Mutations in AGBL5, Encoding α-Tubulin Deglutamylase, Are Associated With Autosomal Recessive Retinitis Pigmentosa

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PURPOSE. AGBL5, encoding ATP/GTP binding protein-like 5, was previously proposed as an autosomal recessive retinitis pigmentosa (arRP) candidate gene based on the identification of missense variants in two families. In this study, we performed next-generation sequencing to reveal additional RP cases with AGBL5 variants, including protein-truncating variants.

METHODS. Whole-genome sequencing (WGS) or whole-exome sequencing (WES) was performed in three probands. Subsequent Sanger sequencing and segregation analysis were performed in the selected candidate genes. The medical history of individuals carrying AGBL5 variants was reviewed and additional ophthalmic examinations were performed, including fundus photography, fundus autofluorescence imaging, and optical coherence tomography.

RESULTS. AGBL5 variants were identified in three unrelated arRP families, comprising homozygous variants in family 1 (c.1775G>A:p.(Trp592*)) and family 2 (complex allele: c.[323C>G; 2659T>C]; p.[(Pro108Arg; 887Argext1)1]), and compound heterozygous variants (c.752T>G:p.(Val251Gly) and c.1504dupG:p.(Ala502Glyfs*15)) in family 3. All affected individuals displayed typical RP phenotypes.

CONCLUSIONS. Our study convincingly shows that variants in AGBL5 are associated with arRP. The identification of AGBL5 and TTLL5, a previously described RP-associated gene encoding the tubulin tyrosine ligase-like family, member 5 protein, highlights the importance of polyand deglutamylation in retinal homeostasis. Further studies are required to investigate the underlying disease mechanism associated with AGBL5 variants.

Keywords: retinitis pigmentosa, whole exome sequencing, AGBL5, post-translational modification

Retinitis pigmentosa (RP) (Mendelian Inheritance in Man [MIM]268000) encompasses a clinically and genetically heterogeneous group of inherited retinal dystrophies (IRDs) with a worldwide prevalence of approximately 1 in 4000 individuals. Retinitis pigmentosa is characterized by initial rod photoreceptor degeneration resulting in night blindness, followed by midperipheral visual field loss and deterioration of central vision due to cone photoreceptor degeneration.1 The clinical presentation of RP is highly variable in terms of onset, disease progression, fundus appearance, and associated ocular features. The ophthalmic features indicative for RP include bone spicule pigmentation with atrophy of the retinal pigment epithelium (RPE), waxy disc pallor, and retinal arteriole attenuation.2,3

To date, 82 genes have been implicated in RP (https://sph.uth.edu/retnet; in the public domain). These genes encode proteins involved in different retinal pathways, including the phototransduction cascade, the retinoid cycle, ciliary transport,
retinal development, and RNA splicing factors. Recent whole-exome sequencing (WES) studies demonstrated that approximately 60% of RP cases can be explained by variants in these genes (Haer-Wigman L, personal communication, 2016), suggesting that several genes are yet to be identified. This study aimed to identify novel genes associated with RP using next-generation sequencing (NGS) technology.

Here, we describe the identification of biallelic variants in AGBL5 in four autosomal recessive retinitis pigmentosa (arRP) patients from three unrelated families.

Subjects and Methods

Subjects and Clinical Evaluation

The study protocol adhered to the tenets of the Declaration of Helsinki and received approval from the respective local ethics committees. Written informed consent was obtained from all participants or parents of children prior to their inclusion in this study. Patients were ascertained from the inherited retinal disease clinics at the Rotterdam Eye Hospital (The Netherlands) and Moorfields Eye Hospital (London, UK).

Each patient underwent a full clinical examination including visual acuity and dilated fundus examination. Retinal fundus imaging was obtained by 35° color fundus photography (Topcon Great Britain Ltd, Berkshire, UK), 55° fundus autofluorescence (FAF) imaging (Spectralis; Heidelberg Engineering Ltd, Heidelberg, Germany), and Spectralis coherence tomography (OCT).

Full-field and pattern electroretinography (ERG, PERG) were available from three patients, performed with gold electrodes, which incorporated the International Society for Clinical Electrophysiology of Vision (ISCEV) standards.9,10 We also studied all retrospective data including Stratus (Carl Zeiss Meditec, Dublin, CA, USA) OCTs.

Genetic Analysis

Genomic DNA was isolated from peripheral blood lymphocytes according to standard salting-out procedures.11 In one unaffected individual from family 1, DNA was extracted from saliva material using the Oragene system (OG-500; Genotek, Ottawa, ON, Canada).

Whole-exome sequencing of the proband from family 1 was performed using Agilent’s SureSelect All Human Exome version 2 kit (50 Mb; Agilent Technologies, Santa Clara, CA, USA), followed by sequencing on a SOLiD4 sequencing platform (Life Technologies, Carlsbad, CA, USA). Reads were mapped against the University of California, Santa Cruz (UCSC) genome browser, human hg19 assembly (build 37) using Lifescope v2.1 software (Lifetechnologies), and variants were called using the Genome Analysis Toolkit (GATK) v2 according to the recommended guidelines (https://software.broadinstitute.org/gatk/best-practices/; in the public domain). Identified variants were filtered based on minor allele frequency (<0.005) (<0.01 in <6000 internal control genomes).

Variant quality scores were then recalibrated according to GATK best practices separately for indels and single nucleotide polymorphisms (SNPs). Resulting variants were annotated using ANNOVAR (http://annovar.openbioinformatics.org/; in the public domain) based on Ensemble (http://www.ensembl.org; in the public domain) gene and transcript definitions. Candidate variants were filtered based on function (non-synonymous, presumed loss of function or splicing, defined as intronic sites within 5 bp of an exon–intron junction) and minor allele frequency (<0.005 minor allele frequency in our internal control group, as well as the National Heart, Lung, and Blood Institute [NHBLI] exome sequencing dataset).

The patient from family 3 underwent whole-genome sequencing (WGS) as part of a large collaborative study: DNA from 599 unrelated patients with inherited retinal disease, ascertained from the Inherited Eye Disease clinics at Moorfields Eye Hospital (MEH), London, underwent WGS as part of the National Institute for Health Research England (NIHR) Bio-Resource–Rare Diseases project, Specialist Pathology Evaluating Exomes in Diagnostics (SPEED). Briefly, peripheral blood mononuclear cell–derived genomic DNA was processed using the Illumina TrueSeq DNA PCR-Free Sample Preparation kit (Illumina, Inc., San Diego, CA, USA) and sequenced using an Illumina HiSeq 2500, generating a minimum coverage of 15× for ~5% of the genome. Reads were aligned to the genome (GRCh37) using Isaac aligner (Illumina, Inc., Great Chesterton, UK). SNVs and indels were identified using Isaac variant caller. Variant examination was performed only on the single nucleotide variants (SNVs) and indels that met the following criteria: passed standard quality filters, predicted to alter the sequence of a protein, and had an allele frequency <0.01 in the 1000 genomes database, the NHHLI GO Exome Sequencing Project (http://evs.gs.washington.edu/EVS/ release 20130513; in the public domain), the UK10K database (http://www.uk10k.org; in the public domain), the ExAC database (http://exac.broadinstitute.org; in the public domain), and allele frequency <0.02 in ~6000 internal control genomes.

Sanger Sequencing

Primers for amplification of coding exons and flanking exon–intron boundaries of AGBL5 (NM_021831.5) were designed with Primer3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; in the public domain). Primer sequences and PCR conditions are available upon request.

Pathogenicity Interpretation of Missense Variant

The Combined Annotation Dependent Depletion (CADD) (http://cadd.gs.washington.edu/; in the public domain) score was used to predict the pathogenicity of missense variants. This scoring system incorporates several widely used in silico tools, such as SIFT (http://sift.jcvi.org/; in the public domain), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/; in the public domain), Mutation Taster (http://www.mutationtaster.org/; in the public domain), PhyloP, and Grantham score. Minor allele frequencies were obtained from the Exome Aggregation Consortium (ExAC) database containing ~60,000 exomes of
Patients from families 2 and 3 initially developed nyctalopia, with a range of onset from 9 to 30 years before subsequent development of peripheral visual field loss. At last review (age range, 24–45 years), there was mild visual acuity loss with best-corrected visual acuity mean 0.34 logMAR (range, 0.30–0.46). All patients developed posterior subcapsular cataract. Patient F2-II:1 had learning difficulties but no other symptoms suggestive of syndromic disease. There were fundus abnormalities consistent with RP including attenuated vessels, macular and midperipheral RPE motting/atrophy, and peripheral intraretinal pigment migration, most prominent in the proband of family 3 (Fig. 2I). Fundus autofluorescence imaging demonstrated a ring of increased autofluorescence parafoveally in F2-II:1, focally increased autofluorescence corresponding to macular edema in F2-II:3, and a partial ring of reduced autofluorescence in F3-II:1 (Figs. 2D, 2G, 2J). All patients had midperipheral reduced autofluorescence in keeping with regions of RPE atrophy and increased pigmentation. On OCT, there was outer retinal atrophy with variable preservation of photoreceptors most apparent in F2-II:1 with a centrally preserved inner segment ellipsoid band (Fig. 2D). Optical coherence tomography imaging demonstrated intraretinal cysts of the inner nuclear layer, which responded to topical carbonic anhydrase inhibitors (Figs. 2E, 2H, 2K). Electrophysiology demonstrated rod and cone system dysfunction (Table 1).

Genetic Findings

Biallelic variants in AGBL5 were identified in four patients from three families. These variants are summarized in Tables 1 and 2. None of them have been previously reported in RP patients. Whole-exome sequencing data analysis did not yield other biallelic variants in the genes known to be mutated in IRD (Retinal Information Network, https://sph.uth.edu/retnet/; in the public domain). The proband of family 1 was homozygous for the c.1775G>A.p.(Trp592*) nonsense variant in the AGBL5 gene (Fig. 3). The resulting transcript is expected to undergo nonsense-mediated decay (NMD), suggesting that this variant is a true loss-of-function allele. This variant was reported in 1/121,398 alleles (0.0008%) in the ExAC dataset.

Two affected siblings from family 2 (GC15894) were homozygous for the nonsense variant c.323C>G.p.(Pro108Arg). All in silico tools predict this variant to be pathogenic (Table 2). The altered proline residue is fully conserved throughout AGBL5 orthologues up to Tetraodon nigroviridis (Fig. 3A) and is located in the core of AGBL5 (Fig. 3B). A change to arginine introduces a larger amino acid, which may destabilize the protein structure. Moreover, proline provides a rigid structure important for interaction with ligands or other proteins. A second homozygous variant, c.2659T>C.p.(887Argex*1), was also identified in the two affected members of this family. This stop-loss variant is predicted to result in a single amino acid residue extension of the protein. Two rare stop-loss variants affecting other nucleotides of the AGBL5 stop codon, c.2661A>G.p.(*887Argex*1) and c.2661A>T.p.(*887Cysex*1), were found in one and seven alleles in the ExAC dataset, respectively.

The compound heterozygous variants c.1504dupG.p.(Ala502fs*15) and c.752T>G.p.(Val251Gly) were identified in a simplex RP patient of family 3. The p.Val251Gly variant alters the valine residue flanking the consensus zinc-binding residues of the M14-like peptidase domain. This valine is conserved throughout paralogues and orthologues of AGBL5 (up to T. nigroviridis). Furthermore, it affects a highly conserved nucleotide with a PhyloP score of 4.75. According to Vissers et al.,12 by using PhyloP score distribution of dbSNP and HGMD variants, this variant can be considered as
## Table 1. Clinical Phenotype of Patients Carrying AGBL5 Variants

<table>
<thead>
<tr>
<th>Family ID, Sex, Origin</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Age at Diagnosis, Recent Exam, y</th>
<th>History</th>
<th>Visual Acuity (Snellen) (logMAR)</th>
<th>Refraction</th>
<th>Ophthalmoscopy</th>
<th>Full Field ERG</th>
<th>OCT</th>
<th>FAF</th>
<th>Other Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1–IV:3 (DNA:249185), M, Turkey</td>
<td>c.1775G&gt;A; p(Trp592*)</td>
<td>c.1775G&gt;A; p(Trp592*)</td>
<td>16, 43</td>
<td>Central vision loss, nyctalopia (2nd decade)</td>
<td>RE: LP, LE: IP–</td>
<td>NA (Pseudophakia)</td>
<td>Mild optic disk pallor, attenuated vessels, macular and (mid) peripheral RPE atrophy with heavy intraretinal bone spicule pigmentation and deep nummular pigmentation</td>
<td>NA</td>
<td>Cystoid maculopathy progressing into complete loss of outer retinal layers and thinning inner retinal layers at fovea</td>
<td>NA</td>
<td>NP</td>
</tr>
<tr>
<td>F2–II:1 (GC:15894.1), F, UK</td>
<td>c.[323C&gt;G]; c.[323C&gt;G]; p.[(Pro108Arg)]; p.[(Pro108Arg)]; (887Argex3.1)]</td>
<td>c.[323C&gt;G]; c.[323C&gt;G]; p.[(Pro108Arg)]; p.[(Pro108Arg)]; (887Argex3.1)]</td>
<td>9, 24</td>
<td>Nyctalopia (age 9)</td>
<td>RE: 6/18 (0.48); LE: 6/12 (0.3)</td>
<td>RE: 0.50/1.00 × 180 LE: 0.50/1.50 × 170</td>
<td>Optic disc pallor, attenuated vessels, macular and midperipheral RPE mottling and occasional hyperpigmented spots</td>
<td>Age 8 y, findings of marked rod system dysfunction with cone system dysfunction R:LE-L. Marked macular involvement on R</td>
<td>Cysts in inner nuclear layer L–R, centrally preserved inner segment ellipsoid band and outer nuclear layers L–R</td>
<td>Parafoveal hyperautofluorescent ring larger on L, R additional ring of hypoautofluorescence surrounding this in macula, midperipheral spots of hypoautofluorescence</td>
<td>Posterior subcapsular cataract, cystoid macular edema (responsive to topical CAI), right amblyopia, right secondary exotropia</td>
</tr>
<tr>
<td>F2–II:3</td>
<td>c.[323C&gt;G]; c.[323C&gt;G]; p.[(Pro108Arg)]; p.[(Pro108Arg)]; (887Argex3.1)]</td>
<td>c.[323C&gt;G]; c.[323C&gt;G]; p.[(Pro108Arg)]; p.[(Pro108Arg)]; (887Argex3.1)]</td>
<td>20, 27</td>
<td>Nyctalopia (2nd decade), central vision loss, visual field loss</td>
<td>RE: 6/18 (0.48); LE: 6/12 (0.3)</td>
<td>RE: 1.50/2.00 × 1 LE: 0.50/1.50 × 1</td>
<td>Optic disc pallor, attenuated vessels, macular and midperipheral RPE mottling, minimal pigment spots</td>
<td>Age 20 y, severe generalized loss of retinal function</td>
<td>Cysts in inner nuclear layer, small foveal region of preserved inner segment ellipsoid band</td>
<td>Frontal hyperautofluorescence surrounded by parafoveal hyperautofluorescent ring, midperipheral spots of hypoautofluorescence</td>
<td>Posterior subcapsular cataract, cystoid macular edema (responsive to CAI), learning difficulties</td>
</tr>
<tr>
<td>F3–II:1 (GC:3687), F, UK</td>
<td>c.752T&gt;G; c.752T&gt;G</td>
<td>c.1504dupG; p.(Ala502Glyfs*15)</td>
<td>59, 65</td>
<td>Nyctalopia, peripheral field loss, (vari: 4th decade)</td>
<td>RE: 6/12 (0.3); LE: 6/12 (0.3)</td>
<td>RE: 0.50/1.00 × 1 LE: 0.50/1.50 × 1</td>
<td>Optic disc pallor, attenuated vessels, macular RPE mottling, heavy midperipheral bone spicule pigmentation</td>
<td>Age 59 y, findings consistent with RP, report not available</td>
<td>Cysts in inner nuclear layer, centrally preserved outer nuclear layer and inner segment ellipsoid band (disrupted), epiretinal membrane</td>
<td>Parafoveal spots of hypoautofluorescence, extensive speckled midperipheral loss of autofluorescence</td>
<td>Cataract, cystoid macular edema (responsive to CAI)</td>
</tr>
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</table>

CAI, Carbonic Anhydrase Inhibitor; DS, Dioptries Sphere; FAF, fundus autofluorescence; L, left; LE, left eye; logMAR, Logarithm of the Minimum Angle of Resolution, to measure metric visual acuity; LP, light perception; NA, not applicable; NP, not performed; OCT, optical coherence tomography; R, right; RE, right eye; RPE, retinal pigmented epithelium.
pathogenic as it is close to the mean PhyloP score (4.7) of the pathogenic de novo variants identified in persons with intellectual disability. All in silico prediction tools indicate that this variant is pathogenic (Table 2). Alteration of this hydrophobic valine to glycine may destabilize the secondary structure of AGBL5 in this domain (Fig. 3B). All variants cosegregated with disease in the family members available for testing (Fig. 1).

**DISCUSSION**

**AGBL5 Is a Novel Gene Associated With RP**

Previous studies by Patel et al.\(^5\) and Kastner et al.\(^{13}\) discovered AGBL5 as a novel candidate gene associated with RP. These studies identified homozygous missense variants (p.Arg276Trp) and p.(Asp295Asn)) in arRP cases from Saudi and Turkish origin.

**FIGURE 2.** Retinal imaging of affected individuals. Color fundus imaging (A, C, F, I) in four patients demonstrated optic disc pallor, attenuated vessels, macular and midperipheral retinal pigment epithelium mottling/atrophy, and peripheral intraretinal pigment migration, heaviest in (A) and (I). FAF imaging in three patients demonstrated a ring of increased autofluorescence parafoveally in F2-II:1 (D) with focal parafoveal increased autofluorescence in F2-II:3 (G), likely representing cystoid macular edema. There was an incomplete ring of reduced autofluorescence parafoveally for F3-II:1 (J). All patients had midperipheral reduced autofluorescence in keeping with regions of RPE atrophy and increased pigmentation. Stratus OCT of the right eye in F1-IV:3 (B) at age 34 showed large cysts at the macula comprising several retinal layers with cysts in the inner nuclear layer of the paramacular region. In the lower scan (at 39 years), complete atrophy of retinal layers at the fovea can be observed. OCT imaging also demonstrated loss of outer retina with partial preservation of the inner segment ellipsoid band in patient F2-II:1 (E), very limited photoreceptor preservation for F2-II:3 (H) and F3-II:1 (K), and intraretinal cysts of the inner nuclear layer for all patients.

<table>
<thead>
<tr>
<th>DNA Variant</th>
<th>Protein Variant</th>
<th>ExAC Allele Frequency, %</th>
<th>SIFT</th>
<th>PolyPhen-2</th>
<th>Mutation Taster</th>
<th>CADD Phred</th>
<th>PhyloP</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.323C&gt;G</td>
<td>p.(Pro108Arg)</td>
<td>0</td>
<td>Deleterious</td>
<td>Probably damaging</td>
<td>Disease causing</td>
<td>24.0</td>
<td>5.45</td>
</tr>
<tr>
<td>c.752T&gt;G</td>
<td>p.(Val251Gly)</td>
<td>0</td>
<td>Deleterious</td>
<td>Probably damaging</td>
<td>Disease causing</td>
<td>27.1</td>
<td>4.73</td>
</tr>
<tr>
<td>c.1775G&gt;A</td>
<td>p.(Trp592*)</td>
<td>0.0008</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>c.1504dupG</td>
<td>p.(Ala502Glyfs*15)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>c.2659T&gt;C</td>
<td>p.(887Argext*1)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not applicable.
In our study, we identified five novel variants in AGBL5 in three unrelated RP cases that further confirm the causality of this gene. The novel variants consist of two missense, two protein-truncating, and one stop-loss variant. To date, all missense variants in AGBL5 with the exception of p.(Pro108Arg) are located within the M14-like carboxypeptidase A domain (Fig. 3A), which highlights the importance of this protein domain for AGBL5 function. This protein domain encodes cytosolic carboxypeptidase-like protein 5; ATP/GTP-binding protein-like 5. Wild-type and mutant residues are shown and colored in cyan and green, respectively. The p.(Pro108Arg) variant, alter proline in position 108 to arginine, is located in the core of the AGBL5 protein. This alteration created a larger amino acid, which may destabilize the protein structure. The p.(Val251Gly) missense variant changed the hydrophobic valine to glycine and may destabilize the structure of AGBL5. These two variants were identified from previous studies by Patel et al.5 and Kastner et al.,13 respectively.

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The two arRP families reported by Patel et al.5 and Kastner et al.13 display a classical RP phenotype, as do our patients. There was some variability in visual function; our eldest patient (age 63) still had relatively well-preserved visual acuity, whereas one subject was blind at age 32. All patients showed retinal cysts in the macula region at some stage of the disease, but this occurs in 18% to 32% of RP patients.15

Interestingly, Kastner et al.13 reported intellectual disability in an affected male and proposed a possible correlation between sex and brain phenotype. In our study, learning difficulties occurred in a female patient, which does not support a correlation between sex and brain phenotypes. Additional RP patients with AGBL5 variants need to be identified to gain more insight into a possible association with brain phenotypes.
Pathak et al. demonstrated that knockdown of Agb5 in zebrafish leads to ciliopathy phenotypes, that is, axis curvature, hydrocephalus, pronephric cysts, and abnormal multicilia motility. However, in humans, variants in AGBL5 are associated with nonsyndromic IRD. These phenotypic discrepancies might be caused by homologous genes in humans that might compensate for the effect caused by these mutations. Alternatively, as the NMD event was not functionally assessed, there is a possibility that in the human patients, truncated proteins with residual activity are being produced. Finally, recent studies show that the use of morpholinos for gene knockdown in zebrafish may lead to off-target effects, thereby explaining additional phenotypes observed in the zebrafish studies.

Imbalance of Poly- and Deglutamylation in Posttranslational Modifications Is Associated With RP

Approximately one-third of IRD-associated genes play roles in maintaining the structure and function of photoreceptor sensory cilium. Previous studies have implicated TTLL5, a gene encoding a polyglutamylase (tubulin tyrosine ligase-like family, member 5) in the pathogenesis of autosomal recessive cone–rod dystrophy. Agb5, also known as CCP5, is known to function in polyglutamylation and deglutamylation of tubulin, and perturbations in this pathway may lead to photoreceptor cell degeneration. Tubulin glutamylation is an essential posttranslational modification associated with stable microtubules, neuronal axons, mitotic spindles, centrioles, and cilia. The tubulin tyrosine ligase-like (TTLL) and CCP deglutamylases are required to maintain the balance of tubulin glutamylation in ciliogenesis. Any imbalance in poly- and deglutamylation might disrupt cilia function. Hyperglutamylation is known to diminish Tetralobymena cilia motility and to induce axonemal microtubules defects. TTLL5 mutant mice displayed a complete loss of retinitis pigmentosa GTPase regulator (RPGR) glutamylation without marked changes in tubulin glutamylation levels. The TTLL5 mutant mouse developed slow photoreceptor degeneration with early mislocalization of cone opsin, features resembling those of Rpgr-null mice. CCP5 knockdown in zebrafish led to hyperglutamylation and reduced the disorganized cilia beat pattern. TTLL5, on the other hand, plays a role in polyglutamylation, which is crucial for microtubule sliding and cilia waveform regulation.

Interestingly, a recent study demonstrates the role of TTLL5 in RPGR<sup>ORF15</sup> glutamylation. The opposite function of AGBL5 (CCP5) in the deglutamylation process may also play an important role in the balance of glutamate chains in RPGR<sup>ORF15</sup>. Posttranslational modifications are regulated by several members of the TTLL and CCP gene families, and we hypothesize that besides AGBL5 and TTLL5, other TTLL (TTLL1 through TTLL11) and CCP (CCP1 through CCP6) genes are plausible candidate genes for IRD.

In conclusion, this study strengthens the role of AGBL5 variants in RP. The involvement of both AGBL5 and TTLL5 in IRDs underlines the critical importance of glutamylation in retinal function.

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