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DATA REPORT

A spectrum of *CYP1B1* mutations associated with primary congenital glaucoma in families of Pakistani descent

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Glaucoma is the second leading cause of blindness, affecting ~65 million people worldwide. We identified and ascertained a large cohort of inbred families with multiple individuals manifesting cardinal symptoms of primary congenital glaucoma (PCG) to investigate the etiology of the disease at a molecular level. Ophthalmic examinations, including slit-lamp microscopy and applanation tonometry, were performed to characterize the causal phenotype and confirm that affected individuals fulfilled the diagnostic criteria for PCG. Subsequently, exclusion analysis was completed with fluorescently labeled short tandem repeat markers, followed by Sanger sequencing to identify pathogenic variants. Exclusion analysis suggested linkage to the *CYP1B1* locus, with positive two-point logarithm of odds scores in 23 families, while Sanger sequencing identified a total of 11 variants, including two novel mutations, in 23 families. All mutations segregated with the disease phenotype in their respective families. These included the following seven missense mutations: p.Y81N, p.E229K, p.R368H, p.R390H, p.W434R, p.R444Q and p.R469W, as well as one nonsense mutation, p.Q37*, and three frameshift mutations, p.W246Lfs81*, p.T404Sfs30* and p.P442Qfs15*. In conclusion, we identified a total of 11 mutations, reconfirming the genetic heterogeneity of *CYP1B1* in the pathogenesis of PCG. To the best of our knowledge, this is the largest study investigating the contribution of *CYP1B1* to the pathogenesis of PCG in the Pakistani population.

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Glaucoma is the second leading cause of blindness, affecting ~65 million people worldwide.[1] It causes irreversible visual field defects, eventually resulting in complete blindness.[2,3] Glaucoma encompasses a range of ocular dystrophies that each exhibit optic disc neuropathy, which is specifically characterized by progressive loss of retinal ganglion cells and optic nerve atrophy.[4,5] The disease is generally classified by age of onset, anatomy of the anterior chamber and etiology. These characteristics define the following three broad categories of disease: open-angle glaucoma, closed-angle glaucoma and primary congenital glaucoma.[6]

The focus of our study, primary congenital glaucoma (PCG), is characterized by developmental defects in the anterior chamber that obstruct the normal flow of aqueous fluid in the eye. Blockage and subsequent buildup of fluid increases intraocular pressure (IOP) and damages the optic nerve.[7–10] Clinical indications of PCG include elevated IOP, buphthalmos (globe enlargement), photophobia, corneal enlargement, epiphora, Haab's striae (Descemet's membrane ruptures), blepharospasm and optic nerve damage.[7,8] PCG occurs in approximately 1 in 10,000 live births, but the incidence varies widely between populations.[6]

PCG is a genetically heterogeneous disorder that occurs both sporadically and in families and is inherited as an autosomal recessive trait. Linkage analysis has identified the following four loci: GLC3A (2p22-p21), GLC3B (1p36.2-36.1), GLC3C (14q24.3) and GLC3D (14q24.2-24.3).[11–14] GLC3A and GLC3D have been cloned, with mutations in cytochrome P450, subfamily B, polypeptide 1 (*CYP1B1*) and latent transforming growth factor β binding protein 2 (*LTBP2*) reported in populations of multiple ethnicities.[15,16] We previously reported four causal mutations identified in large consanguineous families of Pakistani origin.[14]

In an ongoing effort to investigate the genetic basis of PCG and identify novel disease loci and/or causal mutations responsible for the disease phenotype, we have recruited a large cohort of consanguineous families, each including at least one affected individual from a consanguineous mating. Approval for the study was granted by the Institutional Review Boards (IRBs) of the National Centre of Excellence in Molecular Biology (Lahore, Pakistan), the National Eye Institute (Bethesda, MD, USA) and Johns Hopkins University (Baltimore, MD, USA).

Individuals who participated in this study signed an informed written consent, which was approved by each IRB and adheres to the Declaration of Helsinki. Any available medical records were used to compile medical histories for the study participants, but much of the information was provided in interviews with the families. The majority of the enrolled families live in remote areas without access to ophthalmic clinics. Therefore, the precise age of onset of glaucoma or the age at first diagnosis is not well documented. The parents of the affected children, as well as family elders, reported that reduced or abnormal vision was evident in the first year of the children's lives. The consistently early onset of symptoms suggests congenital ocular dystrophy. Common symptoms of the affected individuals were increased

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Table 1. Clinical characteristics of affected individuals manifesting symptoms of primary congenital glaucoma

Family ID	*Individual ID*	*Sex*	*Age at exam (years)*	*IOP (OD/OS)*	*CD ratio (OD/OS)*	*VA (OD/OS)*	*Corneal diameter*	*Other findings*
PKGL001	6	F	45	52/44	NV/NV	NPL/PL	Increased	SD, CE, CH
PKGL014	9	M	4	*16/16*	0.6/0.4	PL/PL	Increased	B/L Bu, B/L MF
PKGL014	10	M	7	*8/20*	0.5/0.7	PL/CF	13 mm	B/L Mc, B/L Ny
PKGL032	11	F	2	40/32	0.7/0.4	PL/HM	Increased	B/L Bu, CO, B/L Ny
PKGL032	12	F	4	NA	NV/NV	PL/PL	Increased	B/L Bu, B/L CO
PKGL040	7	M	12	26/20*	NV/NV	HM/HM	B/L > 14 mm	Bu, B/L CO, Vas, B/L Ny
PKGL040	8	F	16	36/36	1.0/NV	6/60/6/18	Increased	Bu, Rt Pp, Lt PE, CO
PKGL046	10	M	14	NA	NA	NPL/NPL	NA	CO
PKGL046	11	F	10	NA	NA	NA	NA	Bu
PKGL047	8	F	2	44/30	0.8/NV	PL/PL	Increased	B/L Bu, B/L Mc
PKGL047	9	F	5	NA	0.7/0.5	PL/PL	13 mm	B/L Bu, B/L Mc
PKGL051	8	M	2	30/26	0.8/0.6	PL/PL	Increased	B/L Bu, B/L CO
PKGL065	12	M	3	NA/38	NA	PL/PL	Increased	B/L Bu, Mc
PKGL065	11	M	10	25/28	0.8/0.9	NPL/PL	Increased	B/L Bu
PKGL066	14	F	3	22/20*	NV/NV	PL/PL	13.0/14.0 mm	B/L Bu, B/L CO
PKGL068	11	F	10	28/28	1.0/1.0	4/36/4/12	Increased	B/L Bu, B/L CO
PKGL068	12	F	5	Increased	NV/NV	NPL/NPL	Increased	B/L Bu, B/L CH, Php
PKGL071	12	M	13	*19/22	0.9/0.9	CF/CF	Increased	B/L Bu, B/L Ny
PKGL071	13	M	2	*16/12	0.9/0.3	NA	Increased	Rt Mc, Rt CO, B/L MF
PKGL072	11	F	14	38/NA	NV/NV	HM/NPL	Increased	Rt Bu, Lt PE, Rt Ny, B/L Trab
PKGL072	12	F	2	*18/25	0.3/0.9	CF/CF	Increased	B/L Bu, B/L Trab, Lt CO
PKGL072	9	M	5	*18/40	NV/NV	CF/CF	Increased	B/L Bu, B/L Ny, B/L Trab, Lt CH
PKGL073	12	M	12	53/NA	0.7/NV	6/60/NPL	Increased	Rt. Mc, Rt. CH, Lt PE
PKGL073	9	M	4	*18/18*	NV/NV	CF/CF	Increased	B/L HS, B/L Bu, B/L CH
PKGL073	10	F	4	NA	0.3/NV	NA	Increased	B/L Bu, B/L CH
PKGL077	10	F	12	NA	NA	PL/PL	NA	Bu, CO
PKGL079	11	F	10	NA	NA	NPL/NPL	NA	CO
PKGL082	8	M	4	NA	NA	NA	NA	Bu, CO
Control 01		F	28	15/17	0.3/0.3	6/6/6/6	11.5 mm	
Control 02		F	28	17/17	0.3/0.4	6/6/6/6	11.5 mm	

Abbreviations: B/L, bilateral; Bu, buphthalmos; CD Ratio, cup to disc ratio; CE, corneal edema; CF, counting fingers; CH, corneal haze; CO, corneal opacity; HM, hand movement; HS, Haab's striae; IOP, intraocular pressure; Lt, left; Mc, megalocornea; MF, myopic fundus; NA, not available; NPL, no light perception; NV, no view; Ny, nystagmus; OD, oculus dexter; OS, oculus sinister; PL, light perception; PE, phthisical eye; Php, photophobia; Pp, pseudophakia; Rt, right; SD, spheroidal degeneration; Trab, trabeculectomy; VA, visual acuity; Vas, vascularization.
An asterisk indicates IOP is controlled through surgery and medical treatment.

IOP, increased corneal diameter and visual acuity that was reduced to hand movement and/or light perception (Table 1).

All study participants provided a blood sample of ~10 ml that was collected in a tube containing 400 µl of 0.5 mol/l EDTA. Genomic DNA was extracted from white blood cells as previously described.[17] Exclusion analysis, two-point logarithm of odds (LOD) score calculations, and bi-directional Sanger sequencing were completed as previously described, along with the sequences of the primer pairs used for Sanger sequencing.[14]

A total of 23 familial cases localized to chromosome 2p21 (Supplementary Figures 1–23): PKGL001, PKGL014, PKGL028, PKGL032, PKGL040, PKGL046, PKGL047, PKGL050, PKGL051, PKGL058, PKGL060, PKGL065, PKGL066, PKGL067, PKGL068, PKGL069, PKGL070, PKGL071, PKGL072, PKGL073, PKGL077, PKGL079 and PKGL082. Alleles of chromosome 2p21 markers provided evidence of linkage to the GLC3A locus, with positive LOD scores (Supplementary Tables 1–23). Subsequently, we sequenced all coding exons, exon–intron boundaries, and the 5′ and 3′ UTR regions of *CYP1B1*. In 23 PCG families, we identified 11 homozygous variations, including seven missense mutations, one nonsense mutation and three frameshift mutations (Table 2).

We identified a homozygous variation, c.1405C > T (p.R469W), in both PKGL001 and PKGL028. Similarly, we identified a homozygous variation, c.1169G > A (p.R390H), in PKGL040, PKGL046, PKGL060, PKGL065, PKGL066, PKGL067, PKGL069, PKGL070, PKGL071, PKGL073, PKGL077, PKGL079 and PKGL082. Moreover, we identified the homozygous variations c.1300T > C (p.W434R), c.685G > A (p.E229K), c.1331G > A (p.R444Q), c.241T > A (p.Y81N), c.1103G > A (p.R368H), and c.109C > T (p.Q37*) in PKGL014, PKGL047, PKGL050, PKGL051, PKGL058 and PKGL072, respectively (Table 2).

We further identified a homozygous 1 bp insertion, c.736_737insT (p.W246Lfs81*), and a homozygous 1 bp deletion, c.1325delC (p.P442Qfs15*), in PKGL047 and PKGL068, respectively (Table 2 and Supplementary Figure 24a,b), and a 10-bp homozygous duplication, c.1200_1209dupTCATGCCACC (p.T404Sfs30*), in PKGL032 (Table 2). Each of these mutations results in a frameshift and eventually leads to premature termination of *CYP1B1*. Among these variants, seven missense mutations, a frameshift mutation, and a nonsense mutation have been previously reported, whereas p.W246Lfs81* and p.P442Qfs15* are novel. All the above-mentioned pathogenic variations co-segregated with the disease phenotype in their respective families and were absent in 192 ethnically matched control chromosomes.

Next, we examined the evolutionary conservation of the mutated amino acids by aligning the protein sequences of CYP1B1 orthologs. In parallel, we investigated the possible effect of amino acid substitution on the structure of CYP1B1 with SIFT (http://sift.jcvi.org), Condel (http://bg.upf.edu/fannsdb/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml). Evolutionary conservation of the substituted amino acids of all seven missense variants show that not only these amino acids but also the amino acids in the immediate vicinity are highly conserved among other primates, placental mammals and vertebrates (Supplementary Figure 24c). SIFT and PolyPhen-2 analyses showed that all 11 pathogenic variants were damaging to the function and enzymatic activity of CYP1B1.

Table 2. Summary of causal alleles identified in our cohort of primary congenital glaucoma

Family ID	*Individuals ascertained*	*Affecteds ascertained*	*Two-point LOD score*	*Mutation (nucleotide change)*	*Mutation (amino acid change)*	*Status*	*In Silico Prediction*		
						Known/ novel	*Condel*	*PolyPhen-2*	*SIFT*
PKGL001	10	5	1.87	c.1405C > T	p.R469W	Known	De (1)	PD (1)	Da (0)
PKGL014	8	4	3.01	c.1300T > C	p.W434R	Known	De (1)	PD (1)	Da (0)
PKGL028	12	6	5.28	c.1405C > T	p.R469W	Known	De (1)	PD (1)	Da (0)
PKGL032	7	2	1.68	c.1200_1209dup	p.T404Sfs30*	Known			
PKGL040	5	2	1.59	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL046	8	5	2.64	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL047	9	4	1.52	c.736_737insT,	p.W246Lfs81*,	Novel,			
				c.685G > A	p.E229K	Known	De (0.605)	PD (0.950)	Da (0)
PKGL050	9	3	2.27	c.1331G > A	p.R444Q	Known	De (1)	PD (1)	Da (0)
PKGL051	4	1	1.03	c.241T > A	p.Y81N	Known	De (0.998)	PD (1)	Da (0)
PKGL058	2	1	0.82	c.1103G > A	p.R368H	Known	De (0.994)	PD (1)	Da (0)
PKGL060	5	2	1.37	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL065	13	4	3.87	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL066	12	6	5.01	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL067	9	3	3.18	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL068	10	7	3.28	c.1325delC	p.P442Qfs15*	Novel			
PKGL069	5	3	1.39	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL070	9	3	2.54	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL071	8	4	3.54	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL072	7	3	2.45	c.109C > T,	p.Q37*	Known			
				c.1103G > A	p.R368H	Known	De (0.994)	PD (1)	Da (0)
PKGL073	9	5	3.34	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL077	7	4	2.53	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL079	9	6	2.66	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)
PKGL082	5	3	2.07	c.1169G > A	p.R390H	Known	De (1)	PD (1)	Da (0)

Abbreviations: Da, damaging; De, deleterious; LOD, logarithm of odds; PD, probably damaging.
Parentheses show scores of prediction algorithms: Condel, PolyPhen-2 and SIFT.

CYP1B1, a member of the cytochrome P450 superfamily, is the largest known enzyme of the human cytochrome P450 pathway that is primarily expressed in the trabecular meshwork, iris, retina and ciliary body. Membrane-bound CYP1B1 has a molecular structure consisting of a 53-residue membrane-spanning domain located at the NH_2 (amino) terminus of the molecule, followed by a 10-residue proline-rich ‘hinge’ region, which allows flexibility between the membrane-spanning and cytoplasmic domains of the protein molecule.[18] The COOH (carboxy) terminus of CYP1B1 is highly conserved, consisting of conserved core structures composed of a J-helix, a K-helix and a heme-binding region.[19,20] Mutations in CYP1B1, either at the NH_2 or the COOH terminus, are expected to interfere with the basic properties of the CYP1B1 molecule, such as its ability to bind heme or to adopt its regular conformation, resulting in impaired enzymatic function.[20,21] The pathogenesis of *CYP1B1* in PCG is still not fully understood, but it is thought to be involved in metabolic pathways related to ocular differentiation (anterior segment and trabecular meshwork formation). A study conducted recently on *CYP1B1*-deficient mice showed that *CYP1B1* deficiency resulted in increased oxidative stress and structural defects in the trabecular meshwork in the early lives of mice.[22]

To date, more than 150 mutations in *CYP1B1* have been associated with PCG, accounting for a significant fraction of the genetic load of familial and sporadic cases of PCG. We found one mutation, R390H, in 13 of the 23 familial cases investigated in this study, accounting for more than half of the families linked to *CYP1B1*, which is consistent with previously published estimates. The R390H allele is the most frequently occurring mutation identified in Chinese, Iranian, Indian and Pakistani populations affected by PCG, while only a small fraction of Caucasians harbor the R390H allele.[23–27]

We further investigated the origin of this mutation by constructing a haplotype exploiting the following six previously reported *CYP1B1* SNPs: rs2617266, rs10012, rs1056827, rs1056836, rs1056837 and rs1800440. All 13 families harboring the R390H allele shared a common haplotype, C–C–G–C–C–G (data not shown). The same haplotype was recently reported in five families of Pakistani origin that harbored the R390H allele.[27]

Interestingly, the W434R allele identified in PKGL014 has previously been implicated in primary open-angle glaucoma (POAG), where a single heterozygous carrier of Indian descent was reported to have POAG.[28] However, no clinical details of the patient were included in the report; therefore, we cannot compare the severity of the causal phenotype of the heterozygous carrier reported to have POAG with that of the affected individuals in PKGL014 who are homozygous for the W434R allele. Surprisingly, the heterozygous carriers of the W434R allele in PKGL014 are phenotypically normal and do not show any signs or clinical symptoms of POAG or any other ocular dystrophy.

In conclusion, we report a broad spectrum of mutations identified in *CYP1B1* that are responsible for PCG in consanguineous families of Pakistani origin, reaffirming the role of *CYP1B1* in the pathogenesis of PCG. To the best of our knowledge, this is the largest study investigating the contributions of *CYP1B1* causal mutations associated with PCG in the Pakistani population, which will add to our understanding of the genetic basis of PCG.

HGV DATABASE

The relevant data from this Data Report are hosted at the Human Genome Variation Database at http://dx.doi.org/10.6084/m9.figshare.hgv.805 (2016), http://dx.doi.org/10.6084/m9.figshare.hgv.808 (2016), http://dx.doi.org/10.6084/m9.figshare.hgv.811 (2016), http://dx.doi.org/10.6084/m9.figshare.hgv.814 (2016),

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AUTHOR CONTRIBUTIONS

BR, BI, SF and SAR: conceived and designed the experiments; BR, BI and SF: recruited human subjects; SNK, TH, SR, JA and SAR: contributed reagents/materials/analysis tools; BR, BI, FK and SF: performed experiments; BR, BI, FK, SF, MAN, SNK, TH, SR, JA and SAR: analyzed the data; and BR, BI, FK, SR and SAR: contributed to the writing of the manuscript.

COMPETING INTERESTS

The authors declare no conflict of interest.

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