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Mutations in retinal cyclic nucleotide-gated channels identified in familial cases of inherited retinal dystrophies from Pakistan

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Purpose: Cyclic nucleotide-gated (CNG) channels are ligand-gated ion channels that transduce light signals into electrical signals in the retinal photoreceptors. Pathogenic variants in CNG channel genes are reported to cause inherited retinal dystrophies (IRDs). The current study used targeted panel sequencing to describe the mutational spectrum of CNG channel genes in familial cases of IRDs from eight consanguineous Pakistani families.

Methods: The current study included consanguineous Pakistani families with at least two affected members. DNA was extracted from whole blood samples by the phenol-chloroform method. Two affected members from each family were initially analyzed using targeted panel sequencing of 344 known IRD genes. The pathogenicity of candidate variants was assessed using the American College of Medical Genetics and Genomics guidelines. Segregation testing was performed by Sanger sequencing.

Results: Results of eight IRD families revealed a total of four reported variants in *CNGA3* (c.827A>G, c.955T>C, c.1641C>A, c.1810C>T) and three novel variants, including c.1633A>T, c.800G>T, and c.1153T>C in *CNGA1*, *CNGA3*, and *CNGB3* genes, respectively, segregating in each respective family. Among disease-causing variants identified in our study cohort, 87.5% were missense. Furthermore, one of the reported missense variants (i.e., c.1641C>A in *CNGA3*) was segregating in two unrelated families. All identified variants were homozygous and segregated in an autosomal recessive form.

Conclusions: *CNGA3* was the most frequently mutated gene in our study cohort. Only the c.1641C>A variant of *CNGA3* was repeated in two families, showing genetic diversity. The identification of three novel pathogenic variants in CNG channel genes in the present study reaffirms the allelic and genetic heterogeneity of IRDs in the Pakistani population.

Retinal cyclic nucleotide-gated (CNG) channels are ligand-gated ion channels that belong to the superfamily of the pore-loop cation channel [1]. They are found on the plasma membranes of the outer segment of photoreceptor cells and mediate visual phototransduction by binding cyclic guanosine monophosphate (cGMP) [2–4]. In the absence of light, dark-adapted photoreceptors maintain a high level of cytosolic cGMP, which permits CNG channels to stay open [5,6]. When light activates rhodopsin, a visual pigment in rod photoreceptors, it triggers a cascade of enzymatic reactions that ultimately lead to the breakdown of cGMP by phosphodiesterase. This causes a decrease in cGMP levels, which leads to the closure of ion channels, preventing Na^+ and Ca^{2+} from entering and a change in membrane potential [7,8]. The light-induced CNG channel closure lowers the cytosolic Ca^{2+} concentration that, in turn, modulates the sensitivity of the CNG channel and activates guanylate cyclase to resynthesize cGMP [1,6,9]. A similar Ca^{2+}-dependent CNG channel pathway operates in the cone photoreceptors, but their molecular constituent is somewhat different from its rod counterpart [6].

Cone and rod photoreceptors express distinct CNG channels encoded by homologous genes [10]. In mammals, six homologous genes (*CNGA1*, *CNGA2*, *CNGA3*, *CNGA4*, *CNGB1*, and *CNGB3*) encode for structurally similar A and B subunits that assemble in discrete combinations into cell type–specific heterotetrameric complexes [11]. Each heterotetrameric CNG channel is made by three A subunits and one B subunit (CNGA1 and CNGB1 in rods but CNGA3 and CNGB3 in cones) [2]. Membrane proteins encoded by each of the six CNG channel genes consist of six helical transmembrane segments (S1–S6), a channel core comprising a reentrant pore loop between S5 and S6, and cytosolic N- and C-terminals. The pore domain is formed by S5, S6, and the

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intervening reentrant pore loop [6,11,12]. The intracellular cGMP-binding domain is located at the C-terminus, which is joined to S6 by the C-linker [10].

Four of the CNG channel genes (*CNGA1*, *CNGA3*, *CNGB1*, *CNGB3*) are linked to inherited retinal dystrophies (IRDs) [10]. Mutations in the rod-specific channel genes (*CNGA1* or *CNGB1*) cause rod dystrophies/autosomal recessive retinitis pigmentosa (RP)/congenital stationary night blindness [13–15]. A literature search shows that mutations in *CNGA1* and *CNGB1* account for 1% to 8% and 4% of autosomal recessive RP cases, respectively. Cone-specific channel gene (*CNGA3* or *CNGB3*) mutations cause cone dystrophy or achromatopsia/cone-rod dystrophy [16–18]. Collectively, mutations in *CNGA3* and *CNGB3* are responsible for 69% of achromatopsia cases [19,20].

Due to genetic diversity in IRDs, molecular testing is crucial for a definitive diagnosis and patient management. In an ongoing effort to study the mutational spectrum of IRDs in consanguineous Pakistani families [21,22], here we report pathogenic variants in CNG channel genes identified from familial cases belonging to eight unrelated families along with a summary of previously reported CNG channel gene mutations from cases of Pakistani origin.

METHODS

Ethical approval and enrollment of families: The approval of the presented research work was taken from the Bio-Ethical Review Committee of the Faculty of Biologic Sciences, Quaid-i-Azam University Islamabad, Pakistan, and the Ethical Review Committee, Al-Shifa Trust, Rawalpindi, Pakistan. Clinical assessment of families was done by ophthalmologists at Al-Shifa Trust Eye Hospital, Rawalpindi, Pakistan, to confirm the diagnosis of IRD phenotype in each family. Clinical assessments include detailed family and medical history, physical examination, fundoscopy, slit-lamp exam, and visual acuity testing. Families were recruited following the principles of the Declaration of Helsinki for molecular genetic investigation. Written informed consent was obtained from enrolled families before drawing 3 to 4 ml of venous blood from participating affected and control individuals. Genomic DNA extraction and quantification were performed at the Department of Zoology, Quaid-i-Azam University, Islamabad, Pakistan, as per our previously reported method [21].

Targeted panel sequencing and bioinformatics analysis: DNA of two affected individuals from each enrolled IRD family was used for capture panel sequencing at Baylor College of Medicine, Houston, Texas. Exome-enriched genomic libraries were prepared using the KAPA HyperPrep Kit (Roche, Basel, Switzerland) following the manufacturer's protocol, then combined for targeted enrichment of a panel of 344 known genes of IRD-related genes, as described in our previous article [21] with the SureSelect Target Enrichment System for the Illumina Platform (Agilent, Santa Clara, CA) [23]. Using a Novaseq 6000 (Illumina, San Diego, CA), captured DNA was measured and sequenced. As mentioned in our earlier publications, variant calling, data alignment, and filtering were performed at the Functional Genomics Core at Baylor College of Medicine in the United States [21–23]. Variant annotation and filtration were performed according to the American College of Medical Genetics and Genomics recommendations. The HGMD, ClinVar, and LOVD databases were searched for and used to identify previously known pathogenic variants. Several in silico tools were used to assess novel variations for possible effects on protein function, including REVEL v1.3 [24]. Splice site variations, nonsense, and frameshift variants were categorized as probable loss-of-function alleles. Sequence conservation and bioinformatics predictions were used to assess missense variants.

Sanger sequencing and segregation test: Primer3 web-based resource was used to design primers to validate each pathogenic variant. Genomic DNA of available affected and unaffected members of each family were amplified for the suspected gene(s) and subjected to Sanger sequencing.

RESULTS

The eight families reported in this study (selected from 72 inherited retinal dystrophies segregating Pakistani families) were recruited from Al-Shifa trust eye hospital, Rawalpindi, Pakistan. Among these enrolled families, four (RD001, RD028, RD053, and RP125) were Punjabi, two (RD021 and RD025) were Pashtun, and two (RD004 and RD031) were of Kashmiri ethnicity. Clinical data of the affected individuals of these families recorded at enrollment are detailed in Table 1. Comprehensive interviews of elders of each family revealed ethnicity, age of onset, symptoms, and complete family history for drawing pedigrees (Figure 1, Appendix 1 and Appendix 2). All enrolled families had more than one affected individuals. Among 27 affected members, 11 were males and 16 were females. All the enrolled probands had congenital onset of the disease phenotype. Symptoms including decreased visual acuity, color blindness, photophobia, nyctalopia, and hemeralopia of the proband of each family are listed in Table 1. Enrolled affected cases had nystagmus eyes except RD031 (IV.III) and RD053 (IV.III). Refractive error and hyperopia were present in the affected members of RD004 (IV.III), RD021 (IV.IV), RD028 (VI.IV), and RD031 (IV.II). Other ocular and phenotypic features,

including squint and astigmatism, were recorded for patient of RD021 (IV.I). Affected cases of RD053 had only nyctalopia and hemeralopia. Fundus examination of family RD021 (IV.I; Appendix 3) showed small hyperopic changes with a deep cup, a cup-to-disc ratio of 0.4 to 0.5, bull's-eye maculopathy, and periphery unrecordable pigmentary changes.

Genetic analysis: Targeted panel sequencing followed by Sanger sequencing validation confirmed segregation of seven pathogenic variants in CNG channel genes (i.e., *CNGA1*, *CNGA3*, and *CNGB3* with IRD phenotype in eight screened families). There was one stop gain and six missense variants. From the identified variants, three are novel to the current study, and four have been previously reported (Table 2 and Table 3). All identified pathogenic variants were homozygous, segregated with the disease phenotype in an autosomal recessive form. Detailed pedigree of each with segregation of the identified novel/reported variant is given in Figure 1 and Appendix 1 and Appendix 2, respectively. All the identified variants were classified as per guidelines from the American College of Medical Genetics and Genomics, and their details are provided in Table 2.

Among the three novel variants identified in this study, a missense variant, c.1153T>C, causing p.(Trp385Arg) in *CNGB3*, was observed in a Pashtun family (RD021) with two affected siblings (Table 2). Both children belonged to unaffected consanguineous parents and had congenital disease onset with symptoms positive for the cone-rod dystrophy phenotype. Both affected children also had squinting eyes. This observed variant caused the substitution of tryptophan with arginine. Segregation was tested in one affected and two controls of the family (Figure 1B and Figure 2B). This variant is absent from the gnomAD (v2.1.1) database.

A missense variant c.1633A>T of the *CNGA1* gene was observed in a Punjabi family (i.e., RD053 segregating recessively with the RP phenotype; Table 2). Genotyping of an affected member and two unaffected members confirmed the segregation of identified variants (Figure 1C and Figure 2C). The reported gnomAD (v2.1.1) allele frequency for this variant is 0.00002406.

All missense substitutions of *CNGA3* were identified in exon 8. A novel missense variant c.800G>T in the *CNGA3* gene was found in a Kashmiri family (RD004) having four affected females (Table 2). Segregation of this pathogenic variant in an autosomal recessive manner was confirmed by genotyping three affected females and one unaffected female (Figure 1A and Figure 2A). This variant substitutes glycine for valine at position 267 of the encoded protein. This variant is absent from the gnomAD (v2.1.1) database.

Among three previously reported missense variants detected in the *CNGA3* gene, one (c.1641C>A) segregated in two unrelated Punjabi consanguineous families, RD001 and RD028, with the same clinical phenotype (Table 2). The variant p.(Phe547Leu) caused by a single base transition in exon 8 was segregating in an autosomal recessive manner in eight affected individuals (Appendix 1 and Appendix 4). The family RD001 had five affected cases in two successive generations while RD028 had three cases in a single generation. Cases were homozygous for the variant from a heterozygous carrier parent. This variant is classified as pathogenic or likely pathogenic in ClinVar with a minor allele frequency of 0.0001592 in gnomAD (v2.1.1). A consanguineous Pashtun family RD025 with two affected sisters was homozygous for the c.955T>C variant, which results in p.(Cys319Arg; Table 2). Other ocular findings observed in this family were hemeralopia and nyctalopia. Segregation analysis was performed by genotyping two affected and four unaffected individuals (Appendix 1 and Appendix 4). This missense variant is reported as a pathogenic/likely pathogenic in ClinVar, with a minor allele frequency of 0.00001767 in gnomAD (v2.1.1). Another previously reported missense variant identified in RP125 was c.827A>G causing p.(Asn276Ser) in the *CNGA3* gene. The segregation of this variant was confirmed by analyzing four unaffected females and one affected female (Appendix 1 and Appendix 4). This reported variant is absent from the gnomAD (v2.1.1) database.

In a Kashmiri family (RD031), a stop-gain variant c.1810C>T of the *CNGA3* gene causing premature protein termination (i.e., p.(Gln604*)) was segregating in a homozygous recessive manner with disease phenotype (Table 2). Segregation of this variant was confirmed in five affected and three unaffected members, supporting variant pathogenicity (Appendix 2 and Appendix 4), with a gnomAD (v2.1.1) frequency of 0.000003993. Codes met and curated modified classification of previously reported and novel variants based on the data of the present study are listed in Table 2.

DISCUSSION

Understanding the genetic spectrum of individuals with IRDs is imperative for the development of future suitable treatment strategies due to the involvement of multiple genes for the same disease etiology or the same gene disease-causing variant(s) resulting in altered phenotype(s). In the current study, seven disease-causing variants in three CNG channel-associated genes, *CNGA1*, *CNGA3*, and *CNGB3*, were identified in eight consanguineous Pakistani families. Previous studies have indicated that the prevalence of *CNGA3* and *CNGB3* disease-causing variants varies geographically.

Table 1. Demographic and clinical features of probands of IRDs families described in this study.

Sr. No.	Family ID	Proband ID	Clinical diagnosis	Disease progression	Age at enrollment (years)	Ethnicity	No. of Affected cases in family	Visual acuity		Symptoms					
								OD	OS	Color vision defect	Photo phobia	Nystagmus	Nyctalopia	Hemeralopia	Other/s
1	RD001	V.VI	CD/ACHM	Stationary	39	Punjabi	5	NA	NA	+	+	+	-	+	-
2	RD004	IV.III	Maculopathy/ Complete ACHM	Stationary	10	Kashmiri	4	6/60**	6/60**	+	+	+	+	-	Hyperopia
3	RD021	IV.IV	CRD	Progressive	9	Pashtun	2	6/38**	6/48**	+	+	+	-	-	Hyperopia
4	RD025	IV.I	CRD/ Maculopathy	Progressive	20	Pashtun	2	NA	NA	+	+	+	+	+	-
5	RD028	VI.IV	BVMD	Stationary	8	Punjabi	3	C.F***	C.F***	+	+	+	-	+	Hyperopia
6	RD031	IV.II	CRD	Progressive	11	Kashmiri	6	6/60**	6/60**	+	-	+	-	-	Hyperopia
7	RD053	IV.III	RP	Progressive	45	Punjabi	2	NA	NA	-	-	-	+	+	-
8	RP125	IV.II	CD/CRD/ Incomplete ACMH	Progressive	4	Punjabi	3	NA	NA	+	+	+	-	-	-

Abbreviations: OD: right eye, OS: left eye, C.F: Counting fingers, NA: Not Available, CD: cone dystrophy, CRD: cone-rod dystrophy, BVMD: Best vitelliform macular dystrophy, RP: Retinitis Pigmentosa, ACMH: Achromatopsia. The ** symbol indicates the visual acuity was calculated using Early Treatment Diabetic Retinopathy Study (ETDRS) chart, The *** symbol indicates the visual acuity was measured using Snellen Chart.

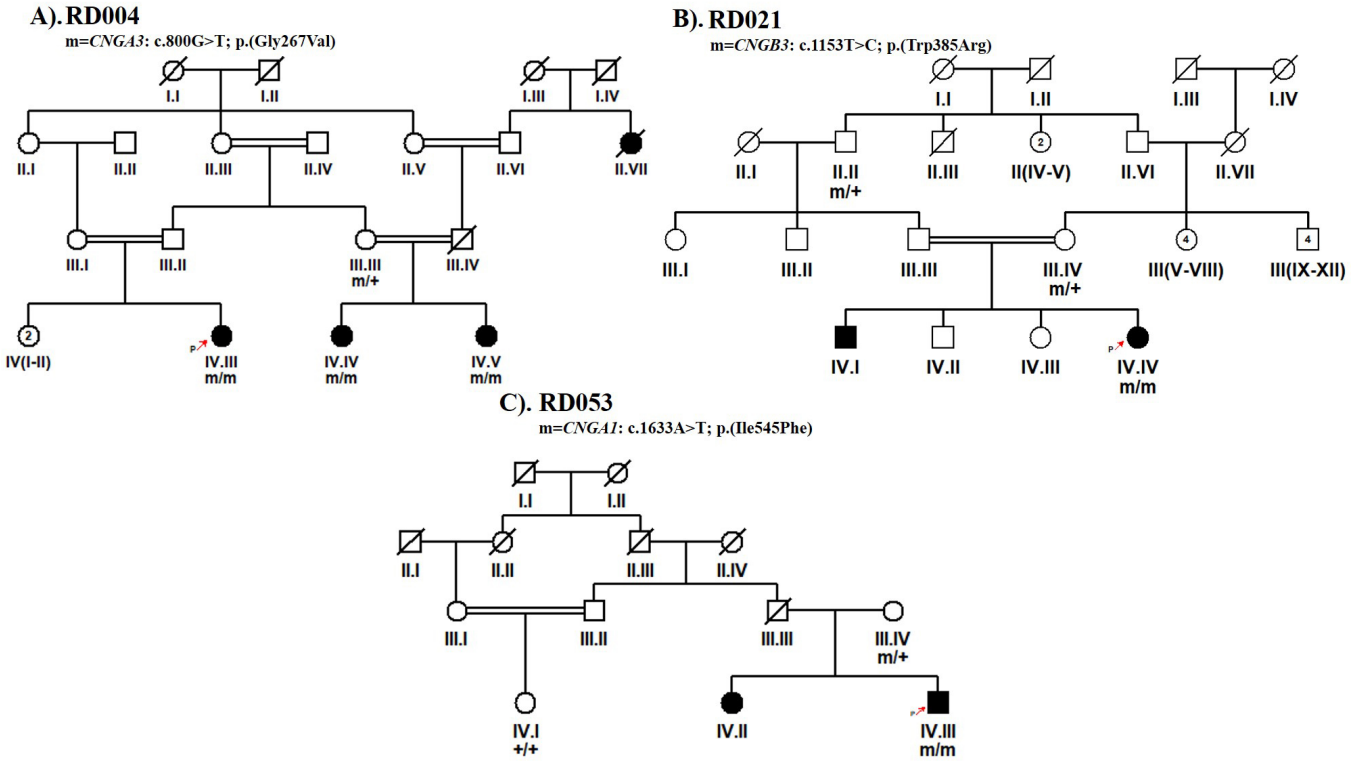

Figure 1. Pedigrees of families with identified novel homozygous variant in CNG channel genes. **A**. Pedigree of family RD004 with a missense variant c.800G>T in the *CNGA3* gene segregating with maculopathy/complete achromatopsia (ACHM)/IRD. **B**. Pedigree of family RD021 in which a novel homozygous pathogenic variant (i.e., c.1153T>C) was identified in the *CNGB3* gene segregating with cone-rod dystrophy (CRD)/IRD. **C**. Pedigree of family RD053 with identified novel homozygous pathogenic variant (i.e., c.1633A>T) in the *CNGA1* gene segregating with RP/IRD. Empty squares and circles show the unaffected males and females, respectively. The filled shapes show the affected individuals. The symbol labeled with a red arrow in the pedigree highlights the proband.

The *CNGA3* variants are more common in Asian populations, whereas *CNGB3* variants are more prevalent in Europe and the United States [25]. This observation is consistent with our current findings, since among eight families analyzed in this study, six were segregating pathogenic variants in the *CNGA3* gene (75%; Table 2). A list of 16 *CNGA3* variants given in the Table 3, including previously published variants from the Pakistani population and those identified in this study, shows that all except two are missense alleles (Table 3). Interestingly, we identified a previously reported missense variant (i.e., c.1641C>A) in *CNGA3* segregating in two unrelated consanguineous Punjabi families, RD001 and RD028 (Table 2). Previously, this variant was reported as a homozygous and heterozygous achromatopsia (ACHM)/cone dystrophy (CD)-causing allele from different populations across the globe [17,26–30], including Pakistan [31,32] (Table 3).

Two families, RD025 and RP125, were identified with two reported missense variants p.(Cys319Arg) and p.(Asn276Ser) in the *CNGA3* gene, respectively (Table 2). Previously, both of these variants p.(Cys319Arg) and p.(Asn276Ser) were reported for the first time as homozygous recessive disease-causing alleles from the Pakistani population [4,18,31,33]. Later on, the variant p.(Cys319Arg) was also reported as a heterozygous allele causing the ACHM phenotype in a Pakistani family in a compound heterozygous manner along with another heterozygous allele, p.(Arg436Trp) [15]. However, in our study, all affected individuals were homozygous for the p.(Cys319Arg) variant, segregating ACHM/cone-rod dystrophy (CRD) phenotype (Table 1 and Table 2).

The homozygous c.1810C>T variant causing p.(Gln604*) in family RD031 is the only stop-gain variant in *CNGA3* identified in this study. This pathogenic variant is reported as likely pathogenic in ClinVar. Variant identification by targeted panel sequencing followed by Sanger sequencing in five affected and three unaffected members supports the variant pathogenicity (Appendix 2 and Appendix 4).

Among the three novel missense variants identified in the current study, one was c.800G>T in the *CNGA3* gene (Table

Table 2. List of variants identified in current study in CNG channels associated retinal dystrophies.

Sr. No.	Family ID	Accession number	Gene	Zygosity	Variant	Exon	Nucleotide change	Protein change	dbSNP ID	ClinVar ID/ Classification	gnomAD	Variant Interpretation (Codes Met)	Curated variant classification
1	RD001	(NM_001298.3)	*CNGA3*	Homo	CHR2: 99013274C>A	8	c.1641C>A	p.(Phe547Leu)	rs104893617	9478/P/LP	0.000159	PM2, PP1, PP2, PP3, PP4	Pathogenic
2	RD004	(NM_001298.3)	*CNGA3*	Homo	CHR2: 99012433G>T	8	c.800G>T	p.(Gly267Val)	Novel	NA	NA	PM2, PP1, PP2, PP3, PP4	Pathogenic
3	RD021	(NM_019098.5)	*CNGB3*	Homo	CHR8: 87656004A>G	10	c.1153T>C	p.(Trp385Arg)	Novel	NA	NA	PM2, PP1, PP2, PP3, PP4	Pathogenic
4	RD025	(NM_001298.3)	*CNGA3*	Homo	CHR2: 99012588T>C	8	c.955T>C	p.(Cys319Arg)	rs753625117	191,120/P/LP	1.77E-05	PM2, PP1, PP2, PP3, PP4	Pathogenic
5	RD028	(NM_001298.3)	*CNGA3*	Homo	CHR2: 99013274C>A	8	c.1641C>A	p.(Phe547Leu)	rs104893617	9478/P/LP	0.000159	PM2, PP1, PP2, PP3, PP4	Pathogenic
6	RD031	(NM_001298.3)	*CNGA3*	Homo	CHR2: 99013443C>T	8	c.1810C>T	p.(Gln604*)	rs753692812	444,512/LP	3.99E-06	PM2, PVS1, PP1, PP4	Pathogenic
7	RD053	(NM_000087.5)	*CNGA1*	Homo	CHR4: 47938866T>A	11	c.1633A>T	p.(Ile545Phe)	rs752905054	NA	2.41E-05	PM2, PP1, PP2, PP3, PP4	Pathogenic
8	RP125	(NM_001298.3)	*CNGA3*	Homo	CHR2: 99012460A>G	8	c.827A>G	p.(Asn276Ser)	rs199474697	156,335/LP/VUS	NA	PM2, PP1, PP2, PP3, PP4	Pathogenic

*Curated variant classification of novel and previously reported variants of uncertain significance is given based on data of present study as per ACMG guidelines. PM2: Pathogenic moderate 2 [Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium] PVS1: Pathogenic very strong [null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene here LOF is a known mechanism of disease)] PP1:[Cosegregation with disease in multiple affected family members in a gene definitively known to cause disease] PP2: Pathogenic supporting 2 [Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease] PP3: Pathogenic supporting 3 [Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)] PP4: Pathogenic supporting 4 [Patient's phenotype or family history is highly specific for a disease with a single genetic etiology]. Sequence variant nomenclature was obtained according to the guidelines of the Human Genome Variation Society (HGVS) by using Mutalyzer. The position of each variant is as per GRCh37/hg19 reference genome assembly.

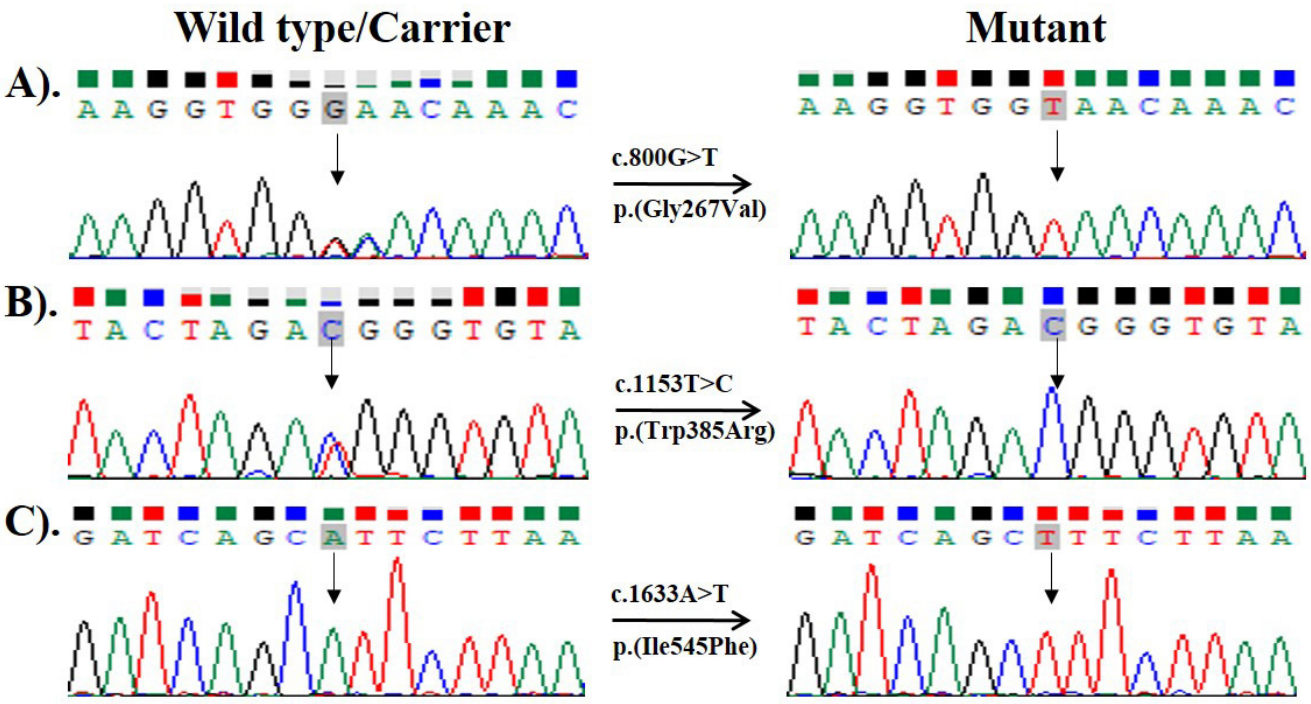

Figure 2. Sequence chromatograms of family RD004, RD021, and RD053 in which a novel homozygous pathogenic variant (i.e., c.800G>T, c.1153T>C, and c.1633A>T) was identified in the *CNGA3*, *CNGB3*, and *CNGA1* gene, respectively. The wild-type/carrier sequence is shown on the left, and the mutated sequence is shown on the right.

2). All three affected sisters with the p.(Gly267Val) mutation had reduced visual acuity, defective color vision, severe photophobia, and nystagmus, with complaints of nyctalopia. This variant substitutes glycine for valine at position 267 in the protein transcript, causing an alteration in protein structure and function. The second novel variant c.1153T>C, causing p.(Trp385Arg), was observed in the *CNGB3* (cyclic nucleotide-gated channel, beta-3) gene, which encodes the beta subunit of a CNG ion channel playing a role in phototransduction [26]. This variant was segregating with disease phenotype and had additional ocular abnormalities in family RD021. With the identification of this novel *CNGB3* missense variant, the total number of disease-causing variants identified to date from Pakistan has increased to six. Among these six, only two are missense variants, whereas the remaining four are null alleles (Table 3). In the present study, the only identified missense variant in the *CNGA1* (cyclic nucleotide-gated channel, alpha-1) gene was c.1633A>T segregated with an autosomal recessive RP phenotype. Previously, homozygous and compound heterozygous mutations in the *CNGA1* gene have been reported to cause RP in cases from East Asian and European populations [14,34–37]. Our literature search indicated that previously, just two missense mutations in the *CNGA1* gene were identified from cases of Pakistani origin, highlighting the rare incidence of *CNGA1* mutations in our population (Table 3). Identification of a novel homozygous missense variant, segregating with the RP phenotype in an affected case of the RD053 family, supports the previous findings of the association of *CNGA1* with rod dystrophies/RP. All these novel variants are found highly conserved in diverse species by using Clustal Omega and Weblogo3 (Figure 3A–D).

Our literature searches to catalog all the disease-causing variants described until December 2024 in the CNG channel genes from Pakistan and identification of the three novel disease-causing variants described in the present study reveal their mutation spectrum and contribution to the total genetic load of autosomal recessive IRDs in Pakistan (Table 2 and Table 3). Furthermore, given the difference in *CNGA1* (in rod) and *CNGA3* (in cone) expression in photoreceptors, we observed a significant difference in the phenotype of patients. The RD053 patient segregated *CNGA1* and had an RP phenotype in which rod photoreceptors were affected first, whereas in cases of families with mutations in the *CNGA3* gene, cone-rod dystrophy was detected. Interestingly, our study cohort included 72 families with IRD, among which we identified disease-causing variants in CNG channel genes (*CNGA1*,

Table 3. A list of CNG channel associated gene mutations in cases of Pakistani origin (previously reported till 31st December 2024 and novel to current study).

Ser No.	Gene	Nucleotide Change	Protein Change	Variant Type	Reported phenotype	Reference
1	*CNGA1*	c.626-627delTA	p.(Ser209fsX26)	Frame shift	RP	[14]
2	*CNGA1*	c.1298G>A	p.(Gly433Asp)	Missense	RP	[35, 37]
3	*CNGA1*	c.1633A>T	p.(Ile545Phe)	Missense	RP	This study
4	*CNGA3*	c.800G>T	p.(Gly267Val)	Missense	Maculopathy/Complete ACHM	This study
5	*CNGA3*	c.822G>T	p.(Arg274Ser)	Missense	ACHM	[38]
					RP	[39]
6	*CNGA3*	c.827A>G	p.(Asn276Ser)	Missense	ACHM	[4, 33]
					CD/CRD/Incomplete ACMH	This study
7	*CNGA3*	c.847C>T	p.(Arg283Trp)	Missense	ACHM	[4, 37]
8	*CNGA3*	c.952G>A	p.(Ala318Thr)	Missense	RD	[40]
9	*CNGA3*	c.955T>C	p.(Cys319Arg)	Missense	Juvenile CRD with maculopathy	[18]
					IRD	[31]
					ACHM	[15]
					CRD/Maculopathy	This study
10	*CNGA3*	c.991G>C	p.(Gly331Arg)	Missense	ACHM	[41]
11	*CNGA3*	c.1279C>T	p.(Arg427Cys)	Missense	ACHM	[4]
12	*CNGA3*	c.1306C>T	p.(Arg436Trp)	Missense	ACHM	[15, 41, 42]
13	*CNGA3*	c.1315C>T	p.(Arg439Trp)	Missense	ACHM	[15]
14	*CNGA3*	c.1443dupC	p.(Ile482Hisfs*6)	Frame shift	IRD	[31]
15	*CNGA3*	c.1540G>A	p.(Asp514Asn)	Missense	ACHM	[42]
16	*CNGA3*	c.1556T>C	p.(Met519Thr)	Missense	IRD	[31]
17	*CNGA3*	c.1600G>A	p.(Gly534Arg)	Missense	IRD	[31]
18	*CNGA3*	c.1641C>A	p.(Phe547Leu)	Missense	IRD	[31, 32]
					CD/ACHM/BVMD	This study
19	*CNGA3*	c.1810C>T	p.(Gln604*)	Stop gain	CRD	This study
20	*CNGB1*	IVS-1G>A	p.?	Splice Site	RD	[43]
21	*CNGB1*	c.412-1G>A	p.?	Splice Site	RP	[39]
22	*CNGB1*	c.413-1G>A	p.?	Splice Site	RP	[41]
23	*CNGB1*	c.852_874+25del	p.(Ile286Aspfs*9)	Frame shift	RP	[31]
24	*CNGB1*	c.2284C>T	p.(Arg762Cys)	Missense	RP	[39]
25	*CNGB1*	c.2493-2_2495delinsGGC	p.(Ser831Argfs*2)	Frame shift	IRD	[44]
					CSNB	[15]
26	*CNGB1*	c.2493–2A>G	p.?	Splice Site	RP	[35]
27	*CNGB3*	c.646C>T	p.(Arg216X)	Stop gain	ACHM	[41]
					RD	[43]
28	*CNGB3*	c.1148delC	p.(Thr383Ilefs*13)	Frame shift	ACHM	[45]
					Progressive CD	[46]
					ACHM	[46]

Ser No.	Gene	Nucleotide Change	Protein Change	Variant Type	Reported phenotype	Reference
29	*CNGB3*	c.1153T>C	p.(Trp385Arg)	Missense	CRD	This study
30	*CNGB3*	c.1208G>A	p.(Arg403Gln)	Missense	Progressive CD	[46]
31	*CNGB3*	c.1574_1575del	p.(Phe525Ter)	Stop gain	MD	[37]
32	*CNGB3*	c.1825delG	p.(Val609Trpfs*9)	Frame shift	ACHM	[38]
					RP	[39]

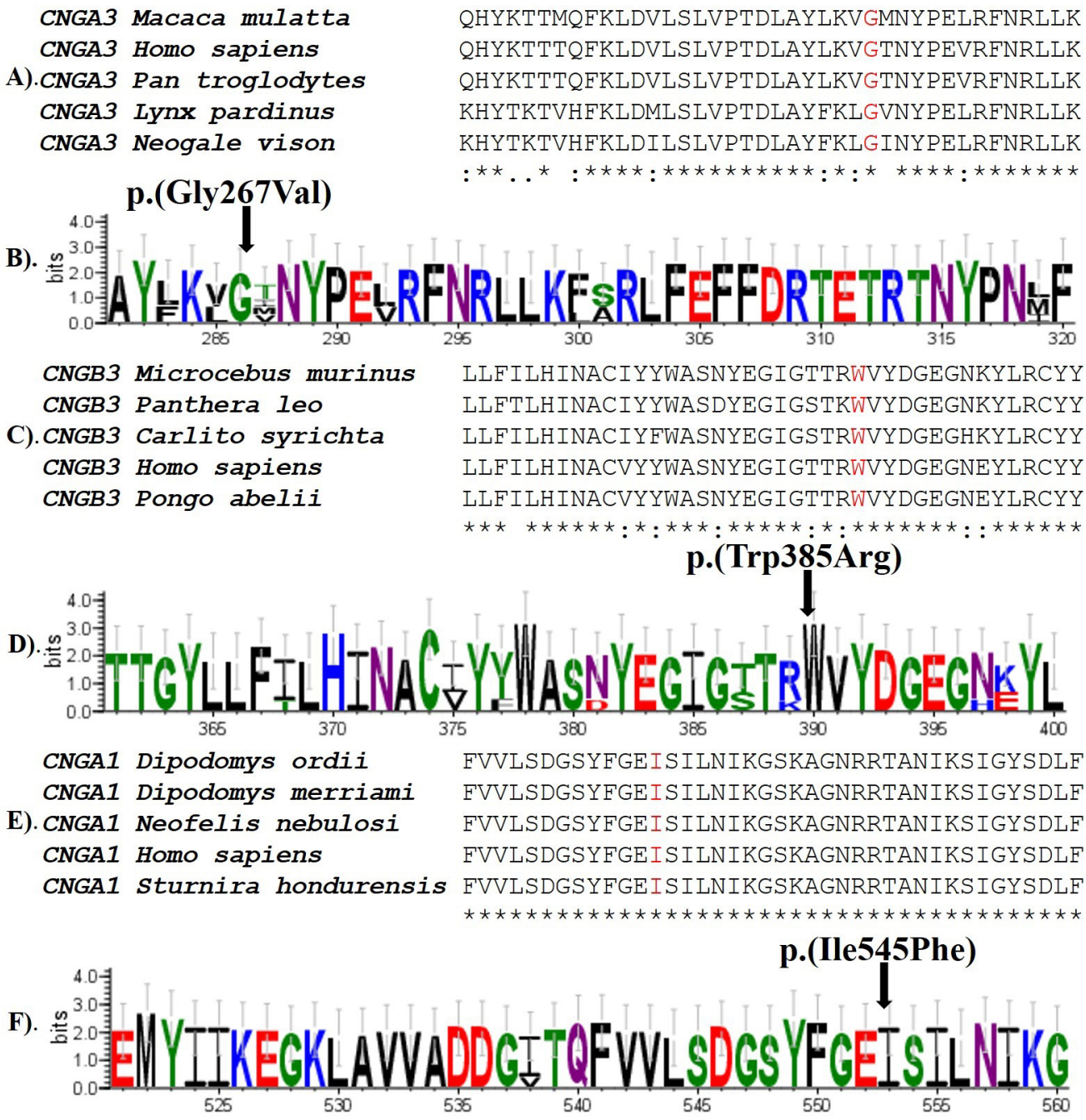

Figure 3. A comparison of *CNGA3*, *CNGB3*, and *CNGA1* gene conservation among different homologs for novel variants identified in this study. **A**, **C**, **E**. Clustal Omega multiple sequence alignment (highlighted in red) for p.(Gly267Val), p.(Trp385Arg), and p.(Ile545Phe) detected in RD004, RD021, and RD053, respectively, showing high sequence conservation in diverse species. **B**, **D**, **F**. WebLogo3 results of three novel variants showing comparison of conservation among homologs. All identified variants show 100% conservation among different species (large size of amino acid abbreviation letters shows full conservation while small size shows less conserved position among homologs).

CNGA3, and *CNGB3*) in eight families (11.1%), but we did not identify any mutation in the *CNGB1* gene. The main limitation in our study explaining genetic variants to phenotypic diversity is the unavailability of electroretinography reports due to the lack of this facility in the collaborating hospital. Our study highlights the need to invest in better diagnostic equipment in Pakistan to capture more comprehensive clinical data for future research. Nevertheless, this study

contributes to the genetic spectrum of the CNG channel-associated phenotypes within the Pakistani population and provides practical implication of genetic screening in the specific diagnosis of IRD subtypes.

APPENDIX 1. PEDIGREES OF FOUR FAMILIES (RD001, RD025, RD028 AND RP125) IN WHICH MISSENSE REPORTED VARIANTS IN *CNGA3* GENE WERE IDENTIFIED.

To access the data, click or select the words "Appendix 1." Empty squares and circles show the unaffected males and females, respectively. The filled shapes show the affected individuals. The symbol labeled with a red arrow in each pedigree highlights the proband. Consanguineous unions are indicated by double lines. A) Pedigree of RD001 family showing the segregation of the *CNGA3* single nucleotide substitution i.e., c.1641C>A in an autosomal recessive manner in five affected cases. B) Pedigree of RD025 family showing the segregation of *CNGA3* single nucleotide substitutions i.e., c.955T>C in two affected cases. C) Pedigree of RD028 family showing the segregation of the *CNGA3* single nucleotide substitution i.e., c.1641C>A; p.(Phe547Leu) in three affected individuals. D) Pedigree of RP125 family showing the segregation of variant c.827A>G in *CNGA3* gene.

APPENDIX 2. PEDIGREE OF FAMILY RD031 WITH A STOP GAIN REPORTED VARIANT IN *CNGA3* GENE.

To access the data, click or select the words "Appendix 2." Empty squares and circles show the unaffected males and females, respectively. The filled shapes show the affected individuals. The symbol labeled with a red arrow in each pedigree highlights the proband. Consanguineous unions are indicated by double lines.

APPENDIX 3. FUNDUS PHOTOGRAPHS OF LEFT AND RIGHT EYE OF AN AFFECTED MEMBER OF FAMILY RD021 (IV-I).

To access the data, click or select the words "Appendix 3." These images show small hyperopic changes with deep cup and had cups to disc ratio (CRD) of 0.4–0.5, bull's eye maculopathy and periphery un-recordable pigmentary changes.

APPENDIX 4. SEQUENCE CHROMATOGRAMS OF FAMILIES WITH REPORTED VARIANTS IN CNG CHANNELS-ASSOCIATED GENE I.E., *CNGA3* (A-D).

To access the data, click or select the words "Appendix 4." The wild-type/carrier sequences are given on the left side, and the variant sequences are given on the right side. (A) The missense variant c.1641C>A in two families RD001 and RD028. (B) The missense variant c.955T>C in RD025. (C) The stop gain variant c.1810C>T in family RD031. (D) The missense variant c.827A>G in family RP125.

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