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Homozygous Variant in ARL3 Causes Autosomal Recessive Cone Rod Dystrophy

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PURPOSE. Cone rod dystrophy (CRD) is a group of inherited retinopathies characterized by the loss of cone and rod photoreceptor cells, which results in poor vision. This study aims to clinically and genetically characterize the segregating CRD phenotype in two large, consanguineous Pakistani families.

METHODS. Funduscopy, optical coherence tomography (OCT), electroretinography (ERG), color vision, and visual acuity assessments were performed to evaluate the retinal structure and function of the affected individuals. Exome sequencing was performed to identify the genetic cause of CRD. Furthermore, the mutation’s effect was evaluated using purified, bacterially expressed ADP-ribosylation factor-like protein 3 (ARL3) and mammalian cells.

RESULTS. Fundus photography and OCT imaging demonstrated features that were consistent with CRD, including bull’s eye macular lesions, macular atrophy, and central photoreceptor thinning. ERG analysis demonstrated moderate to severe reduction primarily of photopic responses in all affected individuals, and scotopic responses show reduction in two affected individuals. The exome sequencing revealed a novel homozygous variant (c.296G>T) in ARL3, which is predicted to substitute an evolutionarily conserved arginine with isoleucine within the encoded protein GTP-binding domain (R99I). The functional studies on the bacterial and heterologous mammalian cells revealed that the arginine at position 99 is essential for the stability of ARL3.

CONCLUSIONS. Our study uncovers an additional CRD gene and assigns the CRD phenotype to a variant of ARL3. The results imply that cargo transportation in photoreceptors as mediated by the ARL3 pathway is essential for cone and rod cell survival and vision in humans.

Keywords: ARL3, cone rod dystrophy, retinitis pigmentosa, autosomal recessive

Retinal dystrophies constitute a group of clinically and genetically heterogeneous eye disorders and can cause permanent blindness.1 These disorders include photoreceptor dystrophies (including cone and cone rod dystrophies), macular dystrophies, choroidal dystrophies, and systemic disorders with associated retinal degeneration.2 Cone rod dystrophies (CRD), which result in a loss of cone and rod photoreceptor cells, constitute a very important but rare class of retinal dystrophies. Worldwide prevalence of CRD ranges from 1 in every 30,000 to 40,000 individuals. CRD first clinically manifests during the first two decades of life as poor visual acuity. Severe or advanced cases of CRD also present with nystagmus, photoaversion, and central scotomas. CRD, reportedly, has multiple modes of inheritance, including autosomal recessive, autosomal dominant, and X-linked recessive.3,4 The high variability of clinical symptoms combined with genetic heterogeneity complicates the prognosis. Thus, accurate diagnosis, along with precise recognition of the underlying genetic defect, is crucial for efficient management of this group of retinal disorders.

Here we report findings of two consanguineous families cosegregating an autosomal recessive form of CRD and a homozygous point mutation (c.296G>T; p.Arg99Ile) in ADP-ribosylation factor-like protein 3 (ARL3), a small G protein belonging to ADP-ribosylation factor (Arf) family. In the mammalian retina, ARL3 is mainly localized on the connecting cilium myoid region of inner segments of cone photoreceptors and acts as an allosteric factor for the release of lipidated proteins bound to PDE6β (6 subunit of phosphodiesterase) and UNC119a/b in a GTP-dependent manner.5 The release function of ARL3 is regulated by its interaction with retinitis pigmentosa...
Mutation in ARL3 Causes CRD

2 (RP2) and ARL13b.5,7 Recently, two homozygous missense variants (p.Arg149Cys and p.Arg149His) of ARL3 were reported to cause Joubert syndrome (Online Mendelian Inheritance in Man [OMIM] #213300), which is characterized by hypoplasia of the cerebellar vermis, developmental delays, renal anomalies, and rod-cone dystrophy in human families.3

Furthermore, two independent studies reported a heterozygous variant p.(Ty390Cys) of ARL3 associated with dominantly inherited retinitis pigmentosa in two European-descent pedigrees.9,10 Similarly, impaired development and degeneration of photoreceptors phenotype was observed in Arf3 knockout mice.11–13 In contrast to these previous reports, affected individuals of our two families that are homozygous for p.Arg99Ile have a nonsyndromic cone-dominant retinal dystrophy.

Materials and Methods

Subjects

This observational study was conducted under institutional review board-approved protocols in accordance with the Declaration of Helsinki for the release of clinical information, family history, and blood extraction for genetic testing. Informed consent was obtained after the study risks and benefits were thoroughly explained. We evaluated the medical history via interviews to identify other possible clinical features beyond vision impairment and rule out potential environmental causes. Funduscopy (TRC-50EX; Topcon, Corp., Tokyo, Japan), optical coherence tomography (3D Optical Coherence Tomography, DRI OCT Triton; Topcon, Corp.), color vision using the Ishihara Chart, visual acuity, and full field electroretinography (ERG) using skin sensor strips and a handheld portable device (Test Protocol# ISCEV 5 Step Light First cd, RETeval portable ERG; Konan Medical, Irvine, CA, USA) were performed by an ophthalmologist to characterize the vision disorder segregating in both families. The ERG data were then compared with the normative values of 50 normal controls.

Whole Exome Sequencing and Sanger Confirmation

Whole genomic DNA was extracted from the whole blood using standard methods. Whole exome sequencing using the genomic DNA sample of one affected member from each family was performed as previously described.14 Briefly, genomic libraries were recovered for exome enrichment using the NimbleGen EZ Exome V2 kit (Roche Diagnostics, San Francisco, CA, USA). The libraries were sequenced on an Illumina HiSeq4000 (Illumina, San Diego, CA, USA), and generated approximately 30 million paired end reads that were each 100 bases long. Data analysis was performed using the Broad Institute’s Genome Analysis Toolkit (GATK) (Broad Institute, Cambridge, MA, USA) and the reads were aligned with the Illumina Chastity Filter with the Burrows Wheeler Aligner (BWA) (Broad Institute).15,16 The variant sites were called using the GATK. Single nucleotide variant (SNV) calls were filtered using the variant quality score recalibration method.15 Sanger sequencing was performed using standard methods to determine the cosegregation of the ARL3 variant with vision impairment in both families. Primers for Sanger sequencing were designed using Primer3. PCR amplification and DNA sequencing were performed as previously described.17 Pathogenic variant nomenclature was assigned in accordance with GenBank Accession number NM_004311, with nucleotide position 1 corresponding to the A of the ATG initiation codon.

Bioinformatics Analysis

To evaluate the protein evolutionary sequence conservation, we used clustal omega multiple sequence alignment and the Phyre2 server was used for protein 3D structure.

Bacterial Expression

Human WT ARL3 or the p.Arg99Ile mutant proteins were expressed in BL21(DE3) bacteria that had used the pET3C vector were grown overnight in ZY auto-induction medium.18 The cells were harvested and lysed with two passes through a French press and were then pelleted by centrifugation at 32,000 rpm (100,000g). Equal protein (20 µg) of whole cell lysate (pre-spin), supernatant, and resuspended pellet were run on SDS-PAGE and stained with Coomassie blue.19

Heterologous Cell Expression

Empty vector (pcDNA3.1), human ARL3 in pcDNA3.1, or ARL3 with p.Arg99Ile in pcDNA3.1 were transiently expressed in COS7, or HeLa, using Lipofectamine 2000. The cells were harvested 24 hours after transfection, lysed in buffer containing 1% CHAPS, 100 mM NaCl, and 25 mM HEPES, pH 7.4, and then clarified through centrifugation at 14,000 rpm. Lysates (20 µg) were run on a 15% SDS-PAGE gel. The membrane was blocked with 5% milk and then incubated with our ARL3 antibody and immunoblots developed with horseradish peroxidase-conjugated secondary (sheep anti-rabbit) antibody.20

Results

Clinical Description of CRD Phenotype

Segregating in Two Large Pakistani Families

Two large, consanguineous families, LUSG03 and LUSG04, both affected with vision impairment were enrolled from the Sindh province of Pakistan (Fig. 1A). The neonatal clinical records of the affected individuals were not available at the time of enrollment. According to family medical histories, all eight affected individuals had bilateral vision impairment. We evaluated all LUSG03 and LUSG04 participating members to identify and characterize signs and symptoms of their vision impairment. No cornea opacity, color vision, or night blindness problems were noted among the affected individuals. Visual field assessment revealed normal peripheral vision, but central field vision was compromised among all the affected individuals. Affected individual V:1 (24 years old) of family LUSG03 had both moderate myopia and bilateral maculopathy without RPE atrophy, whereas individual V:5 (29 years old) had RPE atrophy. A third affected individual V:7 (44 years old) only had myopic fundus changes and foveal and outer nuclear layer thinning in the parafoveal region (Figs. 2A, 3). The fundus examination of the youngest affected individual (IV:5; 15 years old) of family LUSG04 was essentially normal, and the OCT imaging revealed foveal thinning with otherwise normal retinal architecture in both eyes (Figs. 2B, 3). Both affected individuals IV:4 (19 years old) and IV:2 (17 years old) of family LUSG04 had bull’s eye maculopathy with no flecks in either eye (Figs. 2B, 4). Last, the oldest affected individual IV:1 (26 years old) of family LUSG04 demonstrated myopic fundus changes without frank myopic degeneration (Fig. 2B); moreover, his OCT imaging further confirmed myopic changes, including excess posterior curvature and retinal and choroidal thinning (Fig. 3).
ERG analysis of individuals within the family LUSG03 demonstrated moderate to severe reduction in photopic responses with preserved scotopic responses (Fig. 4; Table), with the exception of individuals IV:3 and V:5, in whom scotopic responses were mildly reduced (Supplementary Table S1). ERGs from individual IV:05 of family LUSG04 had moderately reduced photopic amplitudes OU and poor scotopic rod responses but normal scotopic combined responses OU (Fig. 4; Table; Supplementary Table S1). However, the length of dark adaptation was not optimal in this case. Color vision
Deficits were observed in affected individuals in both families (Table). Taking into consideration fundus photography (Fig. 2) and spectral domain OCT imaging findings (Fig. 3) including bull’s eye macular lesions, macular atrophy, parafoveal and central photoreceptor thinning, absence of lipofuscin flecks, and normal vision at birth, no corneal haze, and primarily attenuated photopic responses, individuals demonstrated features that were most consistent with CRD. The findings were typically symmetrical between both eyes.

**ARL3 Missense Variant Is Associated With CRD Phenotypes in Both Families**

To determine the genetic cause of this CRD phenotype, whole exome sequencing was performed for individuals V:1 and IV:5 of families LUSG03 and LUSG04, respectively. Autosomal recessive inheritance, both homozygous and compound heterozygous, was assumed during the filtering stages (Supplementary Table S2). A novel transition variant (c.296G>T) was found in the *ARL3* gene for each individual (Fig. 1A).

Sanger sequencing of *ARL3* revealed segregation of the c.296G>T variant with the cone dystrophy phenotype in participating individuals from both families (Fig. 1A). In humans, *ARL3* is composed of six exons, encoding a 182-residue GTP-binding protein (Fig. 1B). The predicted p.(Arg99-Ile) missense change, due to c.296G>T variant, was present in the GTP-binding domain of ARL3 (Fig. 1B). This variant was present in an evolutionarily conserved region (Fig. 1B) and absent in the NHLBI 6500 exome variants and the ExAC and gnomAD databases, as well as in 270 ethnically matched chromosomes, and is predicted to be pathogenic by various in silico algorithms (Supplementary Table S3).

There was no apparent relationship found between the LUSG03 and LUSG04 families. However, as both families had the same pathogenic variant, segregating with the CRD phenotype, we performed a haplotype analysis using 35 single nucleotide polymorphisms surrounding the *ARL3* variant to determine whether Founder’s effect played a role in this reoccurring variant. The results revealed common ancestry for the c.296G>T allele in both Pakistani families (Supplementary Table S4).

**FIGURE 2.** Color fundus photography of the right and left macula, respectively, of individuals with cone dystrophy from families LUSG03 (A) and LUSG04 (B). Myopic fundus changes were variably present in LUSG03 V:5, V:7 (A) and LUSG04 IV:1, IV:4, and IV:5 (B), including tilted optic disc, alpha zone atrophy, mild temporal disc pallor, and parapapillary RPE thinning consistent with early staphyloma formation. Macular atrophy was observed in LUSG03 V:5 and bull’s eye lesions are visible in LUSG03 V:1, V:7 and LUSG04 IV:1, IV:2, and IV:4. Brain magnetic resonance imaging axial T2 image (C) and sagittal T2 image (D) of LUSG03 individual IV:5, demonstrating no increased interpeduncular fossa, normal cerebellar peduncles, and normal vermis.
FIGURE 3. Spectral domain OCT (Topcon) horizontal raster scans (B) with color fundus photography reference images (A), ETDRS retinal thickness maps (C), and en face infrared reference “shadowgram” imaging (D) of right eyes for individuals from families LUSG03 and LUSG04, demonstrating anatomic changes consistent with cone dystrophy. Reference image from LUSG03 V:5 shows frank geographic atrophy with overlying photoreceptor degeneration. The cone outer-segment tip (COST) line is variably diminished, from subtle (LUSG03 V:1, V:7 and LUSG04 IV:1, IV:5) to bull’s eye parafoveal loss with outer nuclear layer thinning (LUSG04 IV:2, IV:4). Retinal thickness maps demonstrate diffuse retinal thinning in all patients, particularly in those with bull’s eye lesions or macular atrophy (LUSG03 V:5). Increased posterior curvature from myopia is seen among LUSG03 V:5, V:7 and LUSG04 IV:1, IV:5. En face photoreceptor layer infrared shadowgram shows hypointense round lesions in LUSG04 IV:2, IV:4, isointense fovea in LUSG03 V:7 and LUSG04 IV:1, and central hyperintensity surrounded by a ring of hypointensity in LUSG03 V:1, V:5 and LUSG04 IV:5.
Missense Variant Affects the Stability of the Encoded Protein

To determine the effect of p.(Arg99Ile) variant on the encoded ARL3, we performed molecular modeling using the Phyre2 software and numerous structures available on the Protein Database (e.g., 1FZQ). The positively charged guanidino side chain of Arg99 is essential for the formations of ionic bonds that stabilize the orientation of the negatively charged side chain of Asp26, which is central to the canonical G-1 motif (aka P-loop) found in GTP-binding proteins, conserved in the ARF family in the sequence GLD26X(A/S)GKT, and also involved in direct binding of the γ-phosphate of GXP (GTP or GDP) and magnesium in the nucleotide binding site. We noticed that both Arg99 and Asp26 were conserved in 27 of the 30 members of the human ARF family, with differences only occurring in currently uncharacterized proteins. Molecular modeling suggested that the p.(Arg99Ile) variant may alter/reduce the affinity of the protein for guanine nucleotides, resulting in an apoprotein that is much less stable in solutions (Fig. 1C).

Next, to functionally evaluate the consequences of the p.Arg99Ile variant on the encoded protein, we expressed, as previously described,20,21 both wild-type and mutant ARL3 proteins in bacteria and mammalian cells (Figs. 5A, 5C). When expressed in bacterial cells, the overwhelming majority (estimated by eye from multiple preparations at >90%) of wild-type ARL3 remains in the supernatant (S100) after centrifugation at 100,000 g and is soluble, whereas almost all of the ARL3[R99I] was found in the pellet (Fig. 5A), which indicates insolubility of the protein. This dramatic redistribution of ARL3 due to the insoluble fraction had not been seen previously from the mutation of several other residues of the closest paralogs, ARL3 and ARL2,22 or of other members of the ARF family, with the exception of ARL13B, which has a relatively low affinity for guanine nucleotides and is insoluble when expressed in bacteria.23 Exogenous expression of ARL3 or p.Arg99Ile in COS7 or HeLa cells revealed that the mutant migrated somewhat faster than either the endogenous or overexpressed wild-type proteins (Fig. 5B). Faster-migrating immunoreactive bands were also evident and consistent with ARL3[R99I] degradation (Fig. 5B). These characteristics are consistent with a less stable protein or one more accessible to proteases. However, no apparent difference in the cytosolic localization of ARL3[R99I] was observed in transiently transfected COS7 or HeLa cells compared with ARL3 (Fig. 5C). Thus, these studies suggest that the p.Arg99Ile variant associated with cone rod dystrophy in LUSG03 and LUSG04...
families affects the stability of the encoded ARL3 and likely consequently negatively impacts its ability to bind guanine nucleotides with high affinity to allow it to remain folded in a solution.

**DISCUSSION**

This study of two families reports a novel missense variant p.(Arg99Ile) in ARL3 affecting seven individuals with CRD. In both families, in which the p.(Arg99Ile) variant segregated, the earliest signs of CRD (foveal thinning; LUSG04 IV:5) were observed during the second decade of life (15 years old). Additionally, the oldest individuals in both families demonstrated retinal thinning. Previous studies have demonstrated the role of ARL3 in the transport of cargo to photoreceptors.23,29 ARL3 acts as a GTPase, switching between GTP-bound (on) and GDP-bound (off) states and is involved with the trafficking of proteins and lipids from their site of synthesis within inner segments to their site of action in outer segments.25

Recently, missense variants in ARL3 were reported to cause Joubert syndrome, characterized by hypoplasia of the cerebellar vermis, developmental delay, renal anomalies, and rod-cone dystrophy in human families.8 Significantly reduced levels of INPP5E and NPHP3 were observed in the cilia of the fibroblast cells derived from the ARL3-deficient patients.8 In collaboration, we further assessed the visual impairment of an affected individual with Joubert syndrome secondary to a ARL3 homozygous p.(Arg149His) variant. Full-field ERGs showed evidence of severe loss of rod and cone function in both eyes, making it impossible to confirm definitively if this was a primary cone-rod or rod-cone dystrophy. Clinical data of our two large, consanguineous human families were consistent with a nonsyndromic CRD phenotype cosegregating with a missense variant p.(Arg99Ile) of ARL3. We re-evaluated affected individuals of our families for additional ciliopathy phenotypes. Detailed clinical evaluation and kidney ultrasound revealed no features of Joubert syndrome. Furthermore, brain magnetic resonance imaging of one subject was normal, and evidence of molar teeth was not present (Figs. 2C, 2D). Brainstem, ventricle, cerebellum, and cerebellar peduncle morphology were essentially normal.

Our bioinformatic analysis and in vitro studies suggest that the p.Arg99Ile variant results in a less stable protein (based on insolubility of the mammalian protein when expressed in bacteria, altered electrophoretic migration, and increased degradation after expression in cultured mammalian cell lines) compared with wild-type ARL3; Fig. 5) and loss of side chain interactions in the folded protein (Fig. 1C), which is predicted to compromise the binding of guanine nucleotides and might also indirectly affect the interaction with RP2 and ARL13b in vivo, leading to defects in the transportation of myristoylated and prenylated proteins in the photoreceptors.26 It is unclear why the ARL3 p.Arg99Ile mutation specifically led to deterioration of the cone and rod photoreceptors rather than a multisystem ciliopathy phenotype such as Joubert syndrome in these families. In contrast to p.Arg99, the p.Arg149Cys/His variants are predicted to have no direct effect on nucleotide binding.24-25 ARL3 family consists of six ARFs, 22 ARLs, and two SARs.28 Although 16 GEFs have been identified to act on the six ARFs, only one GEF has been identified for the 22 GEFs (ARL13b acting as such on ARL3).27 Consequently, we speculate that we will find several other proteins capable of activating ARL3, leading to the regulation of currently unknown pathways; however, making predictions that link specific mutations to specific phenotypes is

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**TABLE. Clinical Details of the Families Segregating the ARL3 Variant**

| ID     | Disease Status | ARL3 Genotype | Age (y) | Onset of Symptoms | Best-Corrected Visual Acuity OS:OD | Refraction | Color Vision | Photophobia OS:OD | ERG Status |
|--------|----------------|---------------|---------|-------------------|-----------------------------------|------------|-------------|-------------------|------------|
| IV:4   | Normal         | WT/M          | 8       | NA                | 6/6;6/6                           | 0.00       | Normal      | Neg               | Normal rod and cone response |
| V:3    | Normal         | WT/M          | 32      | NA                | 6/6;6/6                           | 0.00       | Normal      | Neg               | Normal rod and cone response |
| IV:3   | Affected       | M/M           | 25      | First decade      | 6/36;6/36                         | −5.5/−5.5  | Normal      | ++/++             | Mildly reduced scotopic amplitudes with normal implicit time, severely reduced photopic amplitudes and very delayed implicit times OU |
| V:1    | Affected       | M/M           | 24      | First decade      | 6/36;6/36                         | −5.25/−5.50| Impaired    | ++/++             | Normal scotopic, reduced photopic amplitudes (severe OD, moderate OS), delayed implicit times OU |
| V:5    | Affected       | M/M           | 29      | First decade      | 6/60;6/60                         | −5.75/−5.50| Normal      | ++/++             | Normal scotopic, severely reduced photopic amplitudes OU |
| V:7    | Affected       | M/M           | 44      | First decade      | 6/24;6/24                         | −6.50/−6.00| Impaired    | ++/++             | Normal scotopic, severely reduced photopic amplitudes OU |

LUSG-04

| ID     | Disease Status | ARL3 Genotype | Age (y) | Onset of Symptoms | Best-Corrected Visual Acuity OS:OD | Refraction | Color Vision | Photophobia OS:OD | ERG Status |
|--------|----------------|---------------|---------|-------------------|-----------------------------------|------------|-------------|-------------------|------------|
| IV:1   | Affected       | M/M           | 30      | First decade      | 6/36;6/36                         | −5.0/−5.0  | Normal      | ++/++             | Reduced amplitudes OU |
| IV:2   | Affected       | M/M           | 25      | First decade      | 6/24;6/24                         | −4.0/−4.75| Normal      | ++/++             | Reduced amplitudes OU |
| IV:5   | Affected       | M/M           | 11      | First decade      | 6/9;6/9                           | −3.25/−2.50| Impaired    | ++/++             | Moderately reduced photopic amplitudes OU, poor scotopic rod responses but normal scotopic combined responses OU (possibly insufficient dark adaptation time) |

M, mutant; NA, not applicable; Neg, negative; WT, wild type.
risky. It is plausible that impaired interaction with ARL13b due to p.Arg149Cys/His variants could yield a widespread loss of the ability to activate ARL3 and thus cause Joubert syndrome.

On the other hand, due to the limitations of in vitro studies, including protein folding differences between prokaryotes and eukaryotes, chaperone activity, and cell type-specific interactors, the in vivo impact of the p.Arg99Ile variant cannot be inferred with confidence at this time. Due to restricted phenotypic outcome, it is plausible that in vivo p.Arg99Ile might have a milder impact, perhaps a weakened affinity for nucleotides or a greater tendency toward degradation/protease action, but retention of most functions, and thus may be a hypomorphic variant of ARL3. However, further studies using animal knock-in model will be needed to address the specific roles of ARL3 in the development of retinal sensory epithelium, cytoskeletal organization, and ARL3-mediated signaling in photoreceptor cells and other cell types. In conclusion, our clinical and molecular analysis of ARL3 allele may prove useful for future genetic diagnosis and counseling, as well as for molecular epidemiology studies of CRDs in humans.

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Mutation in ARL3 Causes CRD

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