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Early onset flecked retinal dystrophy associated with new compound heterozygous *RPE65* variants

Satoshi Katagiri,[1] Katsuhiro Hosono,[2] Takaaki Hayashi,[1,3] Kentaro Kurata,[2] Kei Mizobuchi,[1] Tomokazu Matsuura,[4] Kazutoshi Yoshitake,[5] Takeshi Iwata,[5] Tadashi Nakano,[1] Yoshihiro Hotta[2]

[1]Department of Ophthalmology, The Jikei University School of Medicine, Tokyo, Japan; [2]Department of Ophthalmology, Hamamatsu University School of Medicine, Hamamatsu, Japan; [3]Department of Ophthalmology, Katsushika Medical Center, The Jikei University School of Medicine, Tokyo, Japan; [4]Department of Laboratory Medicine, The Jikei University School of Medicine, Tokyo, Japan; [5]National Institute of Sensory Organs, National Hospital Organization Tokyo Medical Center, Tokyo, Japan

Purpose: To report genetic and clinical features of two unrelated Japanese patients with early onset flecked retinal dystrophy.
Methods: Patients underwent comprehensive ophthalmic examinations that included electroretinography (ERG) after 30 min and 24 h of dark adaptation (DA). Disease-causing gene variants were identified with whole exome sequencing (WES), with identified candidates confirmed with direct sequencing.
Results: WES identified compound heterozygous *RPE65* variants in both patients. Variants in patient 1 included c.1543C>T (p.R515W) and c.683A>C (p.Q228P), while patient 2 exhibited c.1028T>A (p.L343*) and c.683A>C (p.Q228P). Although variants p.R515W and p.L343* have been previously reported as pathogenic, variant p.Q228P was reported as uncertain significance. Each unaffected parent carried the variant heterozygously. Both patients had similar ophthalmic findings, including decreased visual acuity with early onset night blindness, numerous dense white dots/flecks occurring mainly outside the vascular arcades, a diffuse and/or disrupted ellipsoid line as shown with optical coherence tomography, and non-recordable rod and combined responses along with decreased cone responses after 30 min of DA. After 24 h of DA, both patients exhibited marked or partial recovery of the combined responses.
Conclusions: The results indicate that the recovery of combined or residual cone responses might be associated with a mild form of *RPE65*-related early onset flecked retinal dystrophy with new compound heterozygous variants.

The retinoid isomerohydrolase (*RPE65*) gene (OMIM 180069), which encodes a 533 amino acid protein that is expressed in the RPE, plays an important role in the conversion of all-trans-retinyl ester to 11-*cis*-retinol in the visual cycle [1,2]. Biallelic *RPE65* variants were initially reported as being the cause of Leber congenital amaurosis 2 (LCA2; OMIM 204100) [3,4] and retinitis pigmentosa (RP) [5,6]. Although the majority of cases with *RPE65* variants have been reported to be associated with the most severe forms of retinal dystrophies, such as LCA and RP [7], there have been a few rare cases with *RPE65* variants that have only exhibited relatively milder forms, namely, fundus albipunctatus (FAP, OMIM 136880) and early onset flecked retinal dystrophy [8-11]. In addition, a few reports demonstrate that transient visual improvement was seen in some child patients with *RPE65*-related retinal dystrophy [12,13].

Correspondence to: Takaaki Hayashi, Department of Ophthalmology, Katsushika Medical Center, The Jikei University School of Medicine, 6-41-2 Aoto, Katsushika-ku, Tokyo 125-8506, Japan; Phone: +81-3-3603-2111; FAX: +81-3-3433-1936; email: taka@jikei.ac.jp

To date, two patients have been reported to have *RPE65*-related FAP [8,10]. FAP is a rare form of stationary night blindness characterized by congenital night blindness, stationary or slow progression, and numerous dense white dots found at the midperipheral retina. Although electroretinography (ERG) in patients with FAP shows diminished or absent rod responses after standard dark adaptation (DA), there is recovery of the rod function after extended DA [8,14,15]. At the present time, however, little is known about *RPE65*-related FAP and/or flecked retinal dystrophy in the Japanese population.

This report presents information on two unrelated Japanese patients who exhibit early onset flecked retinal dystrophy with compound heterozygous variants in the *RPE65* gene. The purpose of this study was to describe the clinical and genetic features of *RPE65*-related early onset flecked retinal dystrophy.

METHODS

The Institutional Review Board of the Jikei University School of Medicine and Hamamatsu University School of Medicine approved the protocol for this study (approval numbers: The

Jikei University 24–232 6997 and Hamamatsu University 14–040). The protocol adhered to the tenets of the Declaration of Helsinki, with informed consent obtained from each participant or his or her legal guardian.

Clinical study: Two patients (JU#1085 and JU#1303) from two unrelated Japanese families (Families A and B) with early onset flecked retinal dystrophy were evaluated at Jikei University Hospital. Each patient underwent a comprehensive ophthalmic examination, including decimal best-corrected visual acuity (BCVA), slit-lamp examination, funduscopy, Goldmann kinetic visual field testing, fundus autofluorescence imaging (FAI; Optos 200Tx, Ultra-Wide Field Retinal Imaging System, Optos, Dunfermline, UK), fluorescein angiography and optical coherence tomography (OCT; Carl Zeiss Meditec AG, Dublin, CA; Spectralis; Heidelberg Engineering, Heidelberg, Germany). Full-field ERG using a light-emitting diode built-in electrode (LE-4000, Tomey, Nagoya, Japan) was recorded in accordance with the protocols of the International Society for Clinical Electrophysiology of Vision [16]. Details on the procedure and experimental conditions have been previously reported [17]. Each patient was dark adapted for 30 min or 24 h in either eye.

Whole exome sequencing and bioinformatics analysis: Before we conducted whole exome sequencing (WES), we performed Sanger sequencing for the *RDH5* gene (OMIM 601617), which is closely associated with FAP, showing that neither patient had any pathogenic *RDH5* variant. Next, WES was performed on the two patients and their unaffected parents. Briefly, construction of paired-end sequence libraries and exome capture were performed using the SureSelect XT Human All Exon V6 kit (Agilent Technologies, Santa Clara, CA), with the captured libraries sequenced using the NextSeq 500 System (Illumina, San Diego, CA). Sequence reads were mapped to the human reference genome sequence (GRCh37/hg19) using the Burrows-Wheeler Aligner software v 0.7.15. ANNOVAR software v 2016Feb01 was used to annotate single nucleotide variants and insertion-deletion polymorphisms. Among rare nonsynonymous and splice site variants, we screened for variants in the known inherited retinal disease (IRD) genes listed in the RetNet database (Accessed August 23, 2017), as mutations in these genes account for approximately 40% of the IRD cases [18]. In each family, there was one affected individual from the unaffected parents, suggesting autosomal recessive inheritance or sporadic cases. Therefore, the analysis was based on the autosomal recessive and de novo dominant model (Appendix 1, Appendix 2, and Appendix 3). The following three computational algorithms were used to evaluate the missense variant pathogenicity: SIFT, PolyPhen2, and MutationTaster. Details of the WES procedure are described in Appendix 1.

Sanger sequencing validation and segregation analysis: Potential pathogenic variants detected with WES were validated using Sanger sequencing. DNA from family members was examined to investigate the cosegregation of potentially pathogenic variants. The following primer sets for the *RPE65* gene were used: exon 7: forward primer 5′-CTG GGT GAT TTT GCA GCT TCA CA-3′, and reverse primer 5′-GTG ATC AGG ATT GGT ATC AAA GGT-3′; exon 10: forward primer 5′-GCA AAA TTG TGC GCA TCT GCA AG-3′, and reverse primer 5′-ACA TGA GGC AGG AGG ACA ATT CCT-3′; exon 14: forward primer 5′-ATG CCA GGT GGT ACA AGA GTC A-3′, and reverse primer 5′-GCA AAA TTG TGC GCA TCT GCA AG-3′.

RESULTS

Clinical characterization: This study investigated two patients from two unrelated Japanese families. Clinical and genetic data are summarized in Table 1.

Family A: A female patient (JU#1085) was diagnosed at 3 years old with early onset flecked retinal dystrophy with night blindness and loss of visual acuity. She had neither nystagmus nor medical history of any systemic disease, and there was no parental consanguinity. From the age of 3 up to 14 years, medical care was provided by her previous doctor. At 6 years of age, her BCVA was 0.25 in each eye. Goldmann perimetry (GP) examinations at the age of 12 showed she had preserved peripheral visual fields in the V-4e isopters but constricted visual fields in the II-4c and I-4e isopters. Fluorescein angiograms showed hyperfluorescent rings (window defect) surrounding central areas of hypofluorescence in both maculae at age 20 (Figure 1A). She was referred to Jikei University Hospital at the age of 23 due to progressive loss of vision and photophobia. Her BCVA was 0.1 (−3.00 cyl 170°) in the right eye and 0.15 (−0.25 sph −2.50 cyl 5°) in the left eye. Slit-lamp examination showed no abnormal findings in the anterior segment and media. Funduscopy showed bull's eye maculopathy, and numerous dense white dots/flecks occurring mainly outside the vascular arcades, although there were no attenuated vessels or bone spicule-like pigmentations observed (Figure 1B–D). FAI showed overall low autofluorescent signals and hyper-autofluorescent rings within the arcades (Figure 1E). OCT showed severe thinning of the outer retinal layers with a blurred and partial disrupted ellipsoid zone (EZ; Figure 2A). Although GP indicated there were preserved peripheral visual fields in the V-4e isopters, there were also constricted visual fields (I-4e isopters) with the relative central scotoma of the II-4c isopters (Figure 3A).

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Table 1. Clinical and genetic findings for the two patients with biallelic *RPE65* variants.

Family	Patient	Gender	Parental consan-guinity	Age of onset (y)	Symptom at onset	Age at first visit (y)	Refractive error, diopters	BCVA at first visit	Age at last visit (y)	BCVA at last visit	Biallelic *RPE65* variants	
											Paternal variant	Maternal variant
Family A	JU#1085	Female	-	3	Night blindness	23	R /-3.00 cyl 170° L −0.25 sph/-2.50 cyl 5°	R 0.1 L 0.15	26	R 0.1 L 0.2	c.638A>C (p.Q228P)	c.1543C>T (p.R515W)
Family B	JU#1303	Male	-	3	Decreased visual acuity	11	R +2.50 sph/-3.00 cyl 180° L +2.50 sph/-3.50 cyl 180°	R 0.9 L 0.7	13	R 0.7 L 0.8	c.1028T>A (p.L343*)	c.638A>C (p.Q228P)

BCVA=decimal best corrected visual acuity, R=right, L=left, sph=spherical, cyl=cylinder

At 26 years of age, the patient's BCVA was 0.1 and 0.2 in the right and left eyes, respectively. Full-field ERG after 30 min of DA showed non-recordable rod and combined responses along with severely decreased cone and 30 Hz flicker responses in the left eye, whereas after 24 h of DA in the right eye, we observed marked recovery of the rod and combined responses (Figure 4). The longitudinal clinical data demonstrated there was a slow progressive deterioration of the visual function.

Family B: An ophthalmic examination performed by the male patient's (JU#1303) previous doctor when the patient was 3 years old showed there was decreased visual acuity. The patient exhibited neither nystagmus nor a medical history of any systemic disease, and there was no parental consanguinity. At the age of 4, BCVA was 0.9 and 0.7 in the right and left eyes, respectively. OCT revealed continuous but blurred EZ in both eyes at the age of 9 (Figure 2B). The patient was referred to Jikei University Hospital at the age of 11 due to night blindness and photophobia. His BCVA was 0.9 (+2.50 sph −3.00 cyl 180°) in the right eye and 0.7 (+2.50 sph −3.50 D Ax 180°) in the left eye. Slit-lamp examination indicated that there were no abnormal findings in the anterior segment and media. Funduscopy showed there were numerous dense white dots/flecks occurring mainly outside the vascular arcades, although there were no attenuated vessels or bone-spicule pigmentations observed (Figure 5A–C). FAI showed overall low autofluorescent signals and relative hyper-autofluorescent areas within the arcades (Figure 5D). OCT found similar findings to those observed at the age of 9 (Figure 2C). The Farnsworth Panel D-15 Color Vision tests revealed tritan defects in both eyes. Although GP showed that he had preserved peripheral visual fields in the V-4e isopters, the fields were constricted, especially in the superior visual fields of the I-4e isopters (Figure 3B). At the age of 13, his BCVA was 0.8 in both eyes. Full-field ERG after 30 min of DA showed there were non-recordable rod and combined responses with decreased cone and 30 Hz flicker responses, whereas after 24 h of DA, we observed partial recovery of the combined responses in the right and left eyes (Figure 4). Longitudinally, the patient exhibited slow deterioration of visual function.

Identification of pathogenic variants: In this study, we performed WES to investigate both patients and their parents. In the first step, we examined the known IRD genes to screen for variants from WES data, which we then analyzed based on the autosomal recessive and de novo dominant model (Appendix 1 and Appendix 2). After common variants and synonymous variants were excluded, a total of seven rare heterozygous variants remained in the two patients (Appendix 1, Appendix 2, and Appendix 3). Subsequently, we then screened the remaining variants that matched the patients' phenotypes and the disease phenotypes known to be caused by the IRD genes. After the screening, three heterozygous variants of *RPE65* (NM_000329.2) remained, with patient JU#1303 shown to be carrying a missense variant c.638A>C (p.Q228P) and a nonsense variant c.1028T>A (p.L343*), while patient JU#1085 carried the variant (p.Q228P) and a missense variant c.1543C>T (p.R515W; Table 1, Appendix 4 and Appendix 5). The p.L343* and p.R515W variants have previously been reported to cause LCA, RP, and flecked retinal dystrophy [9,17,19], whereas p.Q228P has yet to be reported as a pathogenic variant. The p.Q228P variant was registered as uncertain significance (Variation ID: 298023) in ClinVar and as rs886046510 in the NCBI 1000 Genome Browser. The Q228 residue was found to be highly conserved across different species (Appendix 4). Additionally, in silico analysis using three different computational prediction programs (SIFT, PolyPhen2, and MutationTaster) revealed the pathogenicity of the p.Q228P variant (Appendix 4). According to the standards and guidelines of the American College of Medical Genetics and Genomics (ACMG) [20], the p.Q228P variant was considered "likely pathogenic." Details of the filtering steps and remaining variants are described in Appendix 1, Appendix 2, and Appendix 3. Subsequent Sanger sequencing confirmed those variants were cosegregated with the disease phenotype (Appendix 5).

DISCUSSION

In this study, we used WES to examine two Japanese patients with early onset flecked retinal dystrophy and identified three *RPE65* variants (p.Q228P, p.L343*, and p.R515W), one (p.Q228P) of which was not reported as a pathogenic variant. Clinically, these two patients exhibited early onset night blindness, characteristic funduscopic appearance of FAP, and partial recovery of the combined ERG responses following prolonged DA.

Previous studies have reported that the majority of *RPE65* variants lead to severe clinical phenotypes, such as LCA and early onset RP [3-5] whereas several other studies have found a milder form of *RPE65*-related flecked retinal dystrophy, including FAP [6,8-10]. To date, compound heterozygous states have been reported in two patients with *RPE65*-related FAP. One patient exhibited a combination of the splice site variant (c.11+5G>A) and the missense variant (p.I115T) [8], while the other was shown to have a one base insertion variant (c.639_640insA, p.A214Sfs20*) and a missense variant (p.L328F) [10]. The appearance of *RPE65*-associated FAP indicates that certain residual RPE65 functions (enzyme

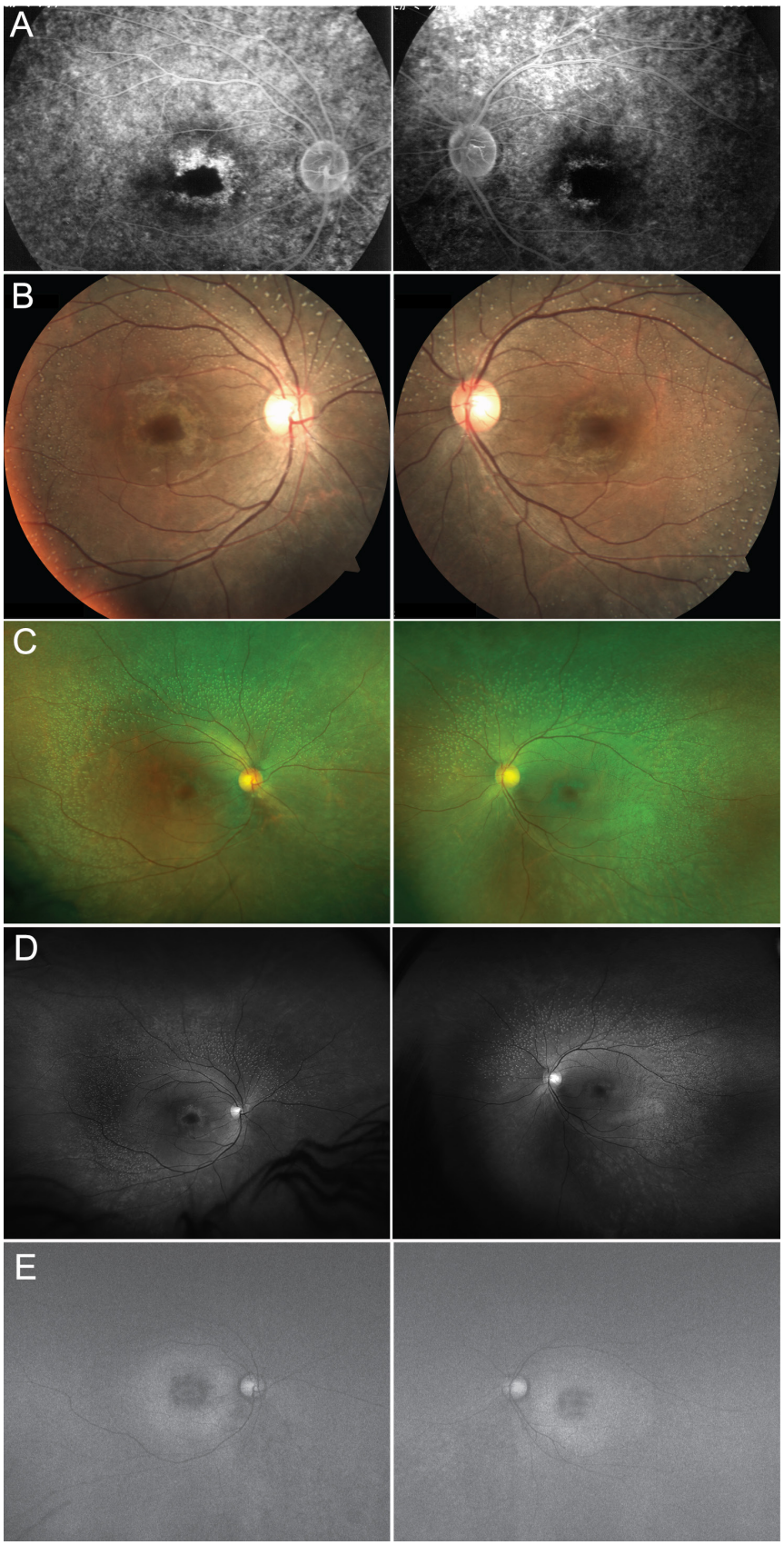

Figure 1. Fundus images of patient JU#1085 (left column: images of the right eye, right column: images of the left eye). **A**: Late-phase fluorescein angiograms show hyperfluorescent rings surrounding central areas of hypofluorescence (20 years of age). Fundus (**B** and **C**) and red-free retinal (**D**) images show bull's eye maculopathy and numerous dense white dots/flecks occurring mainly outside the vascular arcades (23 years of age). **E**: Wide-field fundus autofluorescence images show overall low autofluorescent signals and hyper-autofluorescent rings within the arcades (23 years of age).

activity) encoded by one disease allele with p.I115T or p.L328F are necessary and sufficient for causing the mild phenotype (FAP), as the other disease allele with c.11+5G>A or c.639_640insA has been presumed to be a null function. In the present study, recovery of the rod and combined responses after prolonged DA in patient JU#1085 (Figure 4) was similar to that observed for the patient with *RPE65*-related FAP with compound heterozygous variants (c.11+5G>A and p.I115T) [8]. Collectively, the p.Q228P variant might have a variant-specific phenotypic effect as the p.I115T or p.L328F variant [8,10]. Similarly, two other studies reported finding preserved visual function in multiple patients with *RPE65*-related flecked retinal dystrophy [9,11]. The full-field ERG for patient JU#1303 showed non-recordable rod responses after standard DA and relatively preserved cone responses (Figure 4). These findings were similar to those reported for two other patients with *RPE65*-related flecked retinal dystrophy (one with a homozygous hypomorphic variant [p.P25L] [11] and the other with compound heterozygous variants [c.1067dupA and p.R515W] [9]). Patient JU#1303 also exhibited partial recovery of the combined responses after prolonged DA (Figure 4). In the present study patients, the findings for the residual ERG responses and preserved peripheral visual fields (Figure 3) were similar to those for the mild disease form, *RPE65*-related FAP or flecked retinal dystrophy. These findings suggest that *RPE65* variants rarely result in the appearance of FAP, which exhibits the more severe retinal dysfunction compared to the *RDH5*-related FAP, and thus, generally are associated with good visual acuity and visual fields [21]. In two patients previously reported to have compound

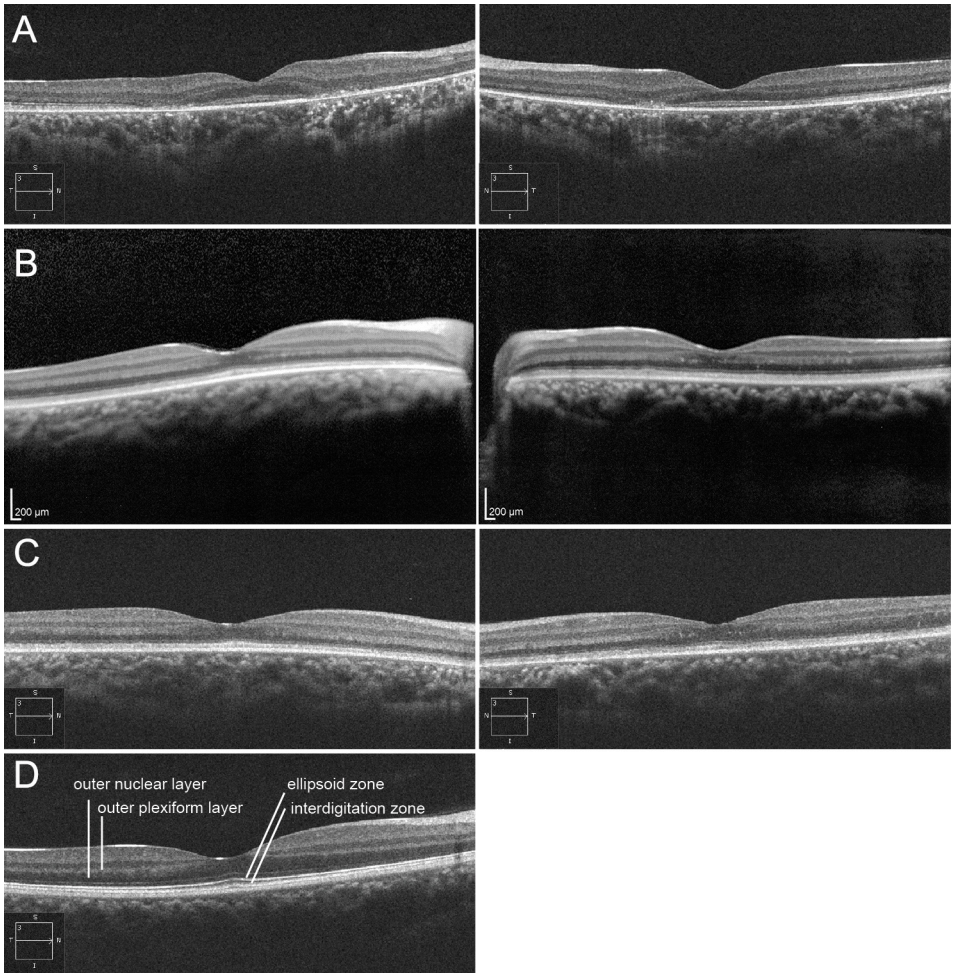

Figure 2. Images of horizontal optical coherence tomography (OCT) macular scan (left column: images of the righty eye, right column: images of the left eye). **A**: OCT images (patient JU#1085) show severe thinning of the outer retinal layers, with a blurred and partial disrupted ellipsoid zone, (23 years of age). **B**: OCT images (patient JU#1303) reveal a continuous but blurred ellipsoid zone, (9 years of age). **C**: OCT images for patient JU#1303 at the age of 11 are similar to those at the age of 9. **D**: The outer retinal layers of the right eye are labeled in a male control without any retinal disease in his early 20s.

heterozygous states (one with c.1067dupA and p.R515W, and the other with c.11+5G>A and p.R515W in *RPE65*), the p.R515W variant was reported to be associated with the residual ERG responses, which suggests that p.R515W might be a hypomorphic variant [9]. Each of the present patients had a missense variant (p.Q228P) in one allele, as well as the p.R515W variant or the nonsense variant (p.L343*) in the other allele (Table 1). These findings indicate that p.Q228P appears to play an important role in the mild phenotype with the residual ERG responses. However, it is uncertain why the ERG findings for the recovery of the rod and residual cone responses were somewhat different between patients JU#1085 and JU#1303. A recent immunohistochemical study demonstrated that the RPE65 protein is expressed not only in the RPE but also in human cones, but not in the rods [22]. Thus, although it is possible that the RPE65 residual enzyme activities associated with hypomorphic mutated proteins might exhibit different effects on cones and rods, the mechanisms underlying *RPE65*-related retinal dystrophies are complex and can occur in various phenotypes (LCA, RP, and FAP, among others).

RPE65-related LCA is the first IRD to have been treated with gene replacement therapy in the first clinical trials, with the outcomes showing the therapies to be safe and effective

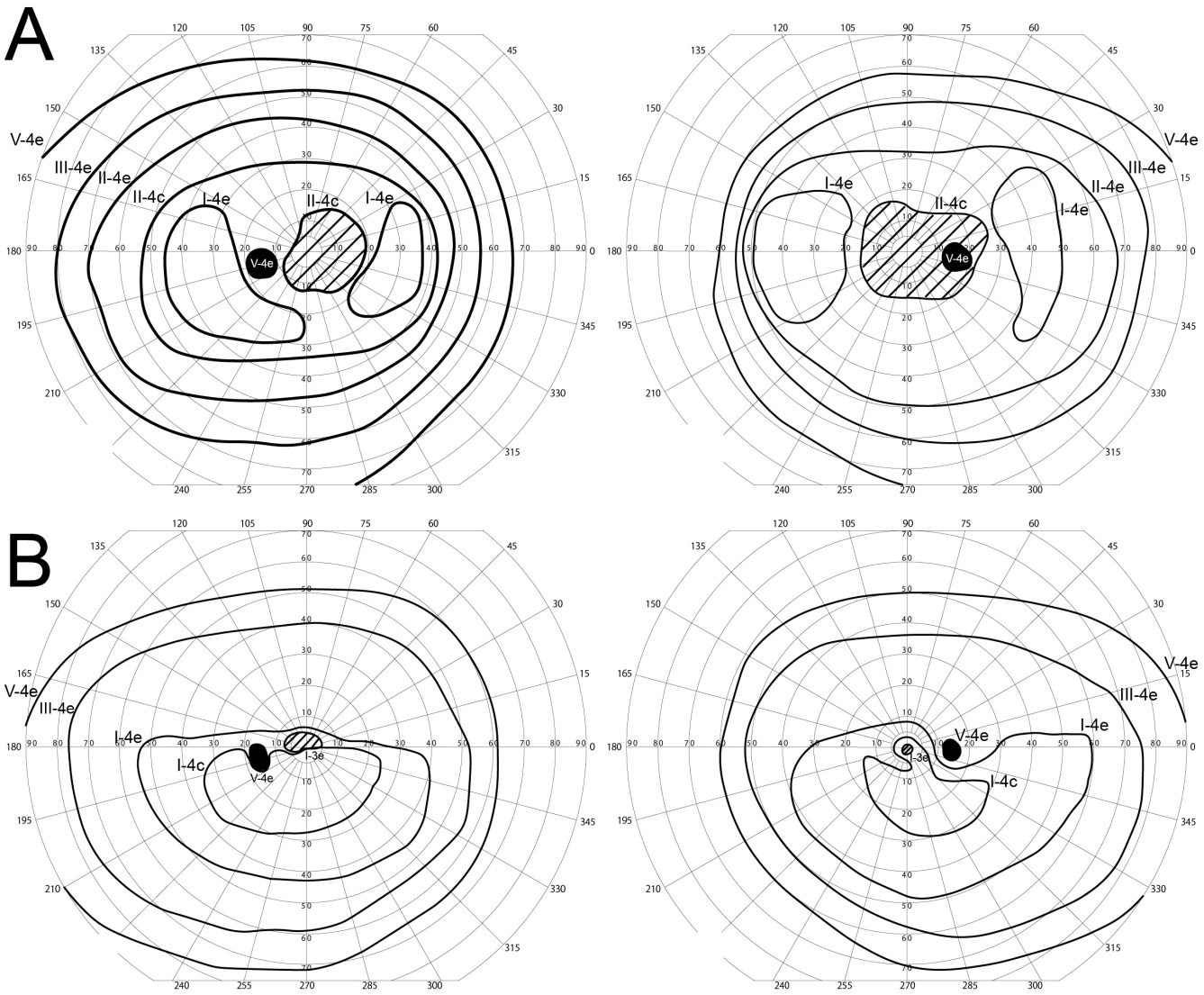

Figure 3. Visual field evaluations with Goldmann perimetry (left column: images of the left eye, right column: images of the right eye). **A**: Patient JU#1085 shows preserved peripheral visual fields in the V-4e isopters, although there were constricted visual fields with central scotoma of the II-4c isopters (23 years of age). **B**: Patient JU#1303 shows preserved peripheral visual fields in the V-4e isopters, although these were constricted especially in the superior visual fields of the I-4e isopters (11 years of age).

[23,24]. Furthermore, subsequent longitudinal follow-up studies have also revealed that some patients achieved improvements in their visual and retinal functions a few months after the treatment [25-27]. Weleber et al. followed patients for 2 years after gene therapy and reported that the greatest improvements in visual acuity were observed in younger patients who had better baseline visual acuities and relatively preserved outer retinal layers, as seen in OCT images [27]. Weleber et al. further reported that a 6-year-old female patient (subject 204 in the original article) exhibited considerable improvements in rod and cone ERG responses in the treated right eye compared to the untreated left eye [27]. The phase 3 trial of voretigene neparvovec (AAV2-hRPE65v2) for patients with *RPE65*-related retinal dystrophy was completed in 2017 [28], and the U.S. Food and Drug Administration has approved voretigene neparvovec, a one-time gene therapy product for the treatment of patients with *RPE65*-related retinal dystrophy. In addition to gene therapy, another study reported that oral *9-cis*-retinoid supplementation therapy was also able to improve visual function in some patients with *RPE65*-related LCA [29]. Based on these previous findings, the present study patients (JU#1085 and JU#1303), who had residual ERG responses and preserved peripheral visual fields, might also be eligible for future clinical trials of *RPE65* gene replacement therapy and/or administration of oral *9-cis*-retinoid supplementation, which have been conducted for patients with LCA with biallelic *RPE65* variants. However, as biallelic *RPE65* variants appear to be rare in Japanese patients with IRDs, a large-scale cohort study will need to be undertaken to elucidate the prevalence and natural history of *RPE65*-related retinal dystrophies in the Japanese population.

In conclusion, we identified a *RPE65* variant (p.Q228P), considered likely pathogenic, in two Japanese patients with early onset flecked retinal dystrophy. The results indicated that the recovery of combined responses or residual cone responses might be associated with a mild form of *RPE65*-related early onset flecked retinal dystrophies caused by the new compound heterozygous variants.

APPENDIX 1 FILTERING STEPS USED TO SELECT FOR AUTOSOMAL RECESSIVE VARIANTS IN THE STUDY.

To access the data, click or select the words "Appendix 1."

APPENDIX 2 FILTERING STEPS USED TO SELECT FOR DE NOVO DOMINANT VARIANTS IN THE STUDY.

To access the data, click or select the words "Appendix 2."

APPENDIX 3 REMAINING VARIANTS AFTER PERFORMING THE FILTERING STEPS.

To access the data, click or select the words "Appendix 3."

APPENDIX 4 CANDIDATE LIST OF THE COMPOUND HETEROZYGOUS VARIANTS.

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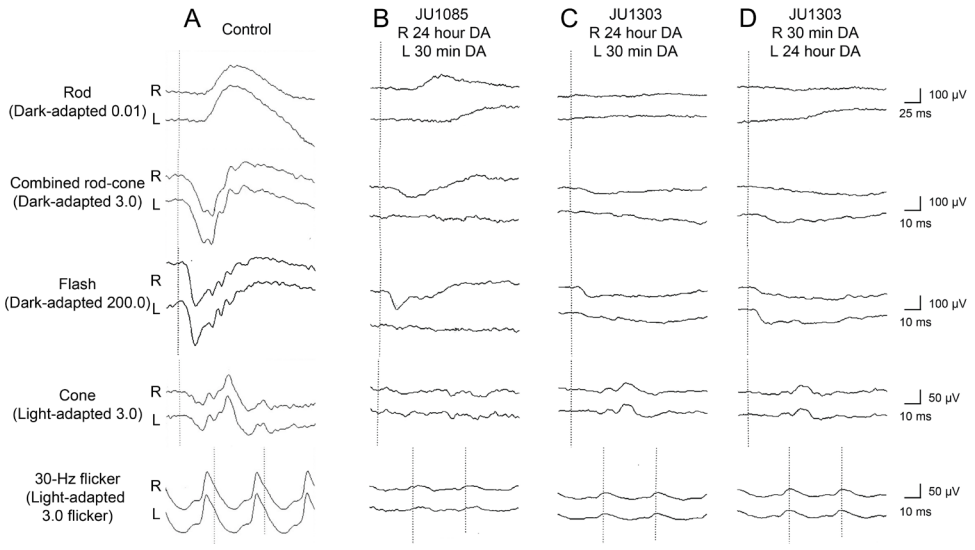

Figure 4. Full field electroretinograms (ERGs). **A**: ERG responses from a control. **B**: In patient JU#1085, the ERG after 30 min of dark adaptation (DA) shows non-recordable rod and combined responses along with severely decreased cone and 30 Hz flicker responses in the left eye (left, L), whereas marked recovery of the rod and combined responses was observed after 24 h of DA in the right eye (right, R; 26 years of age). **C** and **D**: In patient JU#1303, the ERG after 30 min of DA shows non-recordable rod and combined responses along with decreased cone and 30 Hz flicker responses in the left and the right eyes, whereas partial recovery of the combined responses after 24 h of DA are observed in the left and the right eyes (13 years of age).

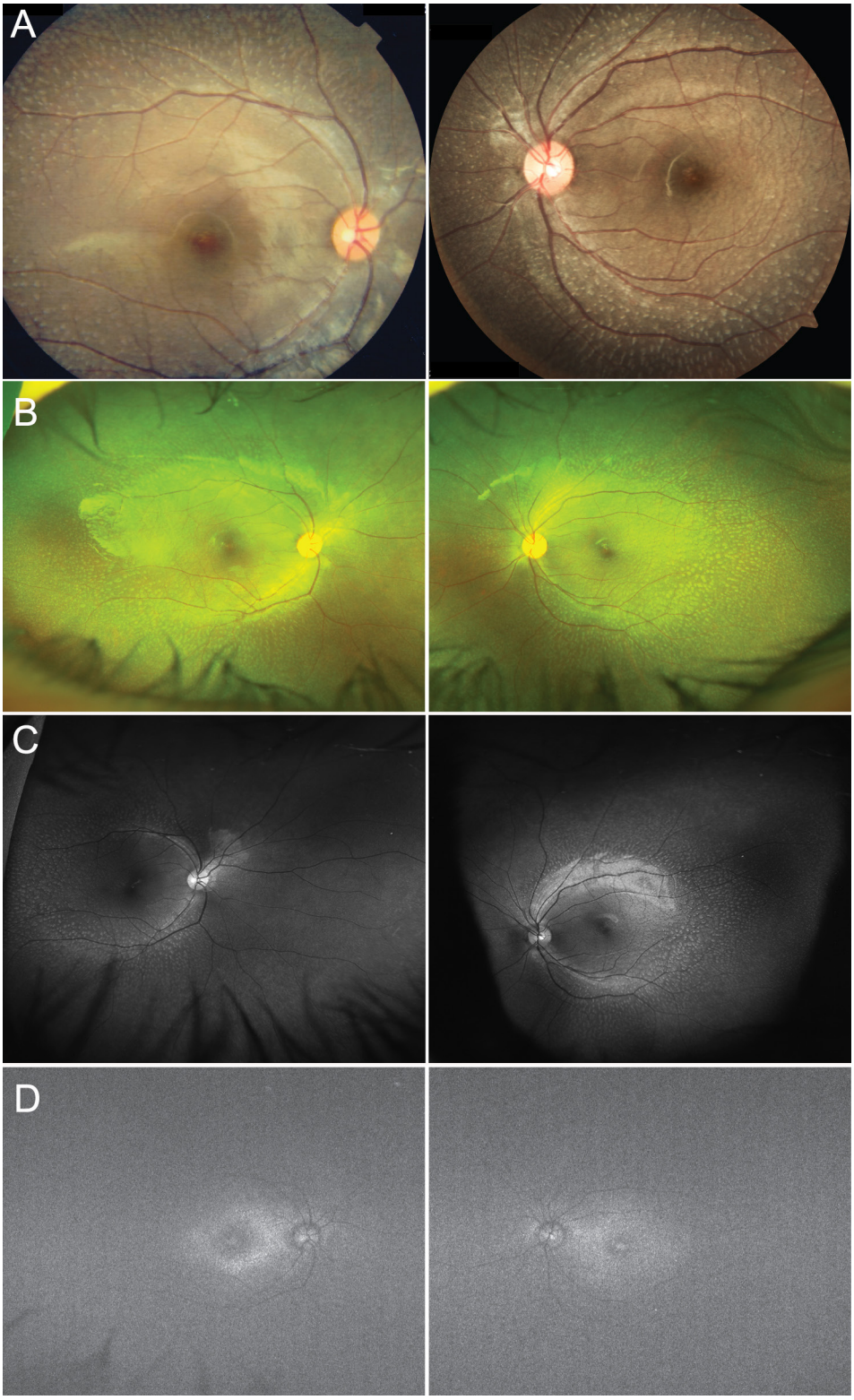

Figure 5. Fundus images of patient JU#1303 (left column: images of the right eye, right column: images of the left eye). Fundus (**A** and **B**) and red-free retinal (**C**) images show numerous dense white dots/flecks occurring mainly outside the vascular arcades (11 years of age). **D**: Wide-field fundus autofluorescence images show overall low autofluorescent signals and relative hyper-autofluorescent areas within the arcades (11 years of age).

APPENDIX 5 NUCLEOTIDE SEQUENCING OF THE *RPE65* VARIANTS IN PATIENTS JU#1085, JU#1303 AND THEIR PARENTS.

To access the data, click or select the words "Appendix 5."

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