSION

This is the first genetic study from India to describe the molecular defect underlying the PCG phenotype and demonstrates the direct association of the *CYP1B1* mutations with this devastating childhood blindness.[1,2] Unknown developmental defects of the trabecular meshwork and anterior chamber angle of the eye cause this disorder.[10,21,24] Inour investigation of five consanguineous PCG-affected Indian families, five pathogenic mutations (including three novel ones) were identified in eight affected members. These include a novel homozygous frameshift mutation resulting in a functional null allele and compound heterozygous missense and known missense mutations (Table 2). That all are disease-causing mutations is shown by the fact that all mutant alleles cosegregate with the disease phenotype and are absent in the normal population and that the mutated residues are highly conserved across various members of the cytochrome P450 superfamily (Fig. 4). In addition, five SNPs were found in the affected families. These were either observed in the general population and/or were found to affect poorly conserved amino acid residues exclusively (Fig. 4). This study also indicates that *CYP1B1* could be the predominant cause of PCG in the Indian ethnic background, because all families analyzed so far have had mutations in this gene.

Pseudodominant inheritance was seen in one family, whereas all others showed autosomal recessive inheritance with full penetrance. All patients inherited two mutant alleles, whereas unaffected members were heterozygous (carriers) for a single mutant allele segregating in that particular family, except in the pscudodominant family (Fig. 2).

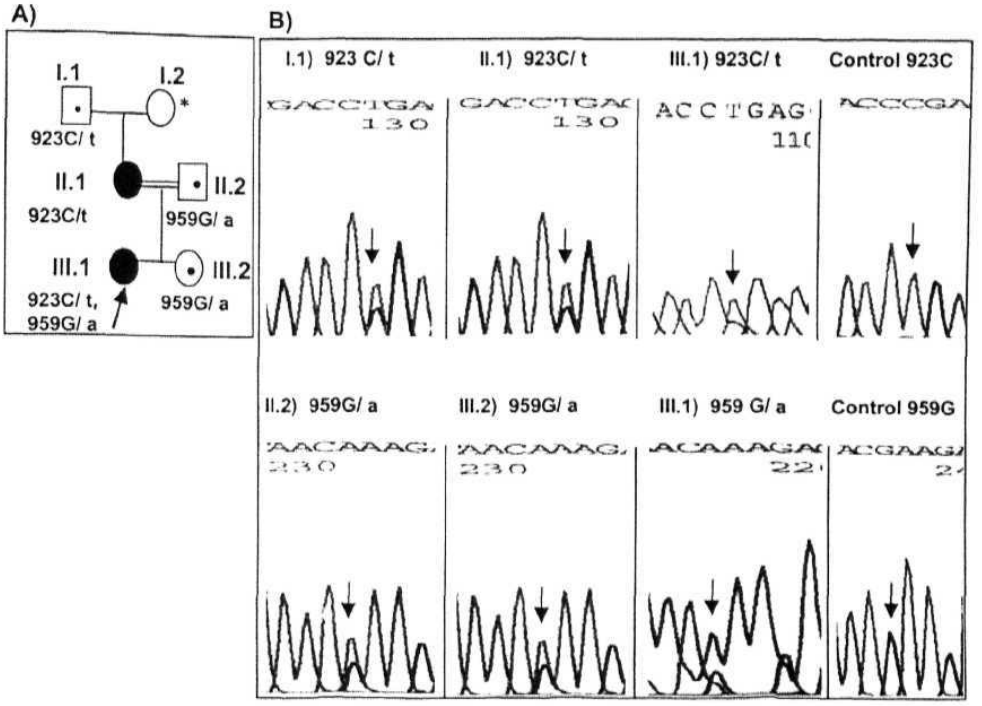

FIGURE 3. (A) Pedigree showing segregation of alleles in PCG1 family. (*) Grandmother's DNA was not available for analysis. Heterozygous alleles arc denoted by a *slash* separating wild type in *uppercase letter's* and mutant residues in *lowercase letters.* (B) Elcctrophcrogram of the sense strand of various members of family PCG 1. Residues arc numbered based on cDNA sequence. *Arrows:* mutated bases.

Of all mutations identified herein, the frameshift mutation resulted in the most severe phenotype. Only the first 10 aa of the 543-aa CYP1B1 protein remain unchanged by the frameshift, whereas the remainder of the protein was replaced by an out-of-framc polypeptide of 222 aa. Despite maximum medical and prompt surgical treatments, both patients in family PCG4 exhibited a most devastating phenotype and were blind (Fig. 5).

In all PCG-pseudodominant families reported so far,[10,12,15] the affected parent has been homozygous and the other a normal carrier; but analysis of the present pseudodominant family (PCG1) indicates that the affected parent (II. 1) is a compound heterozygote. Moreover, an interesting observation is that probably there are three compound heterozygous individuals (II. 1, III. 1, and III.2) in this family, all segregating with different combinations of mutant alleles (Fig. 3) with varying expression, of which one exhibits normal phenotype (unaffected sibling [III.2]—a glaucoma suspect). The exact age of onset of the disease in this case was difficult to ascertain because the affected status of the mother (II. 1) was revealed through her daughter (the proband [III.1]). The presence of llaab's striae in the left eye of the affected mother (Table]) suggests that she had PCG in that eye before 3 years of age, whereas the right eye had late-onset PCG. An asymmetric manifestation of PCG was seen in the affected mother (the left eye became blind at 21 years, whereas IOP in the right eye is under control with medication).

The mother had glaucoma diagnosed at age 30 (Table 1) and had ocular features indicating that disease may have begun in one eye before age 3. However, because the second *CYP1B1* mutation has not been identified in the mother (II. 1), and this missing allele, as passed on to her 8-year-old daughter (III.2), has resulted in a normal phenotype (Fig. 3A), this seems to be a complex situation, for which various plausible explanations

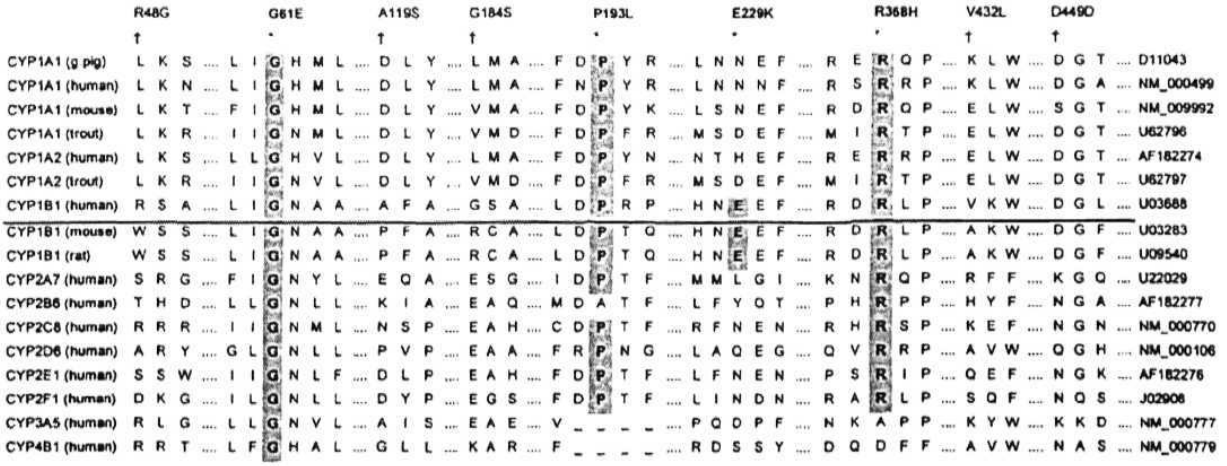

FIGURE 4. Multiple sequence alignment of various members of the cytochrome P450 superfamily. *Bold letters* with *asterisk* and *shading;* conserved residues (when mutated) causing PCG phenotype. †Polymorphic residues. *Right:* sequence accession numbers. The human *CYP1B1* sequence is *underlined,*

TABLE 3. Single Nucleotide Polymorphisms Identified in PCG-Affected Families

Pedigree	Intron/Exon	Sequence Change (genomic/cDNA position in bp)	Codon Change	Mutation Type	Novel* or Reported
PCG1, PCG2, PCG6, and PCG11	Intron I	3793 T→C (−13 bp)†	Not applicable	—	Refs. 12, 15
PCG4	Exon II	488 C→G	R48G	Missense	Refs. 12, 15, 19, 18
PCG6	Exon II	896 G→A	G184S	Missense	Novel*
PCG2	Exon III	1640 G→C	V432L	Missense	Refs. 9, 12, 15, 18, 19
PCG1	Exon III	1693 T→C	D449D	Silent	Refs. 12, 15, 19, 18

* Reported for the first time in this study.
† Indicates —13 bp upstream of ATG or genomic DNA position 3793 bp.

can be considered: (1) The dramatic phenotypic variability observed between the two eyes of the affected mother is possibly the consequence of an as yet unknown mutation within the promoter region (perhaps a promoter deletion), and may indicate that *CYP1B1* is a dosage-sensitive gene. (2) The mother may simply be a carrier of congenital glaucoma who happens also to have an early-onset form of glaucoma caused by mutation at another locus or glaucoma of a nongenetic origin. (3) It may be possible that heterozygosity for the 923C→T mutation causes late-onset disease, although to our knowledge there are no reported instances of development of late-onset disease in earners of the *CYP1B1* mutation. (4) If the mother has a new mutation and is mosaic for the mutation, she could have one eye more affected than the other, because of unequal representation of the defect in the two eyes. It is possible that she has an unaffected child who inherited that chromosome, because of the absence of the mutation in the germ line. Although various roles for *CYP1B1* in eye development have been proposed recently,[23] it is tempting to speculate that the likely role of *CYP1B1* is in the detoxification or elimination of a toxic metabolite, which may be harmful to the normal development of the eye.

Previous studies have indicated that the G61E and R3681I mutations are not fully penetrant in Saudi families,[10,15] whereas in these Indian families, both arc fully penetrant. R368H, reported earlier,[15] maps to helix K, which is one of the highly conserved core structures (CCSs). This homozygous mutation seen in three patients of two unrelated families (PCG 2 and PCG6) shows a very severe phenotype, in either one or both eyes. The CCSs arc suspected to be involved in proper protein folding and in active heme binding.[23] Therefore, any homozygous impairment of this domain could lead to a severe phenotype. The other highly conserved G61E mutation[12] is adjacent to the N-terminal proline-rich region of CYP1B1 and is also likely to affect the proper protein function and result in disease manifestation. The proline-proline-glycine-proline motif may serve to join the membrane-binding N terminus to the globular region of the P450 protein.[9,10,15,23]

Because the anterior chamber angle in humans has undergone some very recent evolutionary changes, this may be a problem in using animal models, especially the *CYP1B1* knockout mice, for studying PCG's pathogenesis.[23] Typical trabecular meshwork can be found only in humans and higher primates, whereas lower species have only a reticular meshwork.[24] Although it may be difficult to extrapolate the findings obtained from the *CYP1B1* null mice, the phenotype obtained in such mice need not be the same as that of the functional *CYP1B1* knockout identified in the present study. This view is in fact substantiated by a study, wherein it was demonstrated that *CYP1B1* null mice did not show any obvious blindness or evidence of glaucoma, as assessed by standard behavioral comparisons with wild-type mice in their response to light and dark.[25] Furthermore, a frequent observation in various knockout studies is that the phcnotypes do not transfer identically across species.

The information derived from this study has both basic and clinical relevance. Genetic counseling can be provided to at-risk families that will aid in the prevention of PCG-related blindness. The characterization of *CYP1B1* and the spectrum of mutations with evidence of pathogenicity and high pen-

TABLE 4. Genotype/Phenotype Effect

Pedigree	Mutation	Laterality	Severity* (OD; OS)	Prognosis (OD; OS)
PCG4				
Proband	Ter@223 aa	Bilateral	OU very severet	OU very poor
Affected sibling	Ter@223 aa	Bilateral	OU very severet	OU very poor
PCG 11, proband	G61E	Bilateral	OU mild	OU good
PCG1				
Proband	P193L and E229K	Bilateral	OU mild	OU good
Affected mother	P193L‡	Bilateral with lute onset OD	OD normal	OD good
			OS very severet	OS very poor
PCG2 proband	R3681I	Bilateral	OD mild	OD good
			OS very severe	OS very poor
PCG6				
Proband	R368H	Bilateral	OD mild	OD good
			OS very severe	OS very poor
Affected sibling	R368H	Bilateral	OU very severe	OU very poor

* Severity of the disease is arbitrarily graded based on the conical changes, IOP, cup-to-disc ratio, and last recorded visual acuity (20/20 is normal; <20/20-20/40 is mild; <20/40-20/200 is severe; <20/200-PL is very severe; NPL is blind; see Table 1.
† Affected individual is blind,
‡ Second mutation in PCG1 mother is unknown.

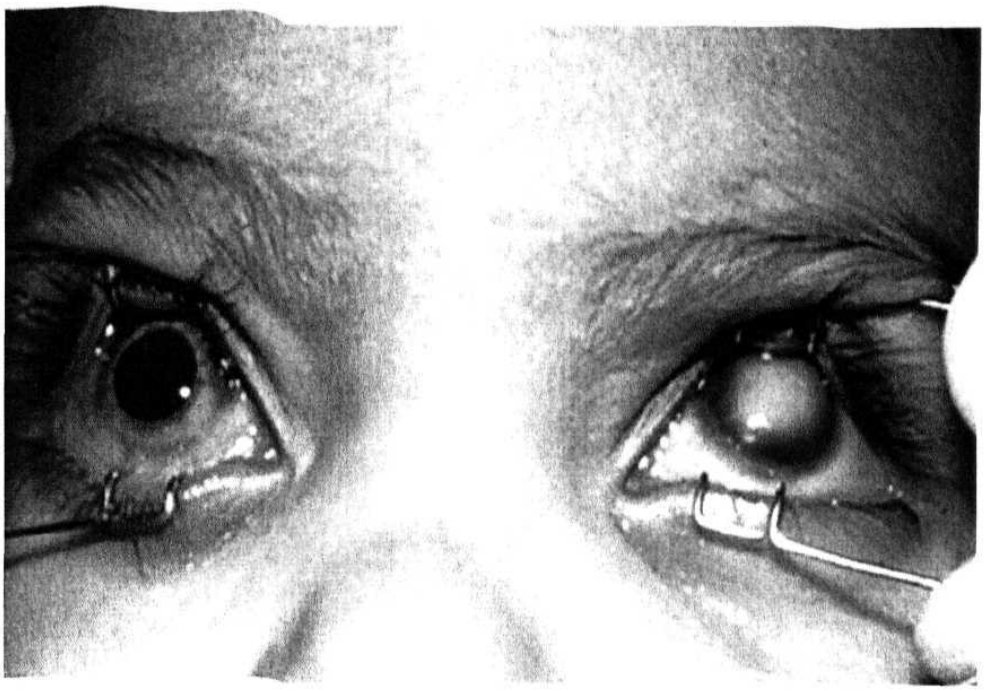

FIGURE 5. Photograph showing blind phenotype in the proband in family PCG4, who had a novel homozygous frameshift mutation (376insA).

etrance could have profound clinical implications in the management of PCG. This will facilitate prenatal diagnosis for this condition, which carries high life-long morbidity. Indeed, further screening oi probands using the simple, fast, and inexpensive PCR-RFLP diagnostic methods developed in this study has enabled us to rapidly identify similar mutations in several other PCG-affected families (Reddy et al., manuscript in preparation). However, further analysis of more families with P(Xi is needed to determine the clinical correlation with the severity of the disease, if any.

Acknowledgments

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Novel Mutation in *FOXCl* Wing Region Causing Axenfeld-Rieger Anomaly

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PURPOSE. TO determine the possible molecular genetic defect underlying Axenfeld-Rieger anomaly (ARA) and to identify the pathogenic mutation causing this anterior segment dysgenesis in an Indian pedigree.

METHODS. The *F0XC1* gene was amplified from genomic DNA of members of an ARA-affected family and control subjects using four novel sets of primers. The amplicons were directly sequenced, and the sequences were analyzed to identify the disease-causing mutation.

RESULTS. A heterozygous novel missense mutation was identified in the coding region of the *FOXCl* gene in all three-patients in this family. Consistent with the autosomal dominant inheritance pattern, the mutation segregated with the disease phenotype and was fully penetrant. The mutation was found in the wing region of the highly conserved forkhead domain of the *FOXCl* gene and resulted in a very severe phenotype leading to blindness.

CONCLUSIONS. This is the first study to demonstrate that a mutation in the *F0XC1* wing region can cause an anterior segment dysgenesis of the eye. This mutation resulted in blindness in the ARA-affected family, and the findings suggest that the *FOXC1* wing region has a functional role in the normal development of the eye. Moreover, this is the first study from India to report the genetic etiology of Axenfeld-Rieger anomaly. Genotype-phenotype correlations of *FOXC1* may help in establishing the disease prognosis and also in understanding the clinical and genetic heterogeneity associated with various anterior segment dysgenesis caused by this gene. (*Invest Ophthalmol Vis Sci.* 2002;43:3613-3616)

The glaucomas are a group of heterogeneous disorders and are a major cause of blindness worldwide. Axenfeld-Rieger anomaly (ARA) is a form of developmental glaucoma, caused by the maldevelopment of the anterior segment of the eye.[1] It is inherited in an autosomal dominant fashion, and glaucoma develops in 50% to 75% of the cases.[2] It consists of a spectrum of developmental defects of the anterior chamber of the eye, with wide variability in expression. Ocular features in ARA include prominent anterior Schwalbe's line, abnormal angle tissue, hypoplastic iris, polycoria, corectopia, and glaucoma.[3] One gene for this disorder has been mapped to chromosome 6 in the p25 region.[4] A few mutations in a forkhead/winged-helix transcription factor gene *FOXCl* (formerly known as *FREAC3* and *FKHL7*) have been implicated in the pathogenesis of this disorder.[4,5]

Although several cases of this disorder with varying severity and manifestations have been identified in India, the genetic etiology was unknown. Therefore an Indian pedigree with multiple affected members in two generations was studied to identify the genetic defect. We herein report the identification of a novel wing mutation in the forkhead domain of the transcription factor gene *FOXCl* that causes the defect, the possible functional role, the diagnostic method developed, and the genotype-phenotype correlation of the mutation.

METHODS

Clinical Evaluation and Patient Selection

The study protocol adhered to the tenets of the Declaration of Helsinki. After providing informed consent, one clinically well-characterized nonconsanguineous ARA-affected family with four members was recruited for the study, This family was selected because it had three affected members in two generations, and all family members were available for the investigation. Patients and family members were evaluated by a glaucoma specialist (AKM) and were followed up for 10 years. Father and both children were affected; the mother was not affected. The clinical data and phenotypes of all three patients are described in Table 1. Ophthalmic examinations included slit lamp biomicroscopy, gonioscopy, measurement of intraocular pressure (IOP) and visual acuity. This family did not exhibit any systemic abnormalities, and no extraocular manifestations were seen.

Mutation Screening and Sequence Analyses

Because mutations in *FOX Cl* are known to cause ARA, the entire-coding region (1.6 kb organized in one exon)[4] was screened for mutations. DNA was extracted from the peripheral leukocytes of the three patients, the mother, and the control subjects. Previous studies had used either 9 or 12 sets of overlapping primers to amplify this highly GC-rich gene.[4,6,7] For mutation screening, these studies had used either single-strand conformation polymorphism (SSCP) followed by sequencing or direct sequencing of polymerase chain reaction (PCR) products, with these sets of overlapping primers. In the present study, mutation screening strategy was simplified by four novel sets of overlapping primers developed by us, Using these primers (spanning the entire exon), we amplified the *FOXCl* gene in patients and control subjects (Table 2). Amplicons were sequenced directly, and patient and control sequences were compared, to identify all sequence variations. The primers used and PCR conditions are described (Table 2). The same sets of primers were used for PCR and bidirectional sequencing.

All PCR reactions were done (PTC 200; MJ Research, Watertown, MA), using 100 ng genomic DNA in 25-μL reactions containing 1 X PCR buffer, 200 μM of the dNTPs, 0.5 μM of each primer, 10% dimethyl sulfoxide (DMSO), and 1 U *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania). All PCR products were purified on separation columns

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TABLE 1. Clinical Data and Phenotypes of Subjects with ARA

Pedigree/ Subjects	Age at Onset	Age at Diagnosis	Corneal Diameter at Diagnosis (mm) (OD; OS)	IOP at Diagnosis (mm Hg) (OD; OS)	Last C/D Ratio (OD; OS)	Last Recorded Vision (OD; OS)	Presence of Schwalbe's Line and Abnormal Angle Tissue (OU)	Presence of Corectopia, Hypoplastic Iris, and Polycoria (OU)	Corneal Changes/ Clarity at Diagnosis (OU)	Age at Surgical Intervention	Treatments (OD; OS)
ARA I.1 (affected father, proband)	By birth	24 y	NA OU	42; 35	NA, total cupping	NPL OD; CF 1 m OS	+	+	Megalocornea, Hazy, Edema	24 y	1× Trab OS; Medical OS
ARA II.1 (affected child)	By birth	2 wk	11; 11.5	24; 26	0.2:0.3	20/360 OU*	+	–	Megalocornea, Hazy, Edema	2 mo	1× Trab/Trab OU
ARA II.2 (affected sibling)	By birth	2.5 mo	12 OU	30; 28	0.4:0.3	20/80 OU†	+	–	Megalocornea, Hazy, Edema	2.5 mo	1× Trab/Trab OU

IOP, intraocular pressure; OD, right eye; OS, left eye; OU, both eyes; C/D, cup-to-disc ratio of the optic nerve; NPL, no perception of light (blind); +, present; –, absent; CF, counting fingers; NA, not available; X, Times; Trab, Trabeculectomy; Trab/Trab, combined trabeculotomy and trabeculectomy; 1 m, one meter; y, year; wk, week; mo, month.

* Best spectacle corrected visual acuity.

† Unaided vision.

(Amicon Microcon PCR; Millipore, Bedford, MA), terminator cycle sequencing was performed (BigDye kit; Applied Biosystems, Foster City, CA), and sequencing reactions were performed on an automated DNA sequencer (ABI Prism 377; Applied Biosystems).

PCR-Restriction Fragment Length Polymorphism Analysis and Coscgrcgation of Mutant Alleles with Disease Phenotype

The novel mutation identified in this stud)' resulted in loss of the *Nla*III recognition site. For determining t he cosegregation of mutant alleles with disease phenotype in the family, the respective fragment harboring the mutation was amplified from all family members, by using set 11 of the primers (Table 2), and an aliquot of amplicon was digested with *Nla*III restriction enzyme (New England Biolabs, Beverly, MA). The fragments were separated on 8% polyacrylamide gel, stained with ethidium bromide, and visualized to distinguish the wild-type and the mutant alleles. Sixty-one ethnically matched volunteer donors without history of eye disorders served as control subjects,

Sequence Alignment

Multiple sequence alignment was performed by submitting various forkhead protein sequences to the European Bioinformatics Institute server. Alignment was performed with Clustal W software (provided in the public domain by the European Bioinformatics Institute, 1 linxton, UK, and available at http://www2.ebi.ac.uk/clustalw).

RESULTS

Identification of a Novel Pathogenic Mutation in the *FOXC1* Wing Region

All three patients (the father and two children) in an ARA-affected family had a heterozygous missense mutation (T→A substitution) at 482 bp (cDNA position) in the highly conserved forkhead domain of the *FOXC1* transcription factor gene. This mutation resulted in the change of amino acid methionine to lysine at 161 amino acid position (M161K) in FOXC1 and also abolished the *Nla*III recognition site in the DNA (Fig. 1). PCR-restriction fragment length polymorphism (RFLP) analysis showed that mutant alleles segregated only with the disease phenotype (Fig. 2) and not with the unaffected mother and the control subjects analyzed. This mutation was also absent in 61 ethnically matched control subjects (data not shown). The mutated methionine residue has been conserved across various species during evolution (Fig. 3).

The forkhead domain contains three α helices and two wing regions. The respective amino acid positions in FOXC1 are α helix-1: 83 to 93; α helix-2: 101 to 110; α helix-3: 119 to 132; wing-1: 143 to **151** and wing-2: 155 to 176.[8,9] Because the amino acid change occurred at position 161, it is in the wing-2 region.

Genotype–Phenotype Correlations

Variable expression of the disease phenotype was noticed between two affected generations of this family (Table 1). This mutation resulted in a very severe phenotype in the father, which, without prompt and early surgical intervention, led to blindness. In contrast, the same mutation with early surgical intervention in the children resulted in moderate severity and reasonably good prognoses (Fig. 4).

DISCUSSION

This is the first study to demonstrate that a mutation in the wing region of the forkhead/winged-helix transcription factor gene *FOXC1* can result in an anterior segment dysgenesis of the eye, ARA. Therefore, the mutation reported herein is a

TABLE 2. Novel sets of *FOXCJ* Primers and Conditions Used for Amplification

Primer Sets Used for Amplification (5′-3′)/Size	Position in cDNA (bp)	Fragment Size (bp)	$MgCl_2$ Used (mM)	Annealing Temperature (°C)
ARA1F - CCCGGACTCGGACTCGGC - 18 mer ARA1R - AAGCGGTCCATGATGAACTGG - 21 mer	−93 to −76 335-315	429	1	62
ARA2F - CCCAAGGACATGGTGAAGC - 19 mer ARA2R - CTGAAGCCCTGGCTATGGT - 19 mer	217-235 926-908	710	1	58
ARA3F - ATCAAGACCGAGAACGGTACG - 21 mer ARA3R - GTGACCGGAGGCAGAGAGTA - 20 mer	676-696 1310-1291	635*	1	58
ARA4F - TACCACTGCAACCTGCAAGC - 20 mer ARA4R - GGGTTCGATTTAGTTCGGCT - 20 mer	1177-1196 1693-1674	517	1.25	58

PCR conditions for all primer sets: initial denaturation at 94°C for 3 minutes followed by (94°C for 30 seconds, annealing for 30 seconds), 72°C for 45 seconds for 35 cycles, with final extension at 72°C for 7 minutes.

* Because of a nonspecific fragment, set 3 PCR fragment was eluted, diluted 1;10, and reamplified for sequencing, with 25 cycles.

novel one. Moreover, this is the fourth mutation in this gene known to cause the ARA phenotype and the first study from the Indian subcontinent to describe the molecular basis of ARA. So far, only three *FOXC1* mutations have been reported to cause this disorder. These include a 10-bp deletion in the upstream of the forkhead domain,[4] a missense mutation (Ile87Met),[4] and a single nucleotide insertion[5] in the al helix region in the forkhead domain of this gene. Consistent with the autosomal dominant inheritance, all three affected members had one mutant allele segregating with the disease phenotype, and the mutation was fully penetrant in the family.

Segregation of mutant alleles with the disease phenotype, absence of mutant alleles in control subjects, and a high degree of conservation of mutated residue across species during evolution imply that the mutation we report is pathogenic. Although various mutations in the *F0XC1* gene have been implicated in the pathogenesis of a spectrum of ocular disorders such as Axenfeld anomaly, Axenfeld-Rieger syndrome, Rieger anomaly, Iris hypoplasia, Peter's anomaly, iridogoniodysgenesis type 1, ARA, and congenital glaucoma, none was found to be in the wing region.[4–8,10] Hence, this is the first wing mutation known to cause any anterior segment dysgenesis related to the *FOXC1* gene.

FOXC1 is a member of the forkhead/winged-helix family of transcription factors. These transcription factors contain a monomeric, 110-amino-acid DNA binding domain (forkhead domain) and have been conserved throughout evolution from yeast to human.[11,12] This DNA-binding motif is a variant of the helix-turn-helix motif and consists of three a helices and two large loops that form wing structures, W1 and W2.[8,9,12] Amino acids 155 to 176 of the forkhead domain constitute the wing-2 region.[8,9] The mutation in this study was found in amino acid position 161, within the wing-2 region (see the Results section). So far, no other mutation has been reported in this region. Therefore, this is the first mutation identified in the wing-2 region of the FOXC1 transcription factor. The forkhead/winged-helix family of transcription factors are essential in a variety of developmental processes, including embryogenesis and tissue specific cell differentiation.[11,12] It has been reported that the W2 region of HNF-γ (a member of the forkhead family of transcription (actors) contacts with the minor groove of DNA.[9] Because FOXC1 is a transcription factor, the W2 mutation could possibly affect the DNA-protein interaction. Haploinsufficiency of forkhead transcription factors has been shown to cause aberrant ocular development.[8,13] Considering these facts, it is tempting to speculate that this mutation may affect the migration and/or differentiation of the mesenchymal cells that contribute to the anterior segment of the eye.[13]

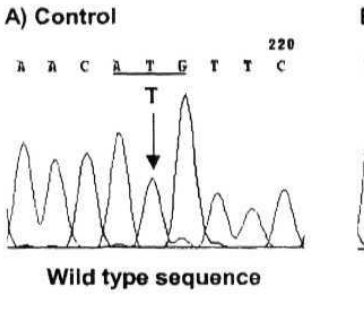

FIGURE 1. Electropherogram of the sense strand of genomic DNA from the ARA proband, showing a novel heterozygous missense mutation. The heterozygous change 482 t→a (M161K) was present in the mutant allele of the proband (**B**) and absent in the control (A). The mutation *(underscore* and *arrow)* results in a change in amino acid at codon 161.

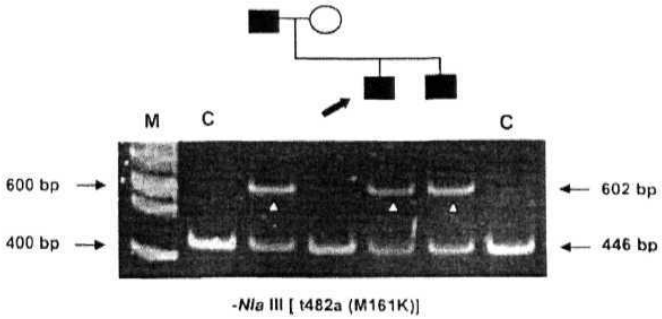

FIGURE 2. PCR-RFLP analysis of cosegregation of the mutant allele with the disease phenotype in an ARA pedigree. (•) Affected male individuals; (○) unaffected female individual; *arrow*: proband. DNA molecular weight marker *{lane M)* in base pairs *(left);* allele sizes *(right)',* control *(lane C)*; mutant allele *(small arrowheads'),* restriction site change and mutation (nucleotide as well as amino acid changes) are shown *below* the gel. The 710-bp *F0XC1* amplification product generated from the *FOXCJ* primer pair 2F/2R (Table 3) was cleaved by *Nla*III into five fragments of sizes 8, 40, 60, 156, and 446 bp in unaffected individuals. The T482A mutation in the affected individuals abolished the *Nla*III site between the 156- and 446-bp fragments, and the resultant 602-bp mutant allele segregates along with the disease phenotype. The normal *Nla*III cleavage products present in affected individuals were generated from the wild-type *F0XC1* allele in these individuals. Fragments less than 446 bp are not shown in this 8% polyacrylamide gel.

The abnormal eye development and the devastating phenotype (blindness in the father) in this family indicates that the FOXC1 wing region has an important functional role in the normal development of the eye (Fig. 4; Table 1). The genotype-phenotype correlation indicates that this mutation resulted in blindness in the father (very severe phenotype and very poor prognosis). The lather had asymmetric manifestations in both eyes (the right eye became blind in childhood; the left eye had very poor vision), whereas the children had uniform manifestations. Late surgical intervention in the father's left eye did not restore vision. Hypoplastic iris, polycoria, and corectopia were present in the father, which indicated an advanced stage of the disease. These manifestations were absent in the children (Table 1). The differences in clinical manifestations between the two affected generations may be attributable to the age of surgical interventions—late surgery in the father (24 years of age) and early surgery in the children (2-2.5 months)—or to the late diagnosis of the disease in father (Table 1).

Several anterior segment dysgenesis show overlapping clinical features and many arc due to different mutations in the *V0XC1* gene.[1,3-8,10] Hence, the clinical and genetic heterogeneity of anterior segment disorders can be understood better with the accumulation of genotype data of the *FOX Cl* gene. It may also help in the classification of these disorders as well as in understanding the prognosis of the disease.

In sum, this study adds one novel mutation to the existing spectrum of mutations that cause anterior segment dysgenesis and also provides insight into the functional dissection of the *F0XC1* gene. The novel sets of primers used in this study have simplified the mutation screening strategy of the *F0XC1* gene. This investigation also suggests that the *FOXC1* gene is possibly defective in other Indian ARA-affected families. However, further analysis of ARA families is needed to establish the genotype-phenotype correlations of this ocular disorder,

Acknowledgments

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M161K

FOXE3 (human)	P	A	A	A	D	M	F	D	N	G	...	AAF82793.1
FOXE1(human)	P	N	A	E	D	M	F	E	S	G	...	AAC51294.1
FOXD2 (human)	P	E	S	A	D	M	F	D	N	G	...	AAC15421.1
FOXD4 (human)	P	A	S	Q	D	M	F	D	N	G	...	AAA92040.1
FOXD3 (rat)	P	Q	S	E	D	M	F	D	N	G	...	AAA41319.1
FOXD3 (zebrafish)	P	Q	S	E	D	M	F	D	N	G	...	AAC06366.1
FOXD1 (human)	P	E	S	A	D	M	F	D	N	G	...	AAC50661.1
FOXC1 (human)	P	D	S	A	N	M	F	E	N	G	...	AAC18081.1
FOXC1 (mouse)	P	D	S	Y	N	M	F	E	N	G	...	AAC24209.1
FOXC1 (zebrafish)	P	D	S	Y	N	M	F	E	N	G	...	AAG44241.1
FOXC2 (human)	P	D	S	Y	N	M	F	E	N	G	...	CAA69400.1
FOXC2 (mouse)	P	D	S	Y	N	M	F	E	N	G	...	CAA63244.1
FOXC2 (rat)	P	D	S	Y	N	M	F	E	N	G	...	AAA41320.1
FKHL18 (human)	P	D	C	H	D	M	F	E	H	G	...	AAC15420.1
Croc (drosophila)	P	D	S	Y	N	M	F	D	N	G	...	AAB35643.1
FOXL1 (human)	P	R	C	L	D	M	F	E	N	G	...	AAG40312.1
FOX11 (mouse)	P	R	C	L	D	M	F	E	N	G	...	CAA63243.1
FOXA1 (zebrafish)	P	D	S	G	N	M	F	E	N	G	...	AAC06367.1
FOXA2 (zebrafish)	P	D	S	G	N	M	F	E	N	G	...	CAA80443.1
Fkh (drosophila)	P	D	S	G	N	M	F	E	N	G	...	AAA28535.1
FOXF1 (human)	P	A	S	E	F	M	F	E	E	G	...	AAC50399.1
FOXF2 (human)	P	A	S	E	F	M	F	E	E	G	...	AAD19875.1
FOXG1C (human)	P	S	S	D	D	V	F	I	G	G	...	CAA52241.1
FOXG1B (human)	P	S	S	D	D	V	F	I	G	G	...	CAA52239.1
FOXG1A (human)	P	S	S	D	D	V	F	I	G	G	...	CAA55038.1
FOXJ1 (human)	P	Q	Y	A	E	R	L	L	S	G	...	CAA67729.1

FIGURE 3. Multiple sequence alignment of human FOXC1 and related FOX protein sequences. *Bold letter* with *asterisk* (*) and *shading* represent the conserved residue methionine M161K (when mutated) that causes the ARA phenotype. *Right:* sequence accession numbers. The human FOXC1 sequence is *underscored.*

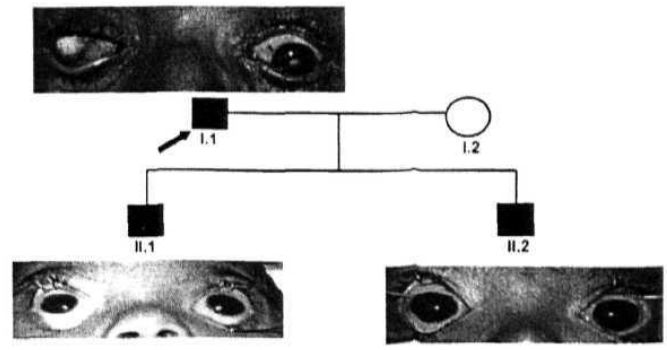

FIGURE 4. Phenotypes of an ARA pedigree after surgery showing varying prognosis. I.1 was the affected father with late surgical intervention showing a very severe phenotype and a very poor prognosis (blind in the right eye and very poor vision in the left eye). II .1 and II.2 were the affected children with early surgical intervention showing clear corneas and reasonably good prognoses. Conical edema completely cleared after surgery. Follow-up periods after surgery were I.1: 10 years, II. 1: 4 years, and II.2: 3 years. For ages of surgical interventions in affected members, refer to Table 1.

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Identification of R368H as a Predominant *CYP1B1* Allele Causing Primary Congenital Glaucoma in Indian Patients

Aramati B. M. Reddy,[1] *Shirty G. Panicker,*[1] *Anil K. Mandal,*[1] *Seyecl E. Hasnain,*[2] *and Dorairajan Balasubramanian*[1]

PURPOSE. TO investigate the predominant mutation in the *CYP1B1* gene in patients in India with primary congenital glaucoma (PCG), using PCR-restriction fragment length polymorphism (RFLP) methods and to characterize the molecular defect in two generations of an affected family.

METHODS. DNA samples from 146 patients with PCG from 138 pedigrees were analyzed for several distinct mutations in *CYP1B1* by PCR-RFLP.

RESULTS. PCR-RFLP screening revealed that 30.8% of patients were positive for any one of the six mutations (376insA, 528G→A, 923C→T, 959G→A, 1449G→A, and 1514C→A), and 17.8% of the patients were found to have the rarely reported mutation R368H (1449G—*A). All mutations were confirmed by DNA sequencing.

CONCLUSIONS. The results suggest extensive allelic heterogeneity in the Indian patients with PCG, with the predominant allele being R368H among the 146 Indian patients tested. It appears possible to use this approach for carrier detection in pedigrees with a positive family history and in population screening. The approach also offers a method for rapid screening of potential carriers and affected individuals. (*Invest Ophthalmol Vis Sci.* 2003;44:4200-4203) DOI:10.1167/iovs.02-0945

Primary congenital glaucoma (PCG) manifests at birth or in early infancy. The phenotype is characterized by elevated intraocular pressure, resulting in photophobia, corneal clouding, and enlargement of the globe, which, if left untreated, results in optic nerve damage and subsequent permanent loss of vision. The incidence of PCG varies geographically. Its incidence is as low as 1 in 10,000 persons in Western countries,[1] and as high as 1 in 1,250 in the Slovak population.[2] In Saudi Arabia, the reported incidence is 1 in 2,500,[3] whereas in the state of Andhra Pradesh in India, the incidence is estimated to be 1 in 3,300.[4] The high incidence in the Eastern populations is thought to be due to consanguineous marriage within these communities. Because PCG is mainly a congenital disorder, early and reliable diagnosis of the disease is vital, so that appropriate and prompt medical and surgical interventions can be initiated in time. This could in turn prevent unwanted visual loss, hence saving the vision in the child.

An autosomal recessive mode of inheritance pattern is well documented for PCG. Even though three different loci have been mapped for PCG[5,6] (Stoilov IR, et al. *IOVS* 2002;43:ARVO E-Abstract 3015), mutations in the *CYP1B1* gene (GLC3A locus[5]) is the most predominant[7] and is reported in various ethnic backgrounds.[7-20] An additional PCCJ locus, GLC3B,[6] has been mapped to the short arm of chromosome 1, region 36, and a third locus, GLC3C (Stoilov IR, et al. *IOVS* 2002;43:ARVO E-Abstract 3015), to 14q24.3, but the genes have not been identified in these two loci. Recently, we showed the association of *CYP1B1* with PCG in the Indian population[18] and detected five distinct mutations.

Although genetic heterogeneity has been shown for PCG, homogeneity in phenotype as well as genotype (E387K) has been reported in the Slovakian Romany people, and common haplotypes (G61E, D374N, R469W) have been associated with the Saudi Arabian population.[11,12] Inbreeding and consanguinity are prevalent in these communities, as in India. Thus, it is of interest to determine which haplotypes are present in the Indian patients. Against this background, we now describe the results of screening for the known mutations in a cohort of 138 pedigrees of 146 patients, by using PCR-RFLP-based simple diagnostic methods.

MATERIALS AND METHODS

Selection and Evaluation of Study Subjects

The study protocol adhered to the tenets of the Declaration of Helsinki. After receiving due informed consent and appropriate clearance from the institutional review board, we recruited 146 patients for the study who were members of 138 pedigrees. The patients were completely unbiased with respect to sex, consanguinity, and familial incidence of the disease. All were clinically evaluated, and diagnosis of PCG was determined by examination with slit lamp biomicroscopy and gonioscopy, measurement of intraocular pressure, and perimetry, in some cases. Blood samples were collected over a period of 2 years in the Children's Eye Care Centre at the Institute. Seventy ethnically matched normal individuals served as control subjects,

Mutation Screening of *CYP1B1*

Genomic DNA was extracted from the peripheral leukocytes of all patients with PCG and control subjects. The translated region (1.6 kb) spanning exons II and III of the gene for cytochrome P4501B1 (*CYP1B1*)[21] from patients and control subjects were amplified by using three sets of primers, as described earlier.[18]

PCR-Restriction Fragment Length Polymorphism and Direct Sequencing

The PCR-RFLP methods described earlier[18] were followed, along with an *Hin*6I(MBI Fermentas, Vilnius, Lithuania) restriction enzyme-based RFLP for 1514C→T (R390C) mutation. DNA samples from 70 volun-

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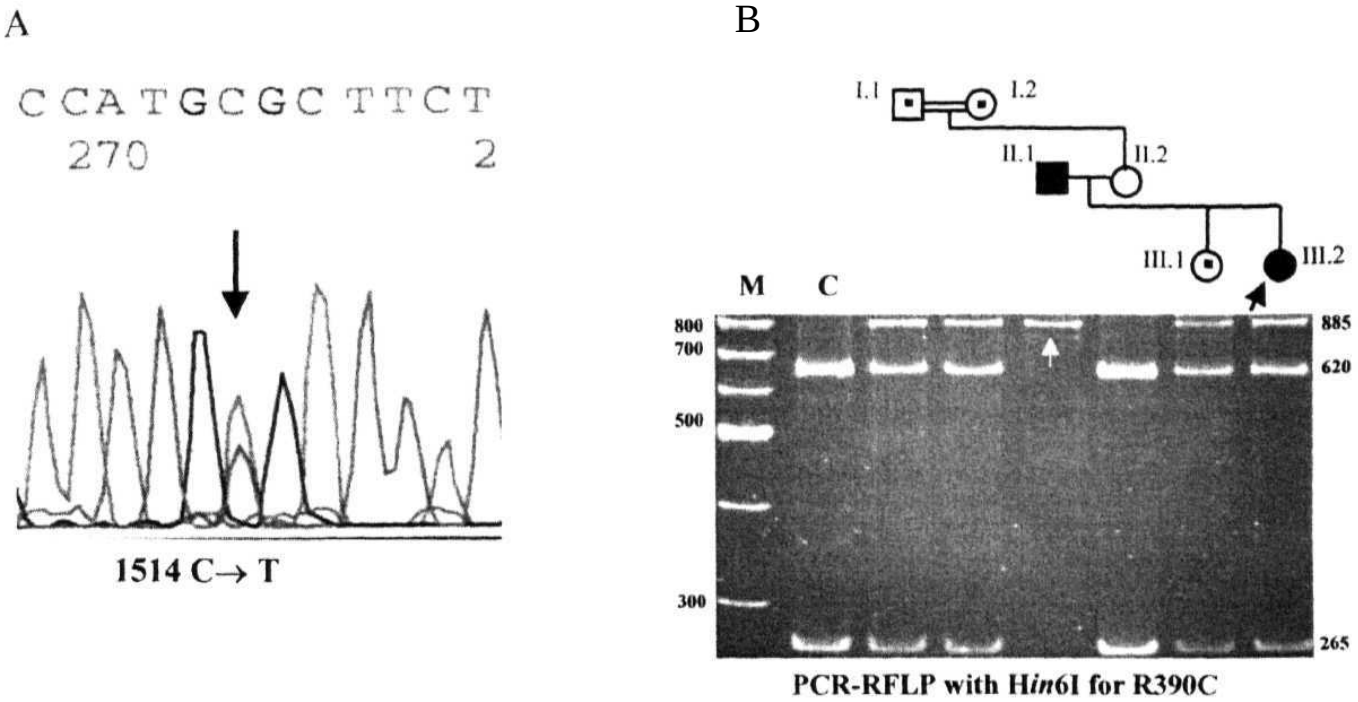

FIGURE 1. (A) Electropherogram of the sense strand of genomic DNA from PCG proband, with novel heterozygous missense mutation. Note the heterozygous change 1514C→T (R390C) in the mutant allele of the proband (*arrow*).(**B**) PCR-RFLP analysis of cosegregation of a mutant allele with a disease phenotype in a PCG pedigree. *Filled square:* affected individuals; *filled circle:* unaffected individuals; *arrow:* the proband. Males and females are shown by *squares* and *circles*, respectively. *I.e/1:* DNA molecular weight marker in base pairs; *right:* allele sizes; *lane C:* control; *arrowhead:* mutant allele, Restriction site change and mutation (nucleotide as well as aa changes) are shown at the *bottom* of the gel. The BHVbp amplification product generated from primer pair 3F/3R[19] was cleaved by *Hin*6I into two fragments of sizes 620 and 26S bp in unaffected individuals. The C1514T mutation in the affected individuals abolished the *Hin* 6I site, and the resultant uncut 885-bp mutant allele segregated along with the disease phenotype. The "normal" *Hin*6I cleavage products present in affected individuals were generated from the nonmutated *CYP1B1* allele in these individuals.

tary donors, without a history of systemic and eye disorders, were used in control experiments. PCR-RFLP-positive samples were sequenced (for reconfirming the respective mutations) using an automated DNA sequencer (Dig Dye Terminator cycle sequencing, ABI Prism 3700; Applied Biosystems, Foster City, CA),

RESULTS

Identification of a Novel Pathogenic Mutation

Direct sequencing of the complete coding region of a proband's DNA from two generations of an affected pseudodominant family (005) revealed a compound heterozygous missense mutation. The first one was a known mutation,[12,18,20] a G→A substitution at base pair position 1449, leading to the amino acid (aa) change arginine to histidine at position 368 (K368H), whereas the second mutation was a novel sequence variation, a C→T substitution at base pair 1514 (Fig. 1A), causing the change arginine to cysteine at position 390 (R390C). The father (Fig. 1B, II. 1) in the same family, also affected by FCG, was homozygous for the novel mutation 1514C→T. The grandparents (I.1 and 1.2) as well as the unaffected sibling (III.1) were heterozygous (carriers) for the mutation. Both mutations, R368H and R390C were found in exon III and resulted in loss of restriction sites *Taa*I and *Hin*6I respectively. The cosegregation of mutations in the family was ascertained by using the PCR-RFLP method. In this pedigree, the grandparents (I.1 and I.2) had a consanguineous marriage, whereas the parents (II. 1 and II.2) were nonconsanguineous (Fig. IB).

PCR-RFLP Analyses of Six Pathogenic Mutations

PCR-RFLP analyses were performed for all six mutations: 376insA, 528G→A, 923C→T, 959G→A, 1449G→A, and 1514C→T. Of the 146 patients in 138 pedigrees, 45 patients in 37 pedigrees were positive for one of these six mutations. All the PCR-RFLP-positive samples were subsequently sequenced to confirm the mutation. More than 30% of the patients were carriers of the respective mutation, as revealed later by sequencing. Among the six mutations, R368H was the predominant PCG allele in this cohort, and 17.8% of the patients were found to be either homozygous or heterozygous for this mutation.

DISCUSSION

PCG is a clinically and genetically heterogeneous disorder, mainly inherited as an autosomal recessive disease, that occurs among various populations of the same ethnic background. Recent molecular genetic studies in various ethnic groups, such as Turkish, Hispanic, Saudi Arabian, Romanian, Brazilian, Canadian, Japanese, Pakistani, German, Lebanese, and Indian, revealed several mutations in the coding region of *CYP1B1*. All these studies have so far repotted approximately 44 different mutations in the entire coding region of *CYP1B1*,[17-20] and the mutations' heterogeneity varies with the population.

The homogeneity-heterogeneity pattern varies with ethnic-groups, as does the phenotypic uniformity of the condition. Whereas the Slovak Romany cases showed allelic homogeneity and phenotypic uniformity,[11] other population studies reported high clinical and allelic heterogeneity. Among these groups, higher homogeneity was present in the Saudi Arabian population (with 72% having the G61E allele and 12% the R469W allele[12]), whereas other populations demonstrated increased genetic heterogeneity. The homogeneity reflects the higher rate of inbreeding in this population. Our PCR-RFLP

TABLE 1. Distribution of Six Mutations in Consanguineous and Nonconsanguneous, Pedigrees

Pedigree ID	Mutation	Consanguineous/ Nonconsanguineous
0001 (2)	P193L (h)	Consanguineous
	E229K (h)	
0002	R368H (H)	Consanguineous
0004 (2)	376Ins A H)	Consanguineous
0005 (2)	R368H (h)	Nonconsanguineous
	R390C (h)	
0006 (2)	R368H (H)	Consanguineous
0011	G61E (H)	Consanguineous
0012 (2)	R390C (H)	Consanguineous
0017	R368H (H)	Nonconsanguineous
0018	R390C (H)	Consanguineous
0022	R368H (H)	Consanguineous
0024	E229K (h)	Consanguineous
0025	R368H (h)	Nonconsanguineous
0035 (2)	R368H (H)	Consanguineous
0037	E229K (h)	Consanguineous
0039	R368H (h)	Nonconsanguineous
0040	R368H (H)	Consanguineous
0051	R368H (H)	Nonconsanguineous
0057	E229K (h)	Consanguineous
0058	G61E (H)	Consanguineous
0067	R368H (H)	Consanguineous
0069	P193L (h)	Nonconsanguineous
0071 (2)	R368H (H)	Nonconsanguineous
0075	R368H (h)	Nonconsanguineous
0076	R368H (H)	Consanguineous
0079	R368H (H)	Nonconsanguineous
0092	R390C (H)	Nonconsanguineous
0093 (2)	G61E (H)	Consanguineous
0095	R368H (h)	Nonconsanguineous
0100	R368H (h)	Consanguineous
0116	E229K (h)	Consanguineous
0125	E229K (h)	Nonconsanguineous
0130	R368H (H)	Nonconsanguineous
0136	R368H (H)	Nonconsanguineous
0137	R368H (H)	Nonconsanguineous
0143	R390C (H)	Nonconsanguineous
0144	R368H (H)	Nonconsanguineous
0150	R368H (H)	Nonconsanguineous

h, Heterozygous mutation; H, homozygous mutation; (2) two patients in the same pedigree.

screening, for six distinct alleles, in a cohort of 146 patients in 138 pedigrees showed a frequency of 16.21% for allele R368H.

This mutation has so far been reported in only a vet*)' few PCG families from Saudi Arabia and Brazil and at a very low frequency ,12.20 In the present study, however, based on the mutation screening, we found it to be a predominant allele associated with PCG in India. This is the highest reported frequency of this mutation of all ethnic backgrounds studied so far, indicating that the frequency of the mutation could vary based on the ethnic origin as well as geographical location. Sequence analysis of the remaining families negative for these six mutations should to be performed to determine whether there are any other predominant alleles in Indian patients with PCG. The possibility of locus heterogeneity in Indian patients with PCC; also should be explored further.

Ethnically matched population screening of 140 chromosomes for these six mutant alleles showed 6.4% and 0.7% carriers for E229K and R368H, respectively. The present data are unlikely to be due to a possible founder effect for the predominant R368H allele, because patients were from ethnically as well as geographically diverse groups in India. Also, these mutations are equally distributed in both consanguineous and nonconsanguineous pedigrees (Table 1). Of the total families recruited, 51.5% belonged to the nonconsanguineous group. Sporadic cases accounted for 80%, and bilateral 88%. Males accounted for 57% of the affected individuals.

Mutations at codon .390, where arginine is changed to cither histidine or serine, have been reported.[10,12] This is the first report of arginine changing to cysteine at the same codon. *Hin*6I-based PCR-RFLP can be therefore used to detect any of these mutations at this codon. Although mutations at codons 368 and 390 have been reported earlier, our report of this combination of mutations is novel, as is the sequence variation in codon 390. This two-generation affected family is also interesting in that whereas the affected father (II. 1) had a homozygous mutation (R390C), the mother (II.2) was a carrier of one of the alleles (R368H; data not shown), and the proband (affected child 111.2) had a compound heterozygous mutation (R390C and R368I1). Consistent with the autosomal mode of inheritance, the affected individuals (II. 1 and III.2) in this pedigree had two mutant alleles from their respective parents; the mutations were completely penetrant.

(Consanguineous marriages and marriages within a distinctive caste or community increase the predisposition and incidence of recessively inherited and multifactorial diseases in the population. It is important to know the carrier status of unaffected members in the families with a positive history to identify the at-risk individuals in such families. Earlier studies have reported that 30% to 35% of blind children in India show a history of hereditary disorder.[22] In the higher socioeconomic levels of developed countries, 22% to 55% of children with genetic disease show an autosomal recessive mode of inheritance.[23] Hence, development of techniques such as PCR-RFLP, the amplification refractory mutation system (ARMS)-PCR, allele-specific oligonucleotide (ASO) blot analysis, and other methods are important for segregation analysis in families with a positive history and for possible prenatal diagnosis and genetic counseling. Moreover, because this disease carries high and life-long morbidity, development of strategies that are noninvasive, rapid, and cost-effective arc very useful in screening populations with a high incidence of this disease. This could in turn help in identifying individuals at risk and also assist in preventing unwanted visual loss in the afflicted families. An earlier study on thalassemia major in a Sardinian population showed that genetic screening and counseling helped to reduce the incidence from 1 in 250 live births to 1 in 4000.[24]

Similarly, the molecular diagnostic methods used in the current study could be used as an added clinical tool in decreasing the incidence of the devastating binding disorder PCG in the afflicted families.

Moreover, our clinical experience in PCG has shown that early diagnosis, along with prompt medical and surgical interventions, result in better prognosis.[16] We thus sec the use of the PCR-RFLP molecular diagnosis described in this study as a tool to identify the disease early and to initiate appropriate and prompt treatments, especially in patients with late manifestation and positive family history. Based on this study, we suggest that PCG mutation screening in India should be performed based on the prevalence of the mutation.

Our study shows that 31% of the patients studied had one of the six mutations that we sought in the screening. Whereas only direct screening or methods such as denaturing HPLC can identify all mutations in *CYP1B1,* R368H appears to be the predominant mutant allele causing PCG in the population studied herein. Given this lead, we believe that screening for this mutation should be given priority, and subsequently the other reported mutations should be screened for in the order of prevalence. Thus, the data derived from this study highlight the use of a rapid screening system for mutations that could assist the medical community in the management of this devastating condition

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Mutation spectrum *of FOXCI* and clinical genetic heterogeneity of Axenfeld-Rieger anomaly in India

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Purpose: Axenfeld-Rieger anomaly (ARA) is a form of anterior segment dysgenesis of the eye, mainly caused by mutations in the *FOXC1* gene. We had earlier reported a novel mutation in the wing region of FOXC1 in an autosomal dominant family. The present study was aimed to identify the spectrum of mutations in the *FOXC1* gene in a cohort of Indian ARA patients from different ethnic backgrounds, and to understand its role in the disease pathogenesis.

Methods: Two new autosomal dominant families and seven sporadic cases of ARA from different ethnic backgrounds were screened for mutations by direct sequencing of the coding region of the *FOXC1* gene. Another autosomal dominant ARA family that was previously reported by us was also included for comparative analysis of clinical genetic parameters. The segregation of the mutations in the autosomal dominant families was analyzed by haplotype and restriction analysis. Genotype-phenotypc correlation were also undertaken to study the role of *FOXCI* in phenotypic manifestation in the patient cohort.

Results: Three of the nine ARA cases harbored mutations in *FOXCI,* of which two novel nonsense mutations Q2X and Q123X, resulted in haploinsufficiency of the gene product, The missense mutation (M161K) that we previously reported in an autosomal dominant family was also found in another family, Haplotypc analysis of these two families suggested multiple founders in the same ethnic group. The mutations resulted in variable expressions of phenotype among the patients as assessed from their prognosis based on visual outcomes.

Conclusions: Significant genetic heterogeneity of *FOXCI* was observed in a multi-ethnic population studied in this region of India resulting in variable ARA phenotypes. The different visual outcome seen in the patients suggest a variable expression of FOXC1 and also provide some insight for understanding the gene functions in this population.

Anterior segment anomalies of the eye constitute a complex spectrum of disorders resulting from malformations of endothelial tissues, due to the disruption of migration and differentiation processes in the neural crest [1-3]. Axenfeld-Rieger phenotypes constitute various forms of anterior segment dysgenesis based on the presence of ocular and extra-ocular symptoms. Axenfeld anomaly, Rieger anomaly and Rieger syndrome comprise a series of overlapping phenotypes with systemic-signs that include umbilical, facial and dental anomalies and are collectively grouped as Axenfeld-Rieger syndrome [1,2,4]. Axenfeld-Rieger anomaly (ARA) on the other hand docs not manifest any systemic abnormalities and is associated with clinical symptoms that include a prominent anteriorly displaced Schwalbe's line, insertion of iris processes in the stroma, hypoplasia of the iris, corectopia and pseudopolycoria [4]. The disorder carries with it a 50% risk of developing glaucoma [1,5,6].

Three loci on chromosomes 4q25, 6p25, and 13q14 have been mapped for various Axenfeld-Rieger phenotypes [6]. Of these, mutations in the forkhead winged/helix transcription factor gene *FOXCI* (earlier known as the *FKHL7* gene) on chromosome 6p25 have been implicated in the causation of Axenfeld-Rieger phenotypes, particularly ARA [3,7,8]. Different mutations have been observed in *FOXCI* causing various Axenfeld-Rieger anomalies ranging from frameshift mutations due to deletions resulting in premature termination of translation in the forkhead domain, missense mutations reducing transactivation and protein interactions, and nonsense mutations causing haploinsufficiency of the gene product [1,4,8]. Structure-function analyses of mutations in *FOXCI* have indicated a reduced stability of FOXCI in DNA binding, transactivation of target genes and transcriptional regulation [9].

Four mutations in *FOXCI* have so far been identified in ARA, of which a novel mutation in the wing region of this gene was reported by us, previously in an autosomal dominant ARA family in India [7|. We now report *FOXCI* gene mutations in a series of varied ARA cases and demonstrate the spectrum of clinical genetic heterogeneity with respect to the disease phenotype in the Indian scenario.

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METHODS

Clinical diagnoses and selection of cases: The study consisted of ten unrelated ARA families belonging to various ethnic backgrounds, and from different geographical locales of India, presenting at our Institute at Hyderabad, between 1997

and 2001. Of these, nine were new families, and one was an autosomal dominant family (ARA3) that we had previously reported [7]. The rationale for including the ARA3 family was to have a comparative analysis of the clinical and genetic traits with respect to all the families so far studied at our center. The guidelines of the Declaration of Helsinki were strictly followed and clearance was obtained from the Institutional Review Board prior to undertaking this study. Cases manifesting typical ARA were selected after a thorough clinical evaluation by our ophthalmologist (AKM). All the cases exhibited symptoms of iridocorneal tissue and angle anomaly, a prominently raised Schwalbe's line, corectopia, iris hypoplasia, polycoria, and were without any systemic abnormalities or other extraocular manifestations. Ophthalmic examination included slit-lamp biomicroscopy, gonioscopy, measurement of intraocular pressure (IOP), and visual acuity testing. Each of these patients had a regular follow-up every three months and their detailed clinical findings were recorded. Of the ten families, three had an autosomal dominant mode of inheritance while the rest were sporadic cases. Except for two probands of sporadic cases, the other probands had a congenital onset of the disease. As Axenfeld-Rieger phenotypes normally present overlapping clinical features, differential diagnosis were avoided by restricting the clinical examination and phenotypic assessment of the patients to the same ophthalmologist (AKM). Based on clinical manifestations, 5-10 ml of blood was drawn by venipuncture from the probands, their affected and normal relatives, and controls (after prior informed consent). A total of 72 ethnically matched normal individuals without any prior history of the disease or other systemic illness served as controls.

Mutation screening by sequencing: The human *FOXC1* gene (Genbank accession number AF078096) spans 1.6 kb and its coding region consists of a single exon encoding a 553 amino acid protein [3]. Direct sequencing of this exon in the ARA families screened for mutations. A set of four overlapping primers covering the entire coding region of the *FOXC1* gene (designed by us in the previous study [7]) were used to amplify the DNA samples. Genomic DNA (about 100 ng) were amplified using 1X PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10% DMSO, 0.5 mM of each primer, and 1 unit of *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania) in a 25 µl reaction mixture. PCR was carried out in a PTC 200 thermal cycler (MJ Research, Waltham, MA) and the cycling conditions were as reported in our previous study [7]. The amplicons were purified prior to sequencing using Amicon Microcon PCR columns (Millipore, Bedford, MA). Bi-directional cycle sequencing was performed using the BigDye terminator kit (PE-Applied Biosystems, Foster city, CA) on an automated DNA sequencer AB1 3700 (PE-Applied Biosystems). The sequences were compared with the normal *FOXCI* gene sequence from the database. The segregation of the M161K mutation was analyzed using the restriction enzyme *Nla* III (New England Biolabs, Beverly, MA), as described earlier [7].

Genotyping and Haplotype analysis: In order to understand the founder effect of common mutations and segregation of the disease phenotype, microsatellite markers flanking the *FOXCI* gene locus on chromosome 6p25 were screened in members of three autosomal dominant families. Three markers D6S1574, D6S309 and D6S470 on 6p25 spanning 9.5 cM were selected from the AB1 Linkage mapping set MD-10 (Version 2.5). The markers were amplified following the manufacturer's protocol, electrophoresed on an automated DNA sequencer AB I 377, and analyzed by GENESCAN software (PE-Applied biosystems, Version 2.1). Individuals were genotyped using the GENOTYPER software (PE-Applied biosystems, Version 2.0) and haplotypes were constructed from the genotype data. Multiple repetitions of experiments were done to exclude the possibility of sample contamination.

RESULTS

Mutation screening of the FOXCI *gene:* Direct sequencing of the *FOXC1* coding sequence revealed a C->T nucleotide change at cDNA position 367, resulting in a nonsense mutation (Gln123Stop) in the ARA7 family, causing a truncation

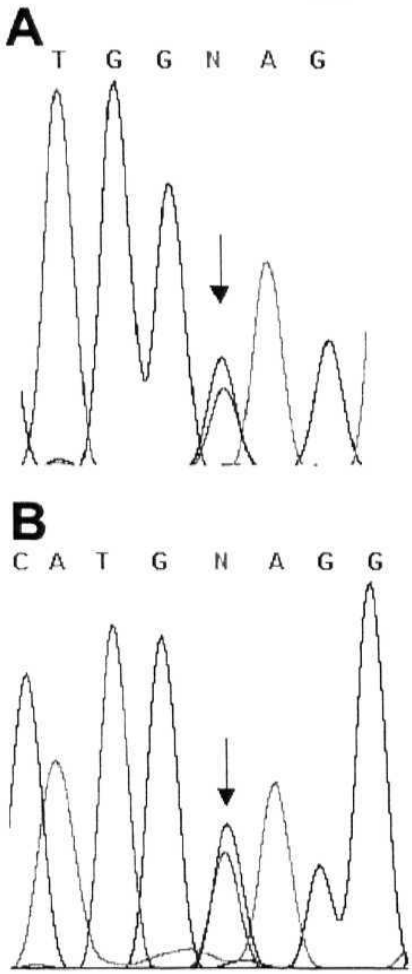

Figure 1. Novel mutations in ARA cases. A: Electropherogram of a sense strand of DNA from the proband of the ARA7 family (ARA7A) showing a C->T change at cDNA postion 367, resulting in a mutation from glutamine to a stop codon (Q123X). **B**: Electropherogram of a sense strand of DNA from the proband of ARA 10 (ARA10A) family showing a C->T change at cDNA position 4, resulting in a mutation from glutaminc to a stop codon (Q2X).

of the FOXC1 protein in the helix 3 region of the forkhead domain (Figure 1A). This mutation has been previously reported in the murine homolog of *FKHL7*, *Mfl*, in a mouse with congenital hydroccphalus phenotype [5]. This is perhaps the first report of the Q123X mutation in human ARA and is found to segregate with the disease phenotype in the three-generation ARA7 family. The unaffected grandmother (ARA7G) of the proband in this family was also found to harbor the same mutation and had the 1-1-1 affected haplotype (Figure 2A).

The M161K mutation in the wing 2 region of *FOXCl*, previously reported in family AR A3 [7], was also seen in family ARA8. The mutation segregated with the disease phenotype in the three-generation ARA8 family and was confirmed by restriction analysis with the enzyme *Nla* III. However, haplotype analysis indicated the segregation of different affected haplotypes, such as 1-3-1 and 3-2-2 in ARA3 and ARA8 families, respectively (Figure 2B).

A sporadic case (ARA 10) exhibited a nucleotide change at cDNA position 4 (C->T), leading to the generation of a stop codon (Figure 1B). To our knowledge, this nonsense mutation is a novel one, leading to premature truncation of the protein considerably before the forkhead domain in the second codon (Gln2Stop). All the mutations observed in this study were absent in the normal controls. The other sporadic cases however did not show any mutations in the *FOXC1* gene.

Genotype-phenotype correlations: Variable phenotypic manifestations were seen in all of the ten families with or without *FOXCl* mutations (Table 1). In family ARA3, early intervention in the offspring (ARA3A and ARA3B) had a better prognosis than their affected father (ARA3C) [7]. In contrast, we found that the proband (ARA8A) of ARA8 family harboring the same mutation did not to show a similar prognosis, perhaps due to her advanced secondary glaucoma at presentation. She underwent transscleral cyclophotocoagulation and attained a visual acuity of 20/260 along with a cup to disc ratio of 0.4 in her right eye. Although her IOP was controlled, her left eye had a strabismus with a dense corneal scar, and she could only gain a visual acuity of "fixes and follows" light (Table 1). Her affected father (ARA8C) and grandfather (ARA8G) also had severe glaucoma and had developed monocular phthisis bulbi at presentation. After medical intervention their visual acuity was "no light perception." However, in their better eye, their IOPs were controlled, corneas were clear, and they had a visual acuity of 20/30. The father (ARA3C) of the proband in ARA3 had severe secondary glaucoma and was blind in his right eye at presentation. In spite of surgical intervention, he had a poor visual outcome with almost total cupping (0.9), corneal haze and edema and diminished vision in the left eye [7]. We also noticed that all the affected members in family ARA8 had a severe loss of vision in one of their eyes, which was not recovered even after surgical intervention and medical treatment (Table 1).

Early intervention in the proband with the nonsense mutation (Q123X; ARA7A) resulted in a relatively fair prognosis with controlled IOP, normal cup to disc ratio, and visual acuity of 20/670 in both eyes, better than his affected mother (ARA7B) and uncle (ARA7U, Table 1). These two relatives

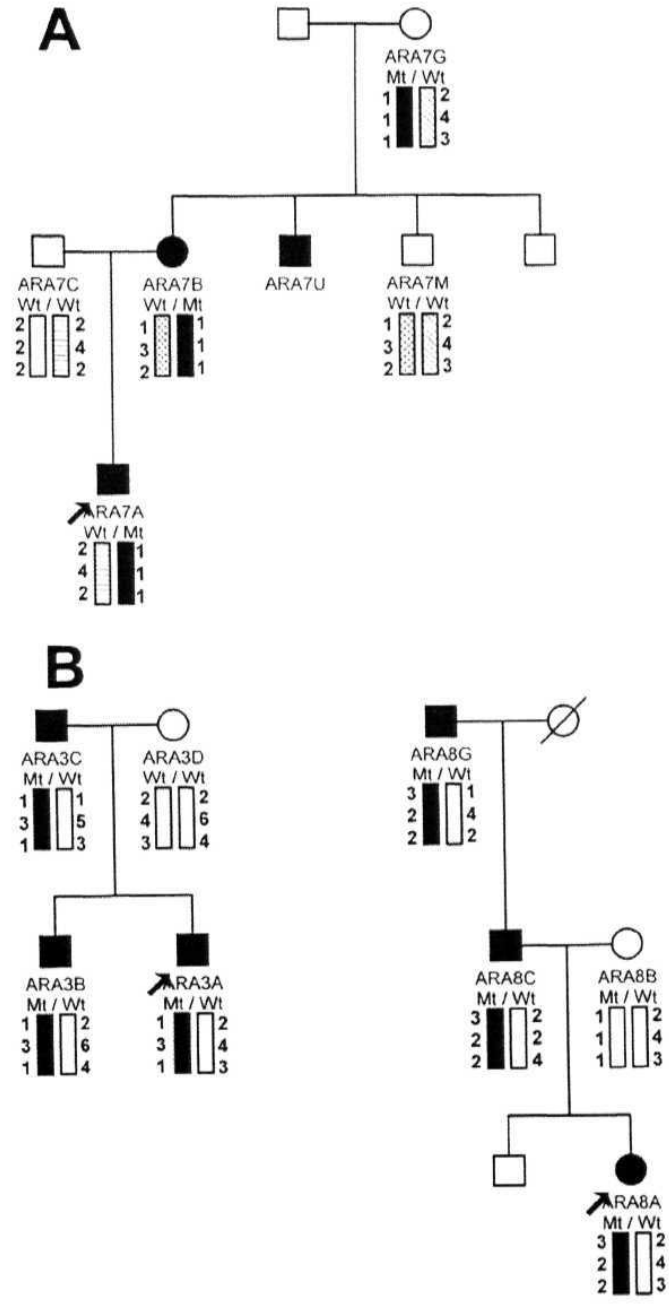

Figure 2. Haplotypes of ARA families. A: Haplotype of family ARA7 exhibiting the Q123X mutation. Darkened bars indicate the affected haplotype. The order of the markers from telomere to centromere and their inter-marker distances arc: D6S 1574-4.7 cM-D6S309-4.8 cM-D6S470. Note that the affected haplotype 1-1-1 is segregating in this family, and the grandmother (ARA7G) is also carrying the same haplotype. "Mt" and "Wt" represent the mutant and wild type chromosomes, respectively. **B**: Haplotype of family ARA3 and ARA8 exhibiting the M161K mutation. Darkened bars indicate the affected haplotype. The order of the markers from telomere to centromere and their inter-marker distances arc: D6S 1574-4.7 cM-D6S309-4.8 cM-D6S470. Note the affected haplotypes 1-3-1 and 3-2-2 segregating in ARA3 and ARA8 family, respectively. "Mt" and "Wt" represent the mutant and wild type chromosomes, respectively.

were diagnosed with severe secondary glaucoma at 10 and 8 years of age, respectively, and were undergoing medical treatment. They, too, had developed phthisis bulbi in one of their eyes at presentation at a later age. In spite of surgical intervention, they had raised IOPs, total cupping, and very little vision in their relatively better eye. The grandmother of the proband (ARA7G) in this family also harbored the same mu-'ation, but did not show any symptoms of ARA or glaucoma nd had a normal IOP (14 mm Hg in both eyes), along with 'isual acuity of 20/30 in both eyes.

The proband of a sporadic case (ARAIOA) with the other lonsense mutation (Q2X) presented at 10 years of age and lid not have any signs of secondary glaucoma. Although his cup to disc ratio was on the borderline, his cornea was clear and his IOP was controlled. On medical treatment, he exhibited good prognosis along with a visual acuity of 20/20 in both the eyes. Five of the six probands of sporadic cases, who did not show any mutation in the *FOXCl* gene, were intervened very early (between 2-9 months) and had fair prognosis with relatively better visual acuity (Table 1). One (ARA4A) had a very high cup to disc ratio in both the eyes (0.9) and in spite of late surgical intervention at 19 years of age exhibited a fair prognosis. The IOP in these probands are under control and they are being regularly monitored for development of glaucoma.

DISCUSSION

Mutations in the forkhead transcription factor gene *FOXCl* have been associated with anterior segment dysgenesis of the eye with various phenotypes [1,4,10]. Four mutations in different regions of *FOXCl* have been implicated in ARA [7]. The present study has identified three different mutations, two of which are novel, and points out the clinical genetic heterogeneity of the *FOXCl* gene in Indian ARA populations (Table 2).

The nonsense mutation (Q123X) in the forkhead region of *FOXCl*, we believe, has been identified for the first time in a human ARA family. The segregation of this mutation with the disease phenotype, its absence in normal individuals, and conservation of its normal residue across species implies its pathogenic nature. This mutation in the third helix is likely to disrupt the sequence-specific contacts with the major groove of the core target sequence [1]. Intriguingly, the normal grandmother (ARA7G) of the proband also carried the same mutation and the affected haplotypc (Figure 2A). We wonder whether she might have inherited a modifier locus along with the mutation, which prevents the expression of the phenotype. However, she has transmitted the mutation through her germline, which resulted in the ARA phenotype in subsequent generations. As this mutation truncates the protein in the forkhead domain, it must be lethal for the expression of the phenotype. We therefore presume that she must be a somatic mosaic for the modifier locus that suppresses the expression of the phenotype. Alternately, the modifier locus may be non-penetrant, as a rare dominant locus may result in 50% of clinically unaffected individuals carrying the affected genotype. A

TABLE 2. DIFFERENT MUTATIONS OBSERVED IN ARA FAMILIES

Family ID Number	Nucleotide change	cDNA position	Mutation	Type of mutation
ARAB	T->A	482	M161K	Missense
ARA7	C->T	367	Q123X*	Nonsense
ARA10	C->T	4	Q2X*	Nonsense

Spectrum of mutations in ARA families, along with their locations and associated changes. Mutations marked with an asterisk ("*") are novel.

TABLE 1. CLINICAL PHENOTYPES OF ARA PATIENTS

Patient ID Number	Age at onset	Age at diagnosis/ intervention	Corneal diameter		IOP at diagnosis		IOP at treatment		Last C/D ratio		Last recorded vision		Corneal changes/ clarity (OU)	Treatments
			OD	OS	OD	OS	OD	OS	OD	OS	OD	OS		
ARA1A	Birth	3 months	13	14	32	26	14	14	NA	0.3	PLPR	20/160	Megalocornea, haze, edema	Trab+ OU
ARA2A	2 years	2 years	13	13	31	27	14	14	0.4	0.3	20/125	20/125	Megalocornea, haze, edema	Trab OU
ARA3A	Birth	2.5 months	12	12	30	28	12	14	0.4	0.3	20/80	20/80	Megalocornea, haze, edema	Trab OS
ARA3B	Birth	2 weeks	11	11.5	24	26	11	12	0.2	0.3	20/80	20/80	Megalocornea, haze, edema	Trab+ OU
ARA3C	Birth	24 years	NA	NA	42	35	14	14	NA	0.9	NLP	CF-1m	Megalocornea, haze, edema	Trab OS; Medical OS
ARA4A	15 years	19 years	12	12	54	54	09	13	0.9	0.8	20/20	20/20	Clear cornea	Trab+ OU
ARA5A	Birth	3 months	10.5	10.5	12	12	10	10	0.2	0.2	FF	FF	Clear cornea	Observation
ARA6A	Birth	9 months	10.5	10.5	14	16	14	14	0.3	0.3	FF	FF	Clear cornea	Observation
ARA7A	Birth	18 days	12	12.5	28	30	20	18	0.2	0.4	20/670	20/670	Haze, edema	Trab+ OU
ARA7B	Birth	10 years	NA	12	NA	NA	PB	30	NA	0.9	NLP	20/200	Clear cornea	Trab OS
ARA7U	Birth	8 years	13	NA	NA	NA	36	PB	NA	NA	LP	NLP	Clear cornea	Trab OD
ARA8A	Birth	3 days	13	12	32	36	12	13	0.4	NA	20/260	FF	Megalocornea, haze, edema	Trab+ OD; TsCPC OS
ARA8C	Birth	28 years	12.5	NA	21	NA	18	PB	0.7	NA	20/30	NLP	Clear cornea	Medical OD
ARA8G	Birth	54 years	NA	12.5	NA	16	PB	16	NA	0.3	NLP	20/30	Clear cornea	Observation
ARA9A	Birth	3 months	12.5	12.5	32	30	12	14	0.3	0.3	20/130	20/130	Megalocornea, haze, edema	Trab+ OU
ARA10A	Birth	10 years	12	12	12	12	12	12	0.5	0.6	20/20	20/20	Clear cornea	Medical OU

Abbrevations: IOP: Intraocular pressure; C/D ratio: cup to disc ratio; PLPR: Light perception with projection; Trab: Trabeculotomy; Trab+: Trabeculotomy/Trabeculectomy; NLP: No light perception; LP: Light perception; CF: Counts fingers; FF: Fixes and follows light; PB: Phthisis bulbi; TsCPC: Transscleral cyclophotocoagulation; NA: Not available; OD: Right eye; OS: Left eye; OU: Both eyes

similar situation of non-penetrance has been observed in diseases like primary congenital glaucoma with normal individuals carrying a pathogenic *CYP1B1* gene mutation [11]. No extra-ocular tissue involvement was observed in the ARA7 family, unlike the congenital hydrocephalus phenotype in the mouse that first showed the Q123X mutation in its *Mf1* gene [5,12,13]. It may be speculated that the Q123X mutation exhibits heterogeneity in variable expression of phenotype across species. Since this is the first report on the presence of the Q123X mutation in humans, this has not allowed any further comparisons on its phenotypic manifestations. Because of early surgical intervention, the proband (ARA7A) had a relatively better visula outcome than his mother (ARA7B) and uncle (ARA7U, Table 1).

In the two families ARA3 and ARA8, which belong to the same ethnic group, the mutation M161K was observed, however with two different sets of affected haplotypes (Figure 2B). This suggests two independent origins of the M161K mutation in these two families and suggests multiple founders for this mutation in the same ethnic group, as both were Hindu families belonging to the same caste group (Vaishyas) in the same geographical region. Early interventions led to a better prognosis in the probands of the ARA3 family, as opposed to the visual outcomes in ARA8 (Table 1). Moreover, the disease seems to have a severe unilateral manifestation among the affected individuals of the ARA8 family. It appears that the 3-2-2 haplotype for the M161K mutation might manifest a more pronounced phenotype in this family. As seen in an earlier study [8], this is another rare instance of a *FOXCI* mutation replicating in two unrelated ARA families.

The other nonsense mutation (Q2X), seen in the proband of family ARA 10, appears to be novel and is the only mutation so far seen in a sporadic case of ARA. It is expected to produce a functionally null allcle since it truncates the FOXC1 protein ahead of the forkhead domain. Although this mutation is expected to result in a null phenotype, the patient showed a relatively better visual outcome in spite of late medical intervention. This raises the possibility of a modifier locus, which can suppress the expression of the *FOXC1* gene (the situation in family ARA7). Alternately, this may represent a mutation with a variable phenotypic expression. This region of the FOXC1 protein contains poly-Ala repeats, which are seen in the activation domain regions of other transcription factors [14j. Hence it is likely that the Q2X mutation in the N-terminal transactivation domain might be terminating the activation of transcription of FOXCI [9].

The other sporadic cases did not show any mutations in *FOXC1*, leading to the possibility of other genes responsible for ARA. Interestingly the proband in the ARA9 family shared the same affected haplotype with the proband of ARA10 (data not shown), unlike other probands of sporadic cases. There have been reports where *FOXCI* mutations are not observed in Axenfeld-Rieger families [3,15]. Recent studies have shown that chromosomal duplications of the 6p25 region have resulted in anterior segment anomalies due to an increased gene dosage of FOXCI or some unknown genes within the duplicated segment [8,16].

We note that the *FOXC1* gene is involved in 3 of 9 new ARA cases in the present study population. However the frequency varies across populations with respect to Axenfeld-Rieger phenotypes and is particularly high in Axenfeld-Rieger syndromes [8,17]. The M161K mutation was seen in two cases and might represent a common *FOXCI* gene mutation in Indian ARA patients. Also, this is the first report that elucidates the presence of nonsense mutations in ARA that were earlier observed only in Axenfeld-Rieger phenotypes with systemic abnormalities [5]. Haploinsufficiency of transcription factors FOXCI and FOXC2, responsible for maintaining the ocular drainage structures, arc reported to result in anterior segment anomalies in mice with similar clinical abnormalities in different genetic backgrounds [2]. However, our results suggest that haploinsufficiency in *FOXCI* results in variable clinical manifestations in human ARA, as seen in ARA7 and ARA 10 families.

Altered amounts of gene dosage of FOXC1 transcription factor have also been noted in deletions and duplications of 6p25 region in anterior segment abnormalities [18]. Disease causing mutations and chromosomal duplications modulate the levels of FOXCI, thereby hampering its regulatory control for efficient transcriptional activation [14]. The present study documents the heterogeneity *oi' FOXCI* in causing variable phenotypic manifestations and provides some insight to understanding the gene functions in Indian ARA populations.

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PAX6 gene mutations and genotype-phenotype correlations in sporadic cases of aniridia from India

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In order to understand the underlying molecular genetic defect causing aniridia in India, eight probands from sporadic cases were screened for all 14 exons of the PAX6 gene using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). Direct sequencing of the SSCP variants revealed a nonsense mutation (R317X) in the eleventh exon leading to a premature termination of the PAX6 protein in the proline-serine-threonine (PST)-rich domain in two probands. Another proband exhibited an intronic polymorphism (IVS 9-12 C-T). The mutation resulted in loss of function of the PAX6 protein along with variable phenotypic manifestations in the probands. This is the first report describing a PAX6 gene mutation in aniridia cases from India and highlights the variable expressivity in phenotypes due to haploinsufficiency.

Keywords: PAX6 gene , aniridia , mutation , haploinsufficiency , phenotype .