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Utility of In Vitro Mutagenesis of RPE65 Protein for Verification of Mutational Pathogenicity Before Gene Therapy

Unikora Yang, MD; Susan Gentleman, PhD; Xiaowu Gai, PhD; Michael B. Gorin, MD, PhD; Mark S. Borchert, MD; Thomas C. Lee, MD; Adda Villanueva, MD; Robert Koenekoop, MD, PhD; Albert M. Maguire, MD; Jean Bennett, MD, PhD; T. Michael Redmond, PhD; Aaron Nagiel, MD, PhD

IMPORTANCE Next-generation sequencing can detect variants of uncertain significance (VUSs), for some of which gene therapy would not be advantageous. Therefore, the pathogenicity of compound heterozygous or homozygous variants should be confirmed before bilateral vitrectomy and administration of voretigene neparvovec-rzyl.

OBJECTIVE To describe an in vitro mutagenesis assay for assessing the pathogenicity of variants in the *RPE65* gene.

DESIGN, SETTING, AND PARTICIPANTS This case series was conducted at 2 tertiary referral centers. Clinical history, imaging, and electrophysiologic testing results were reviewed from September 5, 2008, to December 31, 2019. Participants were 4 pediatric patients with Leber congenital amaurosis who were evaluated for or met the inclusion criteria for phase 1 to 3 clinical trials or were referred for voretigene neparvovec-rzyl treatment.

MAIN OUTCOMES AND MEASURES A functional assay was used to confirm the pathogenicity of novel *RPE65* VUSs in 4 patients with Leber congenital amaurosis.

RESULTS Four patients with Leber congenital amaurosis had VUSs in *RPE65*. Patients 1 and 2 were siblings with the homozygous VUS c.311G>T p.(G104V). Patient 3 was a compound heterozygote with 1 known pathogenic allele, c.1202_1203insCTGG p.(Glu404AlafsTer4), and 1 VUS, c.311G>T p.(G104V), which segregated to separate alleles. Patient 4 was also a compound heterozygote with 1 pathogenic variant, c.11 + 5G>A, and 1 variant in *trans*, c.1399C>T p.(P467S). In vitro mutagenesis revealed that the G104V and P467S RPE65 proteins were catalytically inactive (0% isomerase activity). Patients 1 and 2 were excluded from participation in a phase 1 trial owing to high Adeno-associated virus 2 capsid-neutralizing antibodies. Patients 3 (G104V) and 4 (P467S) underwent successful surgical gene therapy with voretigene neparvovec-rzyl, and their response to lower white light intensity and visual field increased in fewer than 30 days after gene therapy intervention.

CONCLUSIONS AND RELEVANCE Findings from this study suggest that, in patients with missense mutations in *RPE65*, functional assays of protein function can be performed to assess the pathogenicity of variants in both compound heterozygous and homozygous cases. Given the potential risks of gene therapy operations, in vitro RPE65 activity testing should be considered to avoid the possibility of treating a false genotype.

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Supplemental content

Author Affiliations: Author affiliations are listed at the end of this article.

Corresponding Author: Aaron Nagiel, MD, PhD, The Vision Center, Department of Surgery, Children's Hospital Los Angeles, 4650 Sunset Blvd, MS 88, Los Angeles, CA 90027 (anagiel@chla.usc.edu).

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Inherited retinal dystrophies are a genetically and phenotypically heterogenous group of diseases resulting from mutations in more than 200 genes.[1-3] Leber congenital amaurosis (LCA) is the most common cause of inherited childhood blindness[4] and results in severe visual impairment in infancy and early childhood. Mutations in at least 20 genes have been linked to LCA specifically,[5,6] including *CEP290* (OMIM 610142), *GUCY2D* (OMIM 600179), *CRB1* (OMIM 604210), and *RPE65* (OMIM 180069). Although *RPE65*-mediated retinal degeneration accounts for only 6% to 16% of LCA cases,[7,8] *RPE65* is perhaps the most studied LCA gene since its discovery in 1993[9] and has been associated with the disease since 1997.[10] RPE65 is a key component of the visual cycle, which is required for regenerating the visual pigment chromophore 11-*cis*-retinal after exposure to light.[11] *RPE65*-mediated retinal degeneration has been the subject of extensive gene therapy research for the past 20 years. Proof of principle for gene therapy was first shown in animal models of *RPE65*-null Briard dogs in 2001[12] and mice in 2006,[13] before the first patients with LCA were treated.

In 2008, 2 independent groups, Maguire et al[14] and Bainbridge et al,[15] undertook phase 1 gene replacement trials with subretinal injections of recombinant Adeno-associated virus 2 (AAV2) containing human *RPE65*. A subsequent phase 3 trial of bilateral subretinal injections of voretigene neparvovec (AAV2-hRPE65v2) for patients with biallelic *RPE65* mutations evaluated 1-year visual outcomes (including multiluminance mobility testing, which measures functional vision at defined light levels) and showed that 65% of the intervention group and none of the control group passed at the lowest luminance level.[16] In December 2017, the US Food and Drug Administration approved voretigene neparvovec-rzyl (Luxturna), the first gene therapy product for a hereditary retinal dystrophy or any hereditary disease.

Because of the genetically heterogeneous nature of inherited retinal dystrophies, confirmation of pathogenic *RPE65* mutations is essential for identifying patients for whom voretigene neparvovec-rzyl may be advantageous. The treatment is intended to be performed as a bilateral procedure with vitrectomy followed by subretinal injection. The inherent risks of a surgical procedure and anesthesia, especially in the pediatric population, elevate the burden of proof for confirming the pathogenicity of any identified mutations. The 2 mutations not only should segregate to each of the parents but also should have a sufficiently high suspicion for impairing protein function. When compound heterozygous or homozygous *RPE65* variant of uncertain significance (VUS) is encountered, clinicians should confirm the protein dysfunction in vitro before proceeding with a gene therapy surgical procedure.

In this case series, we used a functional assay to confirm pathogenicity of novel *RPE65* VUS in 4 patients with LCA. These patients were evaluated for or met the inclusion criteria for phase 1 to 3 clinical trials or who were referred for voretigene neparvovec-rzyl treatment after it was approved as a drug by the Food and Drug Administration.

Key Points

Question Can the pathogenicity of *RPE65* variants of uncertain significance be identified before gene therapy?

Findings In this case series of 4 pediatric patients with Leber congenital amaurosis, compound heterozygous or homozygous variants of uncertain significance were found in the *RPE65* gene. An in vitro assay incorporated these mutations into *RPE65* and showed catalytic inactivity, which established eligibility for treatment.

Meaning This study suggests that in vitro testing of RPE65 protein function can be used to confirm the pathogenicity of compound heterozygous or homozygous variants of uncertain significance in the *RPE65* gene to identify patients eligible for gene therapy surgical intervention.

Methods

This case series adhered to the tenets of the Declaration of Helsinki[17] and was approved by the institutional review boards of Children's Hospital of Philadelphia and Children's Hospital Los Angeles after Health Insurance Portability and Accountability Act authorization, including written informed parental permission and child assent, was obtained from the parents of the patients. Four pediatric patients with LCA were referred to 2 tertiary centers. Clinical history, imaging, and electrophysiologic testing results were reviewed from September 5, 2008, to December 31, 2019.

Clinical data included visual acuity, refraction, slitlamp biomicroscopic findings, and ophthalmoscopic findings. Retinal imaging included fundus photography and autofluorescence imaging (Optos California; Optos). Spectral-domain optical coherence tomography (Cirrus 5000 HD-OCT; Carl Zeiss Meditec Inc) of the macula was performed to determine the presence of sufficient viable photoreceptor cells to qualify for gene therapy. Full-field scotopic threshold testing was performed using a standardized protocol (Espion; Diagnosys LLC). Goldmann visual fields were obtained (Haag-Streit Goldmann Perimeter Model 940; Haag-Streit AG), and Goldmann visual field isopters were measured by summing the eccentricity across 24 meridians and subtracting scotomas. The isopter generated by the brightest stimulus that was not full was chosen for analysis (III4e isopter for patient 3, and II4e isopter for patient 4).

Patients 1 and 2 were considered for inclusion in the phase 1 trial and received a molecular diagnosis of LCA with *RPE65* gene mutations at the Clinical Laboratory Improvement Amendments (CLIA)–approved Ophthalmic Molecular Diagnostic Laboratory at the University of Michigan, Ann Arbor (before the 2008 expiration of CLIA certification). Molecular diagnosis of a phase 3 trial participant (patient 3) was performed at the CLIA-approved John and Marcia Carver Nonprofit Genetic Testing Laboratory in Iowa City, Iowa. At both laboratories, the patient's DNA was assayed by automated DNA sequencing of the *RPE65* coding sequences. Patient 4 was screened with a CLIA-certified next-generation sequencing custom panel (Fulgent Diagnostics LLC) that tested 24 genes. Segregation of the compound heterozygous *RPE65* variants was

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confirmed through analysis of parental DNA. Subsequently, patients 3 and 4 received gene therapy with bilateral subretinal injections of 1.5×10^{11} vector genomes of voretigene neparvovec-rzyl in a volume of 300 µL.[16]

Functional Assay for *RPE65* Activity

Plasmids specific for each *RPE65* VUS were generated by site-directed mutagenesis of the *RPE65* open reading frame (ORF) cloned in multigenic plasmid (pVitro2; InvivoGen) using a site-directed mutagenesis kit (QuikChange XL; Agilent). Oligonucleotide primer pairs used for mutagenesis are listed in eTable 1 in the Supplement. Mutants were verified by sequence analysis of DNA minipreps. Validated mutant and wild-type plasmids were grown and purified using purification kits in maxi or mega format, as appropriate (Qiagen). These plasmids were used for transient transfection experiments in HEK293-F cells.

Cell culture methods and transient transfection protocols were the same as previously published.[11] For any given experiment, 3×10^7 HEK293-F cells were transfected with 30 µg of pVitro2 plasmid (containing *RPE65* and *CRALBP* [OMIM 180090] ORFs) and 30 µg of pVitro3 plasmid (containing *LRAT* [OMIM 604863] and *RDH5* [OMIM 601617] ORFs; InvivoGen) in the presence of 40 µL of transfection reagent (293fectin; Invitrogen), for a total volume of 30 mL. Twenty-four hours after transfection, all-*trans*-retinol was added to a final concentration of 2.5µM and the cells were incubated for 7 more hours. At this time, culture fractions (29 mL) of transfected HEK293-F cells were centrifuged, cell pellets were harvested, and retinoids were extracted and saponified following previously described methods.[11] The resultant isomeric retinols were analyzed on 5-µm particle sorbent (LiChrospher; Alltech) normal phase columns (2 × 250 mm) on an isocratic high-performance liquid chromatography (HPLC) system equipped with a diode-array UV-visible detector (Agilent 1100/1200 series; Agilent Technologies), following the method of Landers and Olson[18] as earlier modified.[11] Data were analyzed on ChemStation32 software (Agilent).

Immunoblot Analysis

Cell pellets (approximately 2×10^6 cells) from 1 mL of wild-type or mutant RPE65 transfected HEK293-F culture aliquots were lysed in 200-µL protein extraction reagent (CytoBuster; Novagen), incubated on ice for 10 minutes, and centrifuged at 13 000 × *g* for 10 minutes; the supernatant was harvested for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Denatured samples were separated on 12% Bis-Tris gels (NuPage; Invitrogen) and electrotransferred to nitrocellulose membranes. Expression levels of RPE65 were quantitated using fluorescent Western blot. Gels and blots were prepared using nitrocellulose (Hybond-ECL; GE Healthcare) and protein standard markers (ECL Plex Fluorescent Rainbow; GE Healthcare). Primary antibodies were rabbit anti-RPE65[19] (1:4000) and mouse monoclonal anti-*CRALBP* (1:20 000). Secondary antibodies used were Cy5-conjugated goat anti-rabbit and Cy3-conjugated goat anti-mouse ECL Plex fluorescent antibodies (both 1:2500). Processed blots were scanned (Typhoon 9410 Scanner) and quantitated using image analysis software (ImageQuant TL; GE Healthcare). Wild-type and mutant RPE65 levels were normalized to coexpressed *CRALBP* levels, and mutant levels were calculated relative to wild-type RPE65 expression (set at 100%).

Molecular Modeling

Tertiary structure modeling was done with Swiss-Model, version 36.0003 (Swiss Institute of Bioinformatics), a protein structure homology-modeling server accessed via the ExPASy web server or locally from the program DeepView-Swiss-PdbViewer. The template for modeling RPE65 was the structure of RPE65.[20]

Results

Four patients with LCA were identified to have a VUS in 1 or both *RPE65* alleles. Because these patients were potential candidates for inclusion in gene therapy clinical trials or postapproval treatment with voretigene neparvovec-rzyl, the VUSs were engineered into *RPE65* in vitro to determine their association with RPE65 protein function. Functional assays revealed absent catalytic activity, and patients 3 and 4 subsequently underwent successful bilateral gene therapy surgical procedures.

Case Presentations

Patients 1 and 2

Two siblings were legally blind since birth and received an LCA diagnosis. They had no other history of illness or surgical procedure. The older, teen sibling had hand motion visual acuity OU. The younger sibling had a visual acuity of 20/200 OD and 20/800 OS. They had homozygous VUS, c.311G>T p.(G104V), in *RPE65*. Computational predictions using predictive software were consistent in describing the probable pathogenicity of the VUS as class C65 (Align-GVGD), probably damaging (PolyPhen-2), and deleterious (PROVEAN [Protein Variation Effect Analyzer]). However, the siblings were ineligible for the phase 1 trial owing to an Adeno-associated virus 2-neutralizing antibody titer greater than 1:1000 and did not receive study material.

Patient 3

Patient 3 was a child who was legally blind since birth and had no other illness or surgical procedures. Best-corrected visual acuity was 20/200 OU. Baseline clinical testing revealed severely reduced light sensitivity and constricted visual fields typical of other cases of LCA due to RPE65 deficiency. Genetic testing showed that the child was a compound heterozygote with 1 likely pathogenic allele, c.1202_1203insCTGG p.(Glu404AlafsTer4), and a VUS, c.311G>T p.(G104V), in *RPE65*. Within 1 month after bilateral subretinal voretigene neparvovec-rzyl injections for a phase 3 trial, we observed increased sensitivity to white light and expansion of the visual fields bilaterally (**Figure 1**), which plateaued 90 days after injection.

Patient 4

Patient4 was an otherwise healthy child referred for voretigene neparvovec-rzyl treatment after the drug was approved

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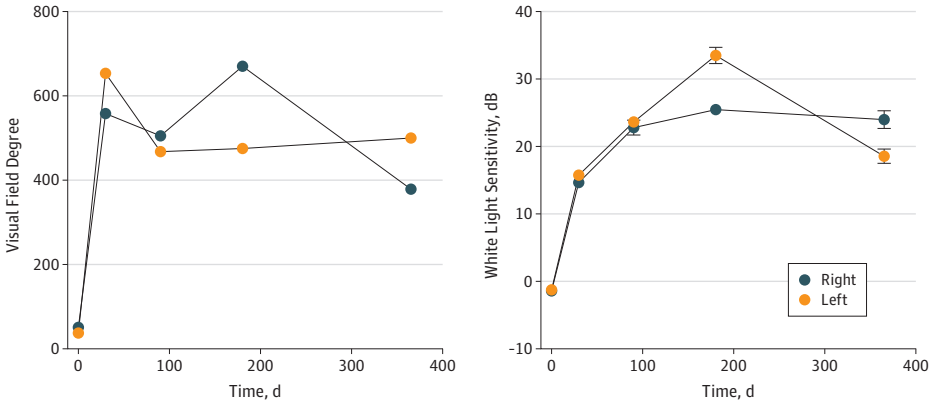
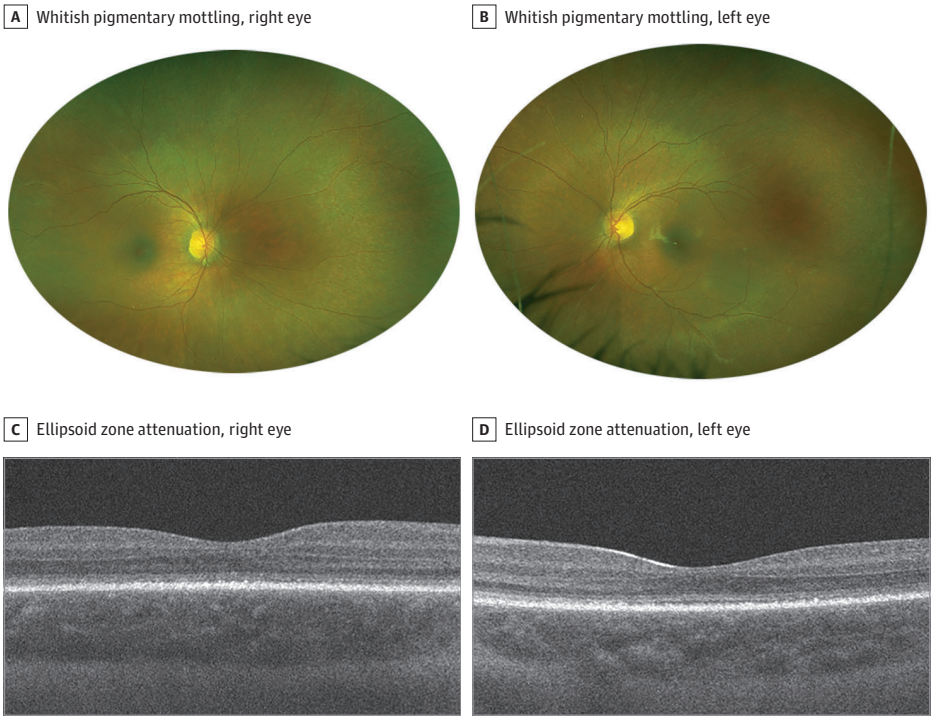
Figure 1. Functional Visual Outcomes in Patient 3 After Gene Therapy



Change is seen from baseline through the 1-year time point in Goldmann visual fields and full-field white light sensitivity after bilateral subretinal injections of voretigene neparvovec-rzyl.

Figure 2. Imaging of Patient 4



Optos ultrawidefield color photos show subtle white pigmentary changes in the periphery of the right (A) and left (B) eyes. Spectral-domain optical coherence tomography of the macula shows diffuse ellipsoid zone attenuation in the right (C) and left (D) eyes.

by the Food and Drug Administration. The patient had poor visual behavior and low-light visual impairment beginning in infancy and received a clinical diagnosis at 18 months, with no detectable response from background noise on electroretinography. The best-corrected visual acuity was 20/150 OU. Baseline visual field testing with Goldmann perimetry showed that the sum total degrees across all 24 meridians for the II4e isopter was 557 OD and 503 OS. Subtle whitish pigmentary mottling was observed in the retinal periphery of both eyes (**Figure 2**A and B). Spectral-domain optical coherence tomography of the macula showed attenuation of the ellipsoid zone band (Figure 2C and D). Genetic testing revealed 2 mutations in *RPE65*: 1 previously reported pathogenic intronic mutation, c.11 + 5G>A,[18,19] and 1 missense VUS, c.1399C>T p.(P467S), not previously reported in either the Human Gene Mutation Database or the Exome Aggregation Consortium data set. Computational predictions for c.1399C>T were probably damaging (PolyPhen-2), class C65 (Align-GVGD), and deleterious (PROVEAN). In vitro mutagenesis of P467S RPE65 was performed before treatment. After treatment, a markedly improved function in dim lighting was observed, and the II4e isopter expanded to 826 OD and 836 OS.

Mutant RPE65 Catalytic Activity

The VUSs c.311G>T p.(G104V) and c.1399C>T p.(P467S) were engineered into *RPE65*. Heterologous expression of these VUSs in the minimal visual cycle culture system[11] showed that the mutated copies of the protein were catalytically inactive

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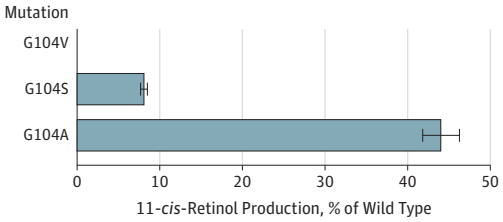
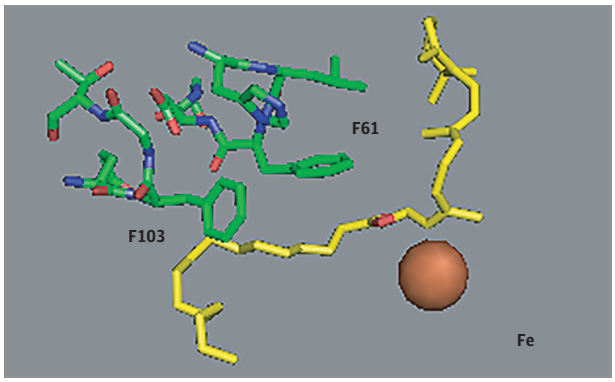
Figure 3. G104V Mutation and RPE65 Activity



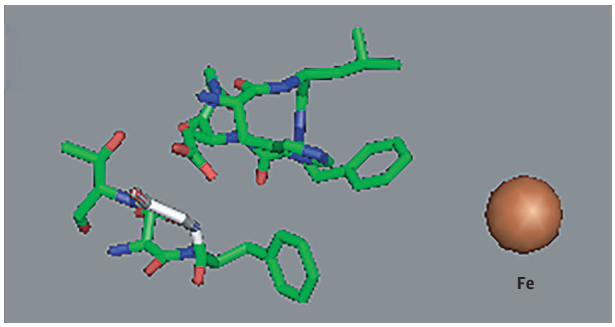
The human RPE65 G104V variant of uncertain significance ended the isomerase activity of RPE65 in vitro, but replacement by residues with smaller side chains moderated this effect. Production of 11-*cis*-retinol in 293-F cells transfected with constructs expressing wild type and mutants of dog RPE65 G104 was identified. Mutant activities are expressed as percentage (SD) of wild-type RPE65 activity (n = ≥4).

Figure 4. G104V Missense Mutation and RPE65 Structure

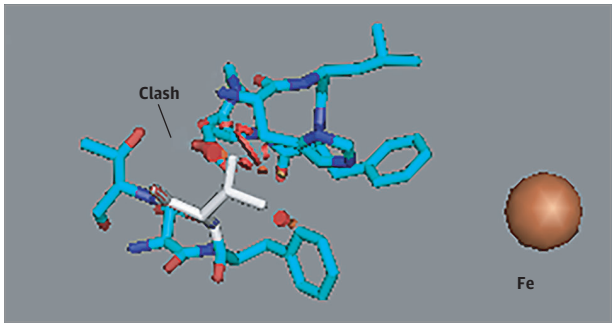
A 11-*cis*-Retinyl palmitate in the RPE65 substrate cleft



B Wild-type structure



C G104V mutant structure



Docking of 11-*cis*-retinyl palmitate in the RPE65 substrate cleft and the positions of the FDG motif (aa59-63) and EFG motif (aa102-105) relative to the cleft are shown (A). The 2 lower panels compare the structure of wild-type structure (B) and G104V mutation (C; both G104 and V104 are depicted in white). The red dots in panel C indicate backbone clashes of the V104 side chain with both H59 and D62. The iron (Fe) atom at the catalytic center of the RPE65 crystal is shown as an orange sphere.

in vitro, confirming the pathogenicity of these variants and establishing patients 3 and 4 as candidates for voretigene neparvovec-rzyl therapy.

G104V Mutation and Isomerase Activity of RPE65

We measured the isomerase activity of cells transfected with pVitro2 plasmid expressing the G104V mutant of RPE65. The RPE65 activity is a combined phenotypic effect of the RPE65 mutant on enzymatic activity and stability, comparable to an in vivo homozygous state. Presence of 11-*cis*-retinol was undetectable in cells transfected with the RPE65 G104V mutant vector (**Figure 3**). RPE65 protein expression was reduced to 16.7% of RPE65 expression in cells transfected with vector for wild-type RPE65 (eTable 2 in the Supplement), indicating substantially reduced stability.

To understand the effect of this mutation, we modeled the region containing G104 on the structure of RPE65[20] (**Figure 4**). G104 is part of a highly conserved EFG motif in metazoan carotenoid oxygenases and completely conserved in RPE65. In addition to G104V, E102K is a known pathogenic mutation.[21,22] From the crystallographic data,[20] G104 is located on a hairpin loop between 2 highly conserved alpha-helical structures and is predicted to fit between 2 β-sheets (Figure 4A). The adjacent residue F103, which is at the tip of the hairpin, covers part of the substrate-binding cavity of RPE65. Any amino acid bigger than glycine or as big as valine may prevent the loop from achieving this fit. A previous study demonstrated that F103 plays an important role in substrate or intermediate interactions in RPE65 isomerase activity.[23] Thus, altering G104 could interfere with the proper positioning of this hairpin loop and displace F103.

We hypothesized that the small size of the glycine (Figure 4B), lacking a side chain, was crucial for positioning, given that the valine side chain would project (Figure 4C) and block proper positioning of F103. To explore this hypothesis, we made additional mutants G104S and G104A, which had side chains smaller than that of valine, and tested them for activity. G104S had 8% of wild-type activity, whereas G104A had 43% of wild-type activity (Figure 3). Protein expression was also enhanced in these mutants (eTable 2 in the Supplement) compared with G104V. Replacing G104 with progressively larger residues (G<A<S<V) progressively reduced RPE65 isomerase activity and expression (G>A>S>V).

P467S Mutation and Isomerase Activity of RPE65

When mutated with P467S, pVitro2 transfection and expression did not produce any 11-*cis*-retinol and had substantially reduced 13-*cis*-retinol production (**Figure 5**). This outcome indicates a severely pathogenic VUS, possibly from loss of protein stability, catalytic failure, or both. Proline residues have

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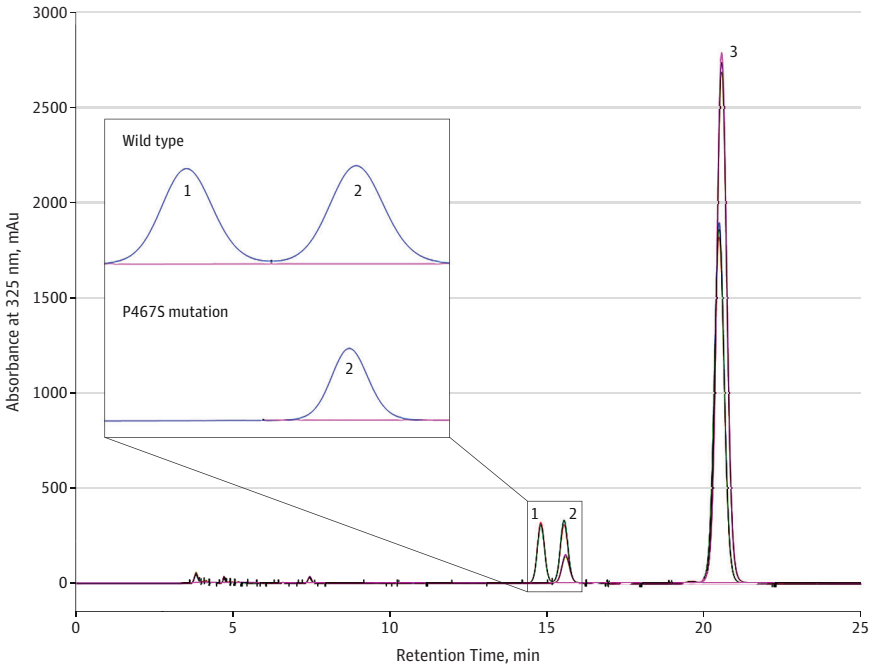
Figure 5. Human RPE65 P467S Mutation and RPE65 Isomerase Activity In Vitro



Overlay of representative normal-phase high-performance liquid chromatography (HPLC) of isomeric retinols extracted and saponified from cell lysates of cells transfected with either wild-type or P467S pVitro2 RPE65 expression constructs. Inset shows an enlargement of HPLC peaks for cells transfected with either wild-type RPE65 (upper panel) or RPE65 P467S mutation (lower panel). Peak identity 1 indicates 11-*cis*-retinol; 2, 13-*cis*-retinol; and 3, all-*trans*-retinol. 13-*cis*-retinol is present in the P467S mutant HPLC profile because of thermal generation, which resulted in some percentage of the retinol being isomerized to 13-*cis*-retinol and reversibly to all-*trans*-retinol. mAu indicates milli-Absorbance units.

unique roles in protein secondary structure elements, such as β turns, and confer rigidity to structures (helices, strands, or turns), meaning that mutations of proline residues can be adverse. P467 is located in a short β strand in the propeller structure of RPE65.[20] The proximity of P467S to E469, a second-shell residue that stabilizes 1 of the 4 first-shell histidines involved in the ferrous iron coordination system, may lead to destabilization or loss of the active center iron and, subsequently, loss of catalytic activity. Missense mutations of first- or second-shell iron ligands have been found in patients with LCA,[24,25] confirming the biochemical studies that demonstrated both first- and second-shell iron ligands are essential for RPE65 isomerase activity.[11,20,26] Similarly, the missense mutation P470L, which is adjacent to E469, is also associated with LCA[27] and may subserve a similar structural role as P467.

Discussion

Advances in the sensitivity and scope of next-generation sequencing have allowed clinicians to search for potential mutations in the whole genome as costs have decreased. However, these unbiased sequencing methods identify increasing numbers of VUSs. Among these candidates, a plausible disease-causing variant may exist, but it is also possible that none result in phenotypic disease. The false genotype rate is the frequency of encountering with whole-exome sequencing a plausible disease-causing recessive or dominant genotype in a healthy patient, which is conceptually similar to the false discovery rate.[28] With use of the sequence data from 60 000 healthy individuals in the Exome Aggregation Consortium, Stone[28] showed a mean of 1.28 plausible disease-causing genotypes per healthy person among the coding sequences of the selected 301 nonmitochondrial candidate retinal dystrophy genes. This false-positive rate can have far-reaching clinical implications, especially in the era of gene therapy.

To meaningfully counsel a patient and ascertain eligibility for gene therapy, clinicians must establish whether a VUS is pathogenic. With retinal dystrophies, genetic and phenotypic heterogeneity can make this task difficult, and evaluations must be performed rigorously. Several strategies exist to enhance the identification of probable causative mutations. In 2015, the American College of Medical Genetics standardized terminology to describe variants (as pathogenic, likely pathogenic, uncertain significance, likely benign, or benign[29]) by referencing single-nucleotide polymorphism databases; filtering variants absent in a control population; and predicting functional relevance according to amino acid changes, introduction of a stop codon, or effects on splicing. Several software tools can be helpful in predicting pathogenicity, including the BLOSUM (Blocks Substitution Matrix) 62,[28,30] PolyPhen-2,[31,32] SIFT (Sorting Intolerant From Tolerant),[33] PMut,[34] PROVEAN,[35] and homology modeling.[36] Most algorithms for missense variant prediction are 65% to 80% accurate when examining known disease variants.[37] For autosomal recessive diseases, parental testing is typically performed to resolve the phase of 2 candidate heterozygous variants. Regardless of phase, unless the variants have been previously reported to be pathogenic with strong genetic or functional evidence, these variants may be classified as VUS despite efforts to improve the prediction software and standardize the variant classification system. This unsolved problem of variant classification has led to challenges and anxiety for both clinicians and patients.[38]

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Ultimately, directed functional assays may be most accurate in estimating the pathogenicity of a specific variant. This case series assessed the utility of a functional assay that was originally designed for research applications[11] and helped recommend 2 patients as candidates for voretigene neparvovec-rzyl treatment. The assay takes approximately 60 hours to complete by 1 trained person over the course of 3 weeks: 2 days for identification and custom synthesis of mutational oligonucleotide primers; 3 days for site-directed mutagenesis, screening of clones, and sequencing; 3 days for culture and purification of mutant plasmids; 3 days for culture and transfection of HEK cells; and 2 days for HPLC analysis. The assay costs are approximately US $1000 for 5 separate mutations and approximately $3000 for analysis by personnel. Given the infrastructure required to run the assay, an opportunity exists for an interested party to provide this critical service to the professional community. Although the gene therapy surgical procedure with voretigene neparvovec-rzyl has a good safety profile, vitreoretinal operation has inherent risks, including retinal breaks, retinal detachment, cataract, elevated intraocular pressure, inflammation, endophthalmitis, or loss of visual acuity. Furthermore, substantial costs are associated with voretigene neparvovec-rzyl and the surgical delivery.[39] Functional protein activity assays for *RPE65*, as well as other candidate genes, should be considered when encountering uncertain pathogenicity from VUSs to avoid the possibility of treating a false genotype.

Limitations

This study was limited by the sample size of 4 patients with *RPE65*-mediated LCA from 2 institutions. The results of gene therapy were limited to 2 patients. However, the findings reflect the rare prevalence of the disease and the recent use of gene therapy intervention.

Conclusions

Findings from this study suggest that, in patients with missense mutations in *RPE65*, functional assays of protein function can be performed to assess the pathogenicity of variants in both compound heterozygous and homozygous cases. Given the potential risks of gene therapy operations, in vitro RPE65 activity testing should be considered to avoid the possibility of treating a false genotype.

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Author Affiliations: Department of Pediatrics, Children's Hospital Los Angeles, Los Angeles, California (Yang); Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, Bethesda, Maryland (Gentleman, Redmond); Center for Personalized Medicine, Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles, Los Angeles, California (Gai); Division of Retinal Disorders and Ophthalmic Genetics, Stein Eye Institute, David Geffen School of Medicine, UCLA (University of California, Los Angeles) (Gorin); The Vision Center, Department of Surgery, Children's Hospital Los Angeles, Los Angeles, California (Borchert, Lee, Nagiel); USC Roski Eye Institute, Department of Ophthalmology, Keck School of Medicine, University of Southern California, Los Angeles (Borchert, Lee, Nagiel); Retina Department Genomics Institute, Mejora Vision MD/Virtual Eye Care MD, Mérida, Yucatán, México (Villanueva); Laboratoire de Diagnostic Moleculaire, Hôpital Maisonneuve Rosemont, Montreal, Quebec, Canada (Villanueva); Department of Paediatric Surgery, Montreal Children's Hospital, McGill University and McGill University Health Centre Research Institute, Montreal, Quebec, Canada (Koenekoop); Department of Human Genetics and Ophthalmology, Montreal Children's Hospital, McGill University and McGill University Health Centre Research Institute, Montreal, Quebec, Canada (Koenekoop); Center for Advanced Retinal and Ocular Therapeutics, University of Pennsylvania, Philadelphia (Maguire, Bennett); F.M. Kirby Center for Molecular Ophthalmology, Scheie Eye Institute, University of Pennsylvania, Philadelphia (Maguire, Bennett).

Author Contributions: Drs Nagiel and Redmond had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
Concept and design: Yang, Redmond, Nagiel.
Acquisition, analysis, or interpretation of data: All authors.
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