Article

Novel Homozygous *TULP1* and *RPE65* Variants Underlies Recessive Retinitis Pigmentosa

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Abstract: Retinitis pigmentosa (RP) clinically and genetically heterogeneous group of inherited retinal disorders (IRD) that result in retinal degeneration. This study aimed to identify the genetic findings of patients with autosomal recessive retinitis pigmentosa (arRP). Whole exome sequencing (WES) was performed in two unrelated Pakistani families underlying arRP. Data analysis and mutation screening was performed for all the known RP genes following bi-directional Sanger sequencing to determine whether any of the candidate variants co-segregated with the disease phenotype in the families. WES data analysis revealed a novel homozygous missense variant (c.1274T>C) in the in Tubby like Protein 1 (TULP1 NM_003322.6) gene in family 1 and a novel homozygous frameshift variant (c.351delC) in the retinoid isomerohydrolase 65 (RPE65 NM_000329.3) gene in family 2. The identified variants perfectly co-segregated with the disease phenotype within the families. Our results strongly suggest that mutations in TULP1 and RPE65 are responsible for the retinal phenotype in the affected individuals. These mutations will increase the mutation spectrum of these genes; furthermore, it will enhance our knowledge and understanding of the underlying molecular mechanisms of retinitis pigmentosa.

Keywords: retinitis pigmentosa; *TULP1; RPE65*; recessive; missense; frameshift

1. Introduction

Retinitis pigmentosa (RP, OMIM: #268000) is a progressive, clinically and genetically heterogeneous group of inherited retinal disorders that result in retinal degeneration [1, 2]. RP affecting about 1:3500–1:5000 people worldwide [1]. Typical clinical features include night blindness during adolescence, side vision during early adulthood, and central vision in later life. The first symptom is progressive night blindness because of the progressive loss of rod and cone photoreceptor cells [2]. Due to rod dysfunction, a gradual loss of the peripheral visual field is followed by a drop in visual acuity due to secondary cone degeneration. RP is classified as non-syndromic RP and syndromic RP. About 20–30% of RP patients have other symptoms, and such cases belong to more than 30 different syndromes [3]. RP is a highly heterogeneous disorder and more than

80 genes are associated with non-syndromic RP [4]. RP can be inherited as an autosomal dominant (AD), autosomal recessive (AR), and X-linked form [1-4]. However, other forms of inheritance patterns such as mitochondrial and digenic RP have also been described in the literature [5]. Advance technologies such as next generation sequencing (NGS) including whole exome sequencing (WES), whole genome sequencing (WGS) and targeted next generation sequencing (TNGS) have identified various genetic causes for human RP.

In this article, we describe two consanguineous Pakistani families having hallmark features of RP. Using WES followed by Sanger sequencing, we found a novel homozygous missense variant (c.1274T>C; p.Ile425Thr) in Tubby like Protein 1 (TULP1 NM_003322.6) gene in family 1 and a novel homozygous frameshift variant (c.351delC; p.Arg118Glyfs*9) in retinoid isomerohydrolase 65 (RPE65 NM_000329.3) gene in family 2 respectively. Both variants perfectly segregate with disease phenotype in all the families members. These finding suggests that TULP1 and RPE65 have an essential function in maintaining adult photoreceptors in human, and also supports the hypothesis that diverse clinical phenotypes can be caused by mutations in the same gene

2. Materials and Methods

2.1. Human Subjects

In the current study, the total number of 16 individuals from two large unrelated Pakistan families was studied. Ten of them had an initial diagnosis with non-syndromic RP. All patients and their families were clinically examined by an ophthalmologist at local hospitals in Pakistan. Comprehensive eye examinations were performed, including visual acuity (VA) and fundus photography. RP diagnosis was confirmed in patients with night blindness, progressive visual field constriction, poor VA in advanced stages and fundus examination [6].

2.2. Ethical Approval

Written Informed consent for genetic testing and publication of these reports were obtained from all participating individuals. This study was approved by the Ethical Review Committee (ERC) of Peking Union Medical College (Beijing, China), China Medical University (Shenyang, China).) and followed Helsinki protocols. All experiments were conducted in accordance with ethical principles.

2.3. DNA Extraction and Quantification

Fresh peripheral blood samples were collected from 7 individuals (4 males and 3 females) of family 1 and 9 individuals (4 males and 5 females) of family 2 (Figure 1 A-B) in ethylenediaminetetraacetic acid (EDTA) vacutainers tube. Genomic DNA was extracted from peripheral blood using the QIAquick DNA extraction kit (Qiagen, Hilden, Germany) and quantified using the Nanodrop-2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

2.4. Library Preparation and Whole Exome Sequencing

Library preparation and whole exome sequencing was performed using the Ion Proton Machine as described previously [7]. The library was prepared using Ion AmpliSeq Library Kit 2.0 (Life Technologies). Whole exome oligos were used to capture the target regions followed by PCR amplification. The PCR conditions were 99°C for 2 minutes, followed by 18 cycles of 99°C for 15 seconds and 60°C for 8 minutes. The primer sequences were partially digested with FuPa reagent. The library was encoded with the Ion Xpress barcode adapter. Magnetic beads were used to purify the resulting libraries. Finally, the Ion PI chip is used for sequencing by the Ion Proton Machine.

2.5. Data Processing

Sequence data was converted from raw data to the FASTQ file, aligned to the GRCh37/hg19 reference sequence. Genotyping of multiple allelic substitutions were performed by Torrent Variant Caller (version 4.4.3). Functionally variants were annotated with ANNOVAR

(http://www.wannovar.usc.edu/). Bam files were visualized with Integrative Genome Viewer (IGV, http://www.broadinstitute.org/igv/). Variant frequencies were determined with Exome Variant Server (http://evs.gs.washington.edu/EVS/), GnomAD (https://gnomad.broadinstitute.org), and 1000 Genomes (http://www.1000genomes.org/). To identify pathogenic variants in the RP patients, we first focused on the known disease-causing genes for RP [OMIM, https://www.omim.org/]. As the pedigrees depicted recessive inheritance pattern thus preference was given to the functional homozygous or compound heterozygous variants, including nonsense variants, missense variants, frameshift indels and splice site variants.

2.6. In-Silico Analysis

For functional effect prediction, the identified variants was subjected to different bioinformatics tools such as Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (SIFT, http://sift.jcvi.org/) , Protein Variation Effect Analyzer (PROVEAN, http://provean.jcvi.org) , Mutation Taster (http://www.mutationtaster.org/) , Varsome (https://varsome.com/) , Mutation assessor (http://mutationassessor.org) , and Combined Annotation Dependent Depletion (CADD, https://cadd.gs.washington.edu/). Finally, for the interpretation of variants, the American College of Medical Genetics and Genomics (ACMG) 2015 guidelines were used [8].

2.7. Candidate Genes Sequencing

Genomic sequences of the detected mutations were retrieved from the University of California, Santa Cruz (UCSC) genome database browser (https://genome.ucsc.edu/). Primer 3 software (http://primer3.ut.ee/) was used for primer design (TULP1; Forward Primer: 5′-GCTCAGGGAGTTGGCTATTT-3′ Reverse Primer 5′-CTGGCAGCTGTGATCTATGT-3′, RPE65; 5′-CTGTGTCCCACCTGCTTAAT-3′ and Reverse Primer 5′- GGATTGCTCCTGTCTATACTCTTC-3′) and Sanger sequencing was performed to validate the candidate variants in the affected and normal individuals in both families members. Chromas Lite (http://technelysium.com.au/wp/) and Codon Code Aligner (https://www.codoncode.com/aligner/) software was used to visualize the chromatograms and identify the sequence variant (Figure 1E and 1F).

2.8. Protein Modeling and Conservation Analysis

The amino acid sequence of TULP1 and RPE65 encoding protein was retrieved from UniProt database (https://www.uniprot.org/) with accession number O00294-1 and Q16518-1 in FASTA format. The 3D modeled structure of the TULP1 and RPE65 proteins for wild and mutant type were prepared using homology modeling in SWISS-MODEL (https://swissmodel.expasy.org/). Structure visualization, measurement of distance, mutagenesis analysis and residue interaction networks to the protein function were performed with the different bioinformatics software's as described previously [9, 10]. The amino acid sequences alignments from different species were downloaded from UCSC genome database browser (https://genome.ucsc.edu/). The sequence alignment was performed with default parameters using Molecular Evolutionary Genetics Analysis (MEGA) software (https://www.megasoftware.net/) (Figure 2).

3. Results

3.1. Clinical Features

Affected individuals of these families initially presented symptoms such as night blindness but subsequently experienced a gradual daytime vision loss. All affected individual from both families was examined by local an ophthalmologist at District Head Quarter (DHQ) Teaching Hospital Bannu (THB), Pakistan.

3.2. Family 1

The members of family 1 live in the Bannu districts of Khyber Pakhtunkhwa Province of Pakistan and the parents are first cousins. Family 1 has four affected individuals (V-1, V-6, V-9 and V-11) having typical features of autosomal recessive RP (Figure 1A). The family's medical history, pregnancy and delivery were uneventful. Parents of the affected individuals were physically and mentally normal and healthy. All affected individuals (V-1, V-6, V-9 and V-11) showed congenital cataract on both eyes, poor vision with night blindness before ten years of age. At the time of clinical examination their age was ranged from 24 to 40 years. Their clinical features include low visual acuity, night blindness, nystigmous, strabismus and progressive loss of vision. The proband (V-1) in this family had congenital cataract which was surgically removed at the age of two year, with no secondary implantation of an intraocular lens and at the age of 8, he gradually reduced eyesight. Fundus examination revealed typical bone-spicule deposits on the periphery and also exhibited attenuation of retinal vessels (Figure 1C). Other clinical features such as intelligent quotient (IQ), height, cardiac, respiratory, skeletal hearing, nose, teeth, nails, skin and hair were observed normal.

3.3. Family 2

Family 2 also originates from the Khyber Pakhtunkhwa province of Pakistan. The family comprises four affected individuals (IV-3, IV-4, IV-10 and IV-11), indicating an autosomal recessive inheritance. All four patients had congenital RP. Last clinical examination, at 38, 40, 42, 46 and 48 years of age for IV-3, IV-4, IV-10 and IV-11, respectively, revealed severe retinal dystrophy, large-size eyes, night blindness, low visual acuity and congenital nystagmus. Prenatal, perinatal and neonatal medical records of all patients were normal. The fundus photographs of the proband (IV-6) show vascular attenuation, peripheral bone spicule pigmentation, accompanied by salt and pepper-like changes, and signs of maculopathy with a yellow perifoveal ring in both eyes (Figure 1D). No cardiac, respiratory, skeletal skin and hearing anomalies were observed. Their vision and was normal. Additional clinical information of the affected individuals is summarized in Table 1.

Table 1. Clinical features of two consanguineous Pakistani RP families.

Clinical Findings	Family 1							Family 2	
Gender	Female	Male	Female	Male	Female	Male	Female	Female	Male
Age (Years)	24	30	36	40	38	40	42	46	48
Height (at time of examination)	161cm	168cm	167cm	164cm	162cm	170cm	165cm	169cm	174cm
Weight (at time of examination)	71kg	73kg	81kg	93kg	84kg	96kg	68kg	72kg	97kg
Night blindness	+	+	+	+	+	+	+	+	+
Retinal dystrophy	+	+	+	+	+	+	+	+	+
Macular degeneration	+	+	+	+	+	+	+	+	+
Pigment deposit	+	+	+	+	+	+	+	+	+
Pale optic disc	+	+	+	+	+	+	+	+	+
Large eyes	+	+	+	+	+	+	+	+	+
Strabismus	+	+	+	+	+	+	+	+	+
Nystagmus	+	+	+	+	+	+	+	+	+
Visual acuity	+	+	+	+	+	+	+	+	+
Developmental delay	-	-	-	-	-	-	-	-	-
Intellectual disability	-	-	-	-	-	-	-	-	-
Microcephaly	-	-	-	-	-	-	-	-	-
Macrocephaly	-	-	-	-	-	-	-	-	-
Speech anamolies	-	-	-	-	-	-	-	-	-
Learning disability	-	-	-	-	-	-	-	-	-
Behavior problem	-	-	-	-	-	-	-	-	-
Psychiatric disorder	-	-	-	-	-	-	-	-	-
Skeletal problem	-	-	-	-	-	-	-	-	-

Skin problem	-	-	-	-	-	-	-	-	-
Epilepsy	-	-	-	-	-	-	-	-	-
Muscular dystrophy	-	-	-	-	-	-	-	-	-
Self-care	+	+	+	+	+	+	+	+	+
Cardian anamolies	-	-	-	-	-	-	-	-	-
Hearing loss	-	-	-	-	-	-	-	-	-
Teeth anamolies	-	-	-	-	-	-	-	-	-
Limb defect	-	-	-	-	-	-	-	-	-

+, present;-, absent.

3.4. Genetic Findings

Molecular analysis revealed a novel homozygous missense variant (c.1274T>C; p.Ile425Thr) in the TULP1 gene in family 1 (Figure 1E) and a novel homozygous frameshift variant (c.351delC; p.Arg118Glyfs*9) in the RPE65 gene in family 2 (Figure 1F). These variants (TULP1: c.1274T>C; p.Ile425Thr and RPE65: c.351delC; p.Arg118Glyfs*9 perfectly segregated in respective families. Affected individuals (V-1, V-6, V-9 and V-11) in family 1 and family 2 (IV-3, IV-4, IV-10 and IV-11) were homozygous whereas unaffected individuals in both families were heterozygous carriers or homozygous normal for the variants. The identified variants were absent in the GnomAD (https://gnomad.broadinstitute.org), and 1000 Genomes (http://www.1000genomes.org/), dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) and 200 ethnically matched control individuals (Table 2).

Table 2. Genotyping in RP patients' variants of the two families.

Category	Family 1	Family 2
Gene	*TULP1*	*RPE65*
Chromosome	chr6	chr1
Cytogenetic location	6p21.31	1p31.3 - 1p31.2
GRCh37	chr6: 35465651-35480673	chr1: 68894505-68915637
Genbank transcript ID	NM_003322.6	NM_000329.3
Ensembl transcript ID	ENST00000229771	ENST00000262340
Zygosity	Homozygous	Homozygous
Variant type	Missense variant	Frameshift variant
DNA changes	c.1274T>C	c.351delC
BP	chr 6: 35471385	chr 1: 68910461
Exon	13	4
Predicted protein changes	p.Ile425Thr	p.Arg118Glyfs*9
ExAC	NA	NA
1000G	NA	NA
HGMD	NA	NA
gnomAD	NA	NA
dbSNP	NA	NA
Mutation Taster	disease causing	disease causing
PolyPhen2	probably damaging; score 0.999	NA
SIFT Score	0.004	NA
PROVEAN Score	Damaging; -4.64	NA
REVEL score	0.964	NA
CADD	28	NA
GERP++	5.16	NA
Human Splicing Finder	No significant splicing motif alteration detected.	Alteration of the WT donor site,

	most probably affecting splicing.

The TULP1 (c.1274T>C) mutation changes a highly conserved isoleucine into threonine (Figure 2A), which is predicted to be pathogenic by different computational algorithms. Orthologous protein sequence alignment shows that the region is highly evolutionarily conserved (Figure 2B, C). Mutant tertiary structure generated by Swiss-model predicts that a change from isoleucine to threonine at amino acid position 425 may have a negative downstream effect on iron coordination (Figure 2D). The RPE65 (c.351delC; p.Arg118Glyfs*9) mutation is predicted as disease causing using different analysis tools (Table 2). The putative read-through protein is 408 amino acids shorter than the wild type RPE65 protein. The RPE65: c.351delC; p.Arg118Glyfs*9 mutation is predicted to bring out anticipatory nonsense-mediated mRNA decay (NMD) and triggering the prevenient degradation. Swiss-model predicts mutant tertiary structure is a single-base deletion at base position 351. It causes significant perturbations for effecting downstream iron coordination and structure of palmitic acid; producing truncated proteins (Figure 2E).

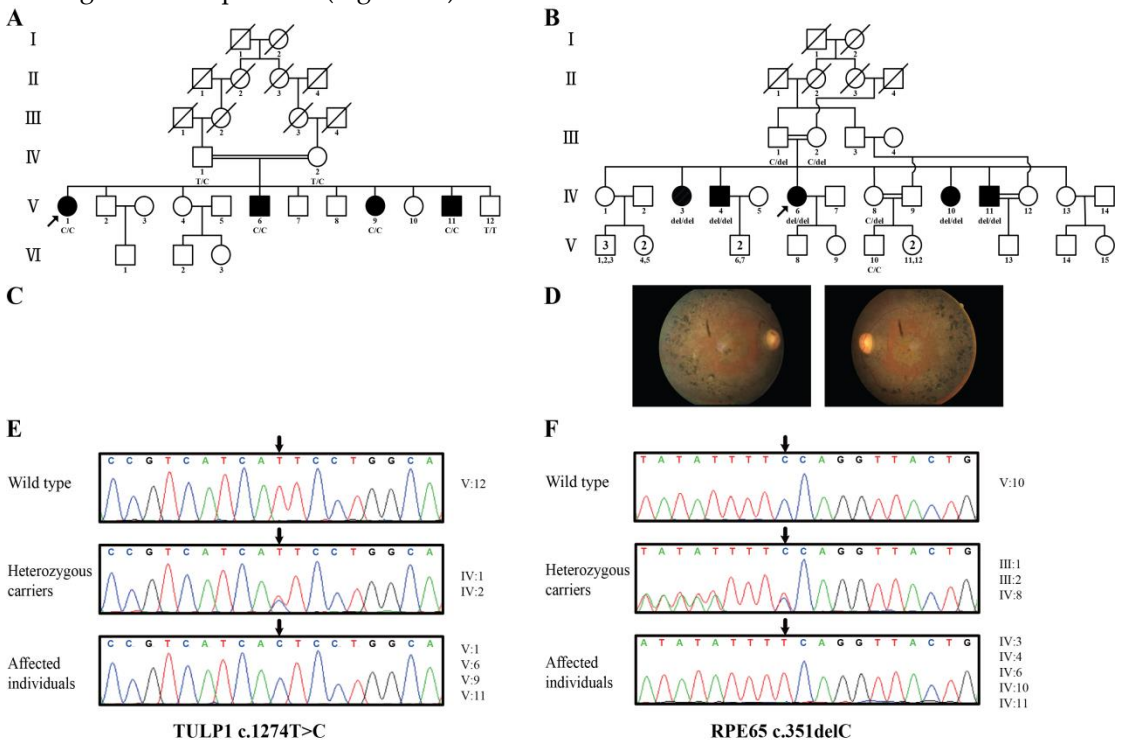

Figure 1. (A, B) Pedigree of families 1 and 2 exhibiting autosomal recessive inheritance pattern. (C) Fundus appearance of affected individual (V-6) from family 1 showing attenuation of retinal vessels with typical bone-spicule deposits on the periphery. (D) Fundus images of affected individual IV-6 of family 2 (right and left eyes). The photographs show vascular attenuation, peripheral bone spicule pigmentation, accompanied by salt and pepper-like changes, and signs of maculopathy with a yellow perifoveal ring in both eyes. (E) Sanger sequencing electrograms of Family 1 (Wild type, heterozygous carriers and affected individuals) having missense variant (c.1274T>C) in the TULP1 gene. (F) Sanger sequencing electrograms of Family 2 (Wild type, heterozygous carrier and affected individuals) having frameshift variant (c.351delC) in the RPE65 gene.

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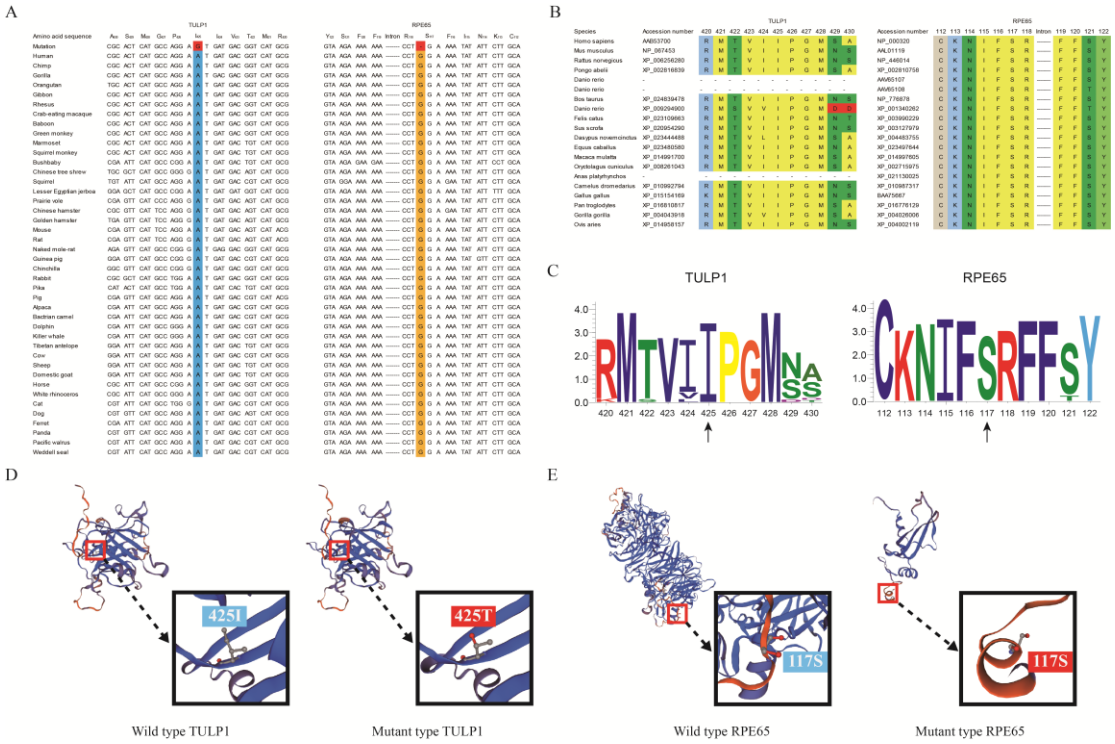

Figure 2. Protein alignment with location of the TULP1 and RPE65 variants. (A) Amino acid alignment of TULP1 and RPE65 orthologues. The amino acid sequences from different species were downloaded from UCSC (https:// http://www.genome.ucsc.edu/). The alignment was performed with default parameters in the MEGA software. (B, C) Amino acid sequences deduced from the nucleotide sequences from different species were downloaded from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). (D, E) Wild type and mutant type of *TULP1* and *RPE65* tertiary protein structures generated by Swiss-model (https://swissmodel.expasy.org/).

4. Discussion

RP belongs to the group of pigmentary retinopathies, which is characterized by retinal pigment deposits visible on fundus examination and primary loss of rod photoreceptor cells followed by secondary loss of cone photoreceptors. Patients typically have night blindness and loss of mid-peripheral visual field. As their condition progresses, they lose their far peripheral visual field and eventually central vision as well.

Herein, we describe two unrelated consanguineous Pakistani families suffering from arRP. The entire patients have night blindness with progressive loss of day time visions. By whole exome sequencing (WES) following, we found a homozygous missense variant (c.1274T>C; p.Ile425Thr) in family 1 in TULP1 and a novel frame shift variant (c.351delC; p.Arg118Glyfs*9) in RPE65 gene in family 2. Both genes (TULP1 and RPE65) are previously described in human as well as in knockout animal models that cause similar RP disease [11-14].

TULP1 is a protein coding gene and a member of the tubby-like gene family (TULPs), encoding tubby-like protein 1 with a cytogenetic location of 6p21.31. It consists of 15 coding exons spanning a 15 kb region and encodes for a 542 amino acid protein, associated with rhodopsin from synthesis site in the inner segments through the connecting cilium to the outer segments [15]. The TULP protein shares a highly conservative C-terminal region containing approximately 200 amino acid residues [16]. It is important for photoreceptor triggering, associating with functional photoreceptor and photoreceptor cells long-term survival [17]. Binding lipids in vitro contribute to the stimulation of phagocytosis of apoptotic retinal pigment epithelium (RPE) cells and macrophages. The variant identified in our patients are located at tubby-like domain (292 to 542aa) of TULP1 protein that might alter the protein function. Previous reports and literature highlighted that mutation in this domain can lead to arRP and Leber congenital amaurosis (LCA) disease [18-20]. Gene Ontology (GO)

annotations of TULP1 include actin filament binding and G-protein coupled photoreceptor activity. To date, about 88 mutations has been reported in human gene mutation database (HGMD, http://www.hgmd.org) (Supplementary table 1) in the TULP1 gene associated with RP and LCA disease. Previous studies showed that TULP1 mutations is associated with different disease such as retinitis pigmentosa 14 (OMIM 600132) and leber congenital amaurosis-15 (OMIM 613843) [15, 21-23].

RPE65 is an isomerohydrolase highly expressed in retinal pigment epithelium and is important for the regeneration of the visual pigment for both rod and cone-mediated vision. Mutations in human RPE65 cause LCA and other forms of arRP which are associated with early-onset blindness. Several RPE65 animal models have been thoroughly characterized to determine the mechanisms that underlie RPE65 associated retinal dystrophies [24]. RPE65 has two forms of regulatory proteins, a soluble form (sRPE65), and a palmitoylated, membrane-bound form (mRPE65) that playing a role in the inhibition of 11-cis retinal synthesis. The sRPE65 binds vitamin A (all-trans-retinol), making it active for LRAT processing to all-trans-retinyl ester; while the mRPE65 protien binds all-trans-retinyl esters, making them available for IMH (isomerohydrolase) processing to all-cis-retinol [29-32]. A homozygous missense mutation (p. Pro363Thr) in the RPE65 was identified for the first time in Germany patient causing autosomal recessive childhood-onset severe retinal dystrophy (arCSRD) [27]. Affected individuals in family 2 carry a novel homozygous frameshift variant (c.351delC; p.Arg118Glyfs*9) in RPE65 gene. Various prediction tools indicating that this alteration might disrupt the protein function leading to RP. RPE65 gene is located on chromosome 1p31.3 - 1p31.2 and contains 14 exons spanning 21 kb region. It encodes a 533 amino acid protein, which is located in the retinal pigment epithelium [25, 26]. RPE65 GO annotations include retinal isomerase, all-trans-retinyl-ester hydrolase, and 11-cis retinol forming activity. Study has shown that RPE65 plays an important role in the production of 11-cis retinal and visual pigment regeneration [28]. To date, 144 different types of mutations such as missense and nonsense (59), splice site (31) and frame shift (27) in RPE65 gene have been reported in human gene mutation database (HGMD, http://www.hgmd.org) (Supplementary table 1) causing RP and LCA phenotypes. Previous study described that genetic alteration in RPE65 can cause Leber congenital amaurosis type 2 (LCA2, OMIM 204100), retinitis pigmentosa 20 (OMIM 613794), and Retinitis pigmentosa 87 (OMIM 618697) with choroidal involvement [28].

In conclusion, the present study further supports previous findings that bi-allelic alteration in TULP1 and RPE65 genes cause arRP. Moreover, our molecular findings through WES expand the knowledge on genotype–phenotype correlations in arRP related to TULP1 and RPE65 variants in Pakistani population.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: HGMD reported mutations in *TULP1* gene with associated disorders, Table S2: HGMD reported mutations in RPE65 gene with associated disorders

Author Contributions: AK: XB, MU, and WRR were involved in the planning of the experiments. AK and XB extracted DNA of the proband and her healthy parents' samples and performed polymerase chain reaction. HSR performed the WES experiment. XB, HSR and XH, analyzed obtained WES results, performed bioinformatics analysis and supervised the findings of this work. AK wrote the manuscript with consultation and support from XB and MU. XZ and WRR provide funding and supervised the manuscript. All authors read and approved the final manuscript.

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