RESEARCH ARTICLE

P.arg102ser is a common *Pde6α* mutation causing autosomal recessive retinitis pigmentosa in Pakistani families

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Abstract

Objective: To explore the genetic cause of autosomal recessive retinitis pigmentosa in consanguineous families.

Methods: The multi-centre study was conducted from July 2015 to June 2018 at Liaquat University of Medical and Health Sciences, Jamshoro, the University of Sindh, Jamshoro, and Islamia University, Bahawalpur, Pakistan, and comprised families affected with non-syndromic autosomal recessive retinitis pigmentosa. Ophthalmological investigations were done to assess the fundus of the patients and the status of the disease. Pedigrees were drawn and family histories were recorded to find out the mode of inheritance. A 10cc sample of whole blood was obtained from each participant and deoxyribonucleic acid was extracted. Homozygosity mapping was performed using three short tandem repeat polymorphisms closely linked to phosphodiesterase 6A gene, and the linked families were Sanger-sequenced for identification of the mutation. Bioinformatic tools were used to design amplification refractory mutation system assay and to assess the protein structure and pathogenic effects of the mutation.

Results: In the 80 consanguineous families, there were 464 individuals, and, of them, 236(51%) were affected with their age ranging between 4 and 80 years. Family history and pedigree drawings revealed autosomal recessive retinitis pigmentosa with early childhood onset. Linkage analysis indicated the homozygosity in 6(7.5%) families. Sanger-sequencing revealed a common mutation c.304C>A (p.Arg102Ser); segregating with the disease in the linked families.

Conclusion: The findings may offer effective genetic counselling and minimise disease penetration in consanguineous families.

Keywords: PDE6a mutations, Retinitis pigmentosa, Pakistan, ARMS assay. (JPMA 71: .816; 2021)

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Introduction

Retinitis pigmentosa (RP) is the most frequently inherited retinal degeneration disorder with a worldwide prevalence of 1 per 4000.[1] It is the leading cause of the early blindness, causing progressive loss of normal function of rod photoreceptor cells, followed by degeneration of cone receptors.[2,3] Non-syndromic RP (NSRP) is transmitted through different mode of inheritance; autosomal recessive RP (ARRP) accounts for 50-60%, followed by autosomal dominant RP (ADRP) 30-40% and X-linked inheritance 5-15%.[4] Over 80 genes have been reported for NSRP from ethnically diverse patients around the globe[5] and 35 genes have been associated with ARRP only.

Mutations in phosphodiesterase 6A (PDE6α) gene have been associated with ARRP in different world populations. It encodes an enzyme which plays an active role in visual photo-transduction cascade. PDE6 is a holoenzyme and consists of alpha and beta subunits. PDE6α encodes the α-subunit of cyclic guanosine monophosphate (cGMP), which is the second messenger molecule in photo-transduction pathway and performs an important role in photo-transduction cascade and transfer signal to brain through optic nerve and retina.[6-9] Mutations in PDE6α gene disturbs the function of cGMP and cause retinal degeneration in humans.[10]

PDE6α gene (Online Mendelian Inheritance in Man [OMIM] *180071) is located on the long arm (q) of chromosome 5 at position 32, and it consists of 22 exons which encode 860 amino acids long protein.[8,11] To date, 40 pathogenic mutations have been reported in PDE6α gene; and majority are single base substitutions (65%, 26/40), resulting in mis-sense and non-sense variants.[12] Previous studies from Pakistan have described different pathogenic mutations in PDE6α gene, associated with ARRP in Pakistani population (Table 1).

Pakistani population is genetically heterogeneous and a mixture of people with different ethnic backgrounds. Previous studies have shown extreme genetic heterogeneity for RP segregating in consanguineous pedigrees. Moreover, various disease-causing alleles have

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Table-1: PDE6α mutations found in Pakistani families.

Gene	Variants	No. of families	No. of alleles
PDE6α	c.889C>T, p.Arg256>Ter[13]	1	2
	IVS10-2A>G, p.K470_L491del	1	2
	c.2218-2219insT, p.Y700fsX714	1	2
	IVS16 c.2028–1G>A[8]	1	2
	c.769C4T, p.R257*[14]	1	2
	c.1630 C>T, p.R44W[15]	1	2
	c.304C>A, p.R102S(This study)	6	12

been associated with variable clinical presentations.[12,14] The current study was planned to explore the common disease-causing genes and alleles in consanguineous pedigrees affected with ARRP and to develop economical test for genetic diagnosis.

Patients and Methods

The multicentre collaborative study was conducted from July 2015 to June 2018 by the Liaquat University of Medical and health Sciences, Jamshoro, the University of Sindh, Jamshoro, and Islamia University, Bahawalpur, Pakistan, and comprised consanguineous pedigrees having minimum two affected individuals identified through different eye hospitals of Sindh and Punjab provinces. Approval was obtained from the ethics research committee of Liaquat University of Medical and Health Sciences (LUMHS), Jamshoro. The number of families and samples were ascertained considering the prevalence of the disease along with the structure of pedigrees. After obtaining written informed consent from each participating family, detailed family and clinical histories were recorded and pedigrees were drawn to ascertain the mode of inheritance by visiting the families at their doorstep. All the affected individuals, their parents and siblings were enrolled. Detailed clinical examinations were done to determine the severity and phenotypic presentation of RP, such as low visual acuity (LVA), fundus pigmentations (FPs) and night blindness (NB).

For blood sampling and deoxyribonucleic acid (DNA) extraction, 10ml venous blood was collected from each participant in 50ml falcon tubes containing 0.5ml ethylenediaminetetraacetic acid (EDTA) as anticoagulant and was shifted as per the standard procedure to the Department of Molecular Biology and Genetics, LUMHS for laboratory experiments. Genomic DNA was isolated from leucocytes.[16] Briefly, whole blood was frozen, and then washed thrice with washing buffer containing 10mM Tris Hydrochloric acid (HCL) and 2mM EDTA, and centrifuged at 3000 rpm for 15 minutes to separate leucocytes as palate and supernant containing red blood cells (RBCs). The separated leucocytes were incubated at 37°C in the presence of 20µl/ml of blood 10% sodium dodecyl sulphate (SDS), 10mM Tris HCL buffer, 2mM EDTA, 400mM sodium chloride (NaCl) and 5µl/ml of Proteinase K (10mg/ml) to digest proteins. After overnight incubation, the digested proteins were precipitated by adding saturated NaCl, 100µl/ml of blood and centrifuged at 3000 rpm for 20 min. The quantity and quality of extracted DNA were confirmed by agarose gel electrophoresis and spectrophotometry at 260 and 280nm wavelength.

Linkage analysis was performed by genotyping three short tandem repeat (STR) markers closely linked to PDE6α gene. A tail of 18 nucleotide long, universal M13 primer was added on 5' end of each forward primer. Total 10µl PCR reaction for amplification of STRs was performed by using 0.4pM fluorescently labelled universal M13 primer along with 0.1pM forward primer, 0.4pM reverse primer, 0.3 units of Taq DNA polymerase, 1.5mM magnesium chloride (MgCl2) buffer, 1µl deoxyribonucleotide triphosphate (dNTPs), 100ng DNA and 3.8µl of water. The optimised condition of STR marker was denaturised at 95°C for 5min, annealing temperature varied for each primer pair (Table 2). The amplified products were separated by capillary electrophoresis on genetic analyser (3130 ABI Prism) and alleles were called by using GeneMapper V 4.1 software. Haplotypes was constructed and compared among the families.

For mutation screening, sequencing primers for 22 exons and exon-intron boundaries of PDE6α gene were designed by using Primer 3 web tool. The specificity of primers was checked by in silico PCR, provided by University of California, Santa Cruz (UCSC). The PCR reaction for amplification of exons consists of 15mM MgCl2 buffer, 25mM dNTPs and 0.3 units of Taq polymerase. The thermal conditions were denaturation at 95ºC for 5 min, denaturation at 95ºC for 30s, annealing was variable for each fragment and elongation at 72ºC for 45 seconds. The final extension was done at 72ºC for 5 min. The amplified fragments were purified by using absolute ethanol

Table-2: Primer sequences of short tandem repeat (STR) polymorphisms and amplification refractory mutation system (ARMS) assay primers.

Locus	Primer name	Direction	Primer sequences
STR Primer Sequences			
PDE6α	D5S812	Forward	TACCACAGCAACCACAAAGA
		Reverse	GAGGAAAGCAAAGACCATGA
	D5S2013	Forward	AATGGTCTCCAGAAAAGCA
		Reverse	AGCCAGGTGAGTCTCGTC
	D5S2015	Forward	TTGGCTAATGGGAGGCAACA
		Reverse	GCTACCTAAAGAACACAGTCATGGC
ARMS Assay Sequences			
PDE6α	Wild	Forward Inner	CGCATGAGCCTGTTCATGTACCGGATCC
c.304C>A	Mutant	Reverse Inner	GGTGGCCAGCTCTGCGATGCCATTACT
Mutation	IC	Forward Outer	ACTACCGGGCCAAGCTCATCTCCGACCT
	IC	Reverse Outer	TTCTTAGAGTGTGCGACATGGCCCACGA

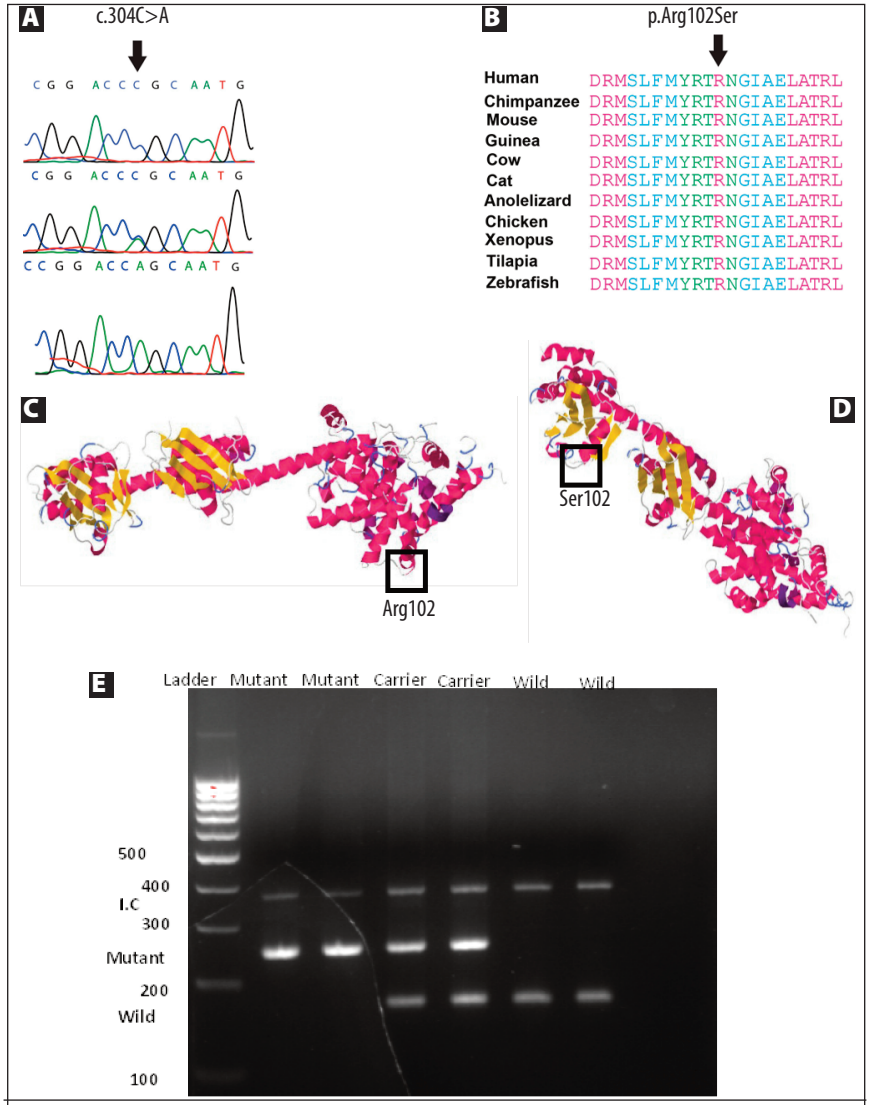

Figure-1: A: The sequence chromatogram of mutation c.304C>A (p.Arg102Ser). B: Multiple sequence alignment of 11 species, showing conservation of Arginine at 102nd position of PDE6a Protein. C & D: The protein model of normal and mutated PDE6α protein respectively. E: The agarose gel image of newly developed amplification refractory mutation system (ARMS) assay polymerase chain reaction (PCR), showing internal control, normal and mutated alleles along with size ladder.

followed by centrifugation at 15000rmp on 14ºC temperature. The Sanger sequencing reaction was performed as described in literature.[17] The chromatograms were analysed by Chromas 1.45.[18]

For bioinformatics analysis, the pathogenicity of identified variant was accessed by using PolyPhen-2, mutation tester and Sift software. The biochemical nature of substituted, wild type amino acid and effect on protein structure was studied by using Have Your Protein Explained (HOPE) tools.[19-22]

For the development of amplification refractory mutation system (ARMS) assay, tetra primers assay was developed for frequent mutation (c.304C>A) using Primer 1 tool.[23] PCR amplification reaction was performed in 20ul volume containing 0.5 pM each outer primer, 0.1 pM of each inner primer, 2mM MgCl2 and 50ng of DNA. The thermal cycle conditions were set as initial denaturation at 95ºC, followed by 30 cycles; each cycle consisting of denaturation at 95ºC for 30s, annealing at 600C for 40s and extension at 72ºC for 50s. Final extension was carried out at 72ºC for 10min. The band size of internal control, wild type (WT) and mutant alleles were noted (Figure 1).

Result

In the 80 consanguineous families, there were 464 individuals, and, of them, 236(51%) were affected with their age ranging between 4 and 80 years. Of the 6 families 3(50%) each belonged to Punjabi and Sindhi ethnic origins. All the affected individuals had complaint of NB and the

Table-3: Clinical findings of the families with autosomal recessive retinitis pigmentosa (RP).

Family ID	Patient ID	Current Age (Years)	Diagnosis Age Years	First symptoms	Night Blindness	Fundus Findings	Visual Acuity	
PKRP-1	1	25	6	Night Blindness	Progressive	MD, P, POD, AA	6/18	6/18
	2	28	8	Night Blindness	Progressive	MD, P, POD, AA	6/24	6/18
	4	32	8	Night Blindness	Progressive	MD, P, POD	6/36	6/40
PKRP-2	1	42	6	Night Blindness	Progressive	MD, P, POD, AA	6/24	6/24
	4	67	12	Night Blindness	Progressive	MD, P ,POD, AA	6/24	6/24
PKRP-3	3	37	10	Night Blindness	Progressive	MD, P ,POD	6/18	6/18
	1	33	7	Night Blindness	Progressive	MD, P ,POD, AA	6/24	6/18
PKRP-4	1	35	8	Night Blindness	Progressive	MD, P ,POD, AA	6/18	6/24
	2	41	10	Night Blindness	Progressive	MD, P, POD, AA	6/24	6/24
PKRP-5	3	26	6	Night Blindness	Progressive	MD, P, POD, AA	6/20	6/20
PKRP-6	1	31	6	Night Blindness	Progressive	MD, P ,POD, AA	6/18	6/18

MD: Macular degeneration, P: Pigmentation, POD: Pale optic disc, AA: Attenuated arteries.

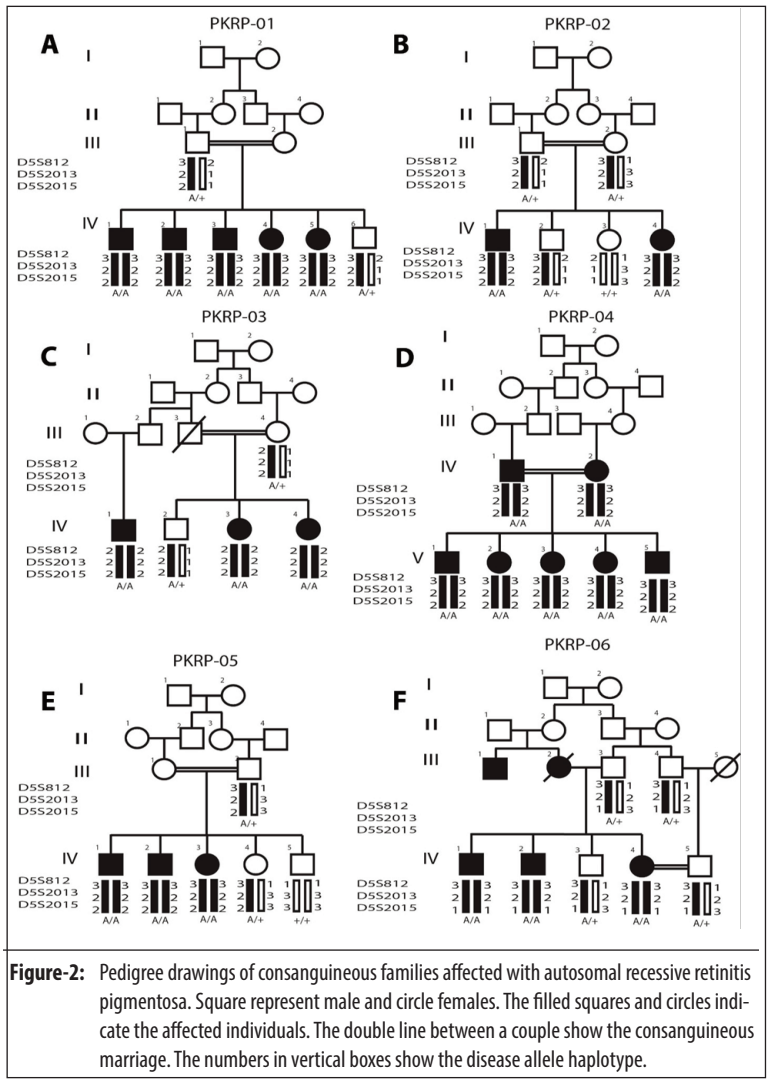

Figure-2: Pedigree drawings of consanguineous families affected with autosomal recessive retinitis pigmentosa. Square represent male and circle females. The filled squares and circles indicate the affected individuals. The double line between a couple show the consanguineous marriage. The numbers in vertical boxes show the disease allele haplotype.

onset of the disease was variable among the families. Clinical examination showed signs of RP with variable severity and pigmentation in fundus. All the normal siblings and parents had normal vision.

Homozygosity mapping showed linkage with three STR markers closely linked to PDE6α gene in 6(7.5%) families (Figure 2). Haplotype analysis showed three different haplotypes across the three markers, indicating the independent origin of the mutation. The direct sequencing of all exons and exon-intron boundaries of PDE6α gene in 5(6.25%) families revealed c.304C>A transition, resulting in a substitution of arginine into serine at 102nd amino acid of PDE6α protein. All the available parents and siblings were sequenced to confirm the segregation of the variant and its association to disease phenotype. In silico functional analysis of c.304C>A transition confirmed the pathogenic status of the variant and multiple sequence alignment revealed that arginine at 102 was highly conserved residue in 12 different orthologue species. Furthermore, arginine is polar amino acid, and its substitution with serine affects the normal structure and function of the protein.

The fundus of all patients carrying c.304C>A (p.Arg102Ser) were assessed. The typical signs of RP were observed in all the examined fundi, showing macular degeneration, deposition of pigments in mid and far periphery regions and attenuation of arteries. The severity of pigmentation, level of degeneration of macula and arterial attenuation were variable among patients of different families (Figure 3). The diagnosis age of the patients carrying c.304C>A (p.Arg102Ser) ranged 6-12 years (Table 3).

Discussion

The current study was planned to explore the frequent RP-causing gene and to develop a cost-effective method for genetic screening and counselling. To the best of our knowledge, it is the first study describing a frequent mutation of PDE6α gene responsible for RP in 6 unrelated Pakistani families from a cohort of 80 families. Besides, a cost-effective and rapid diagnostic method based on tetra primer ARMS assay was developed to screen the common mutation. This will be helpful for targeted screening of ARRP genes and may help to prioritise the detection of mutation for proper genetic counselling.

The 80 consanguineous families with more than two patients of ARRP belonged to different ethnic groups and castes of Pakistan. This is the largest cohort of RP-affected Pakistani families, enrolled from Sindh and southern Punjab, and screened for disease-causing genes. A common PDE6α gene mutation, c.304C>A, p.Arg102Ser, was found segregating with the disease phenotype in 6 families (7.5%; 95% CI: 0.8-11.2%). Three different haplotypes were found in 6 families. Four families shared the haplotype across the three genotyped STRs, while two families had two different haplotypes. It indicated the independent origin of c.304C>A, p.Arg102Ser in the families belonging to different castes. All the 6 families carried the same mutation, but manifested variable phenotypic presentation, possibly due to the ethnic differences or epigenetic influences.

Total 40 pathogenic mutations have been reported worldwide in PDE6α gene. The c.304C>A, p.Arg102Ser was first reported from Danish population[14,24] and 6 other different PDE6α mutations have been identified in Pakistani patients. It includes non-sense, mis-sense and

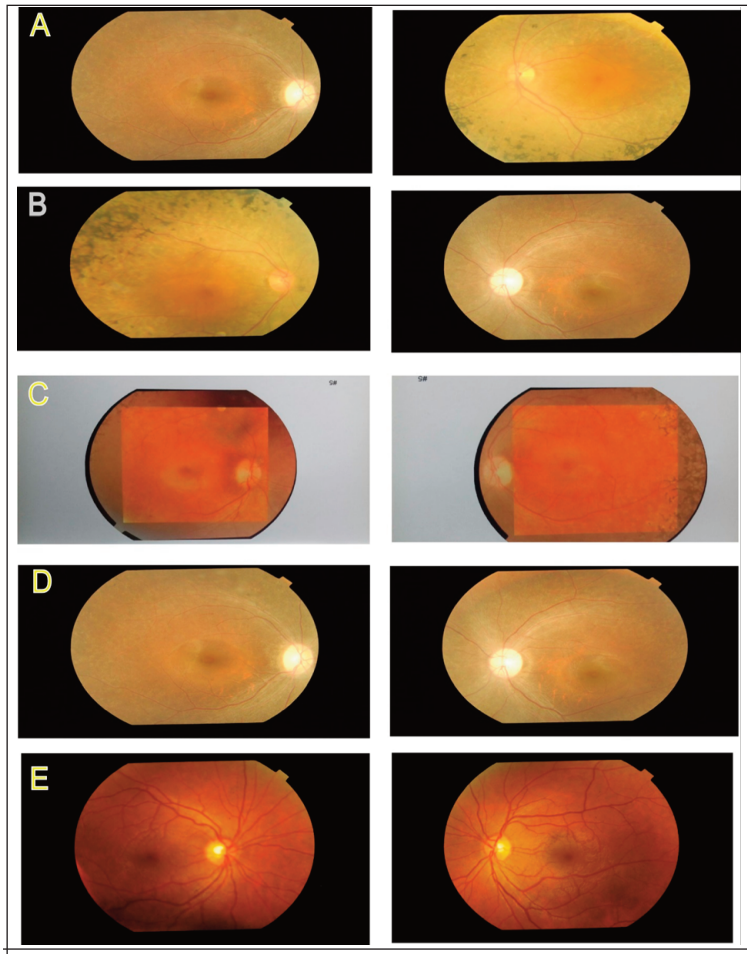

Figure-3: Representative fundus photographs of patients of families affected with Retinitis Pigmentosa, showing macular degeneration, deposition of pigments, pale coloured optic disc and attenuation of arteries. A: OD and OS of affected individual of PKRP-1, B: PKRP2, C: PKRP03, D: PKRP04 and E: PKRP06.

splicing-site variants. A study on 58 consanguineous families from Pakistan found only one RP-affected family carrying c.304C>A, thus suggesting its minimal contribution to inherited RP in Pakistani patients.[8,24] However, it is contradictory to our results, which revealed c.304C>A as the common PDE6α variant in the cohort of 80 families. This variation may be due to different ethnic backgrounds of the patients.

Previously, three different mutations have been identified on the codon 102 of the PDE6α gene in different populations and it is the only codon with multiple amino acid substitutions.[24,25] The arginine at 102 is highly conserved in different species and constitutes the cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA (GAF) domain of PDE6α protein. It is substituted by three different amino acids, serine (p.Arg102Ser), histidine (p.Arg102His) and cysteine (p.Arg102Cys). All the substitutions affect the normal protein function and result into retinal anomalies. The bioinformatics analysis of p.Arg102Ser revealed that there were differences in charge, size and hydrophobicity of WT and mutant amino acids. The mutant residue had negative charge and was smaller in size, thus could cause loss of interaction of GAF with cGMP-binding domain motif. In addition, the mutant amino acid was more hydrophobic and could result in loss of hydrogen bonds and disturb protein folding.

The findings of the current study may be used to structure a policy for the identification of common pathogenic variant of RP in consanguineous pedigrees at risk for identification of carriers and management of the disease.

Conclusion

The findings of the current study may lead to effective genetic counselling and minimising the disease penetration in consanguineous families in Pakistan.

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Conflict of Interest: None.

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