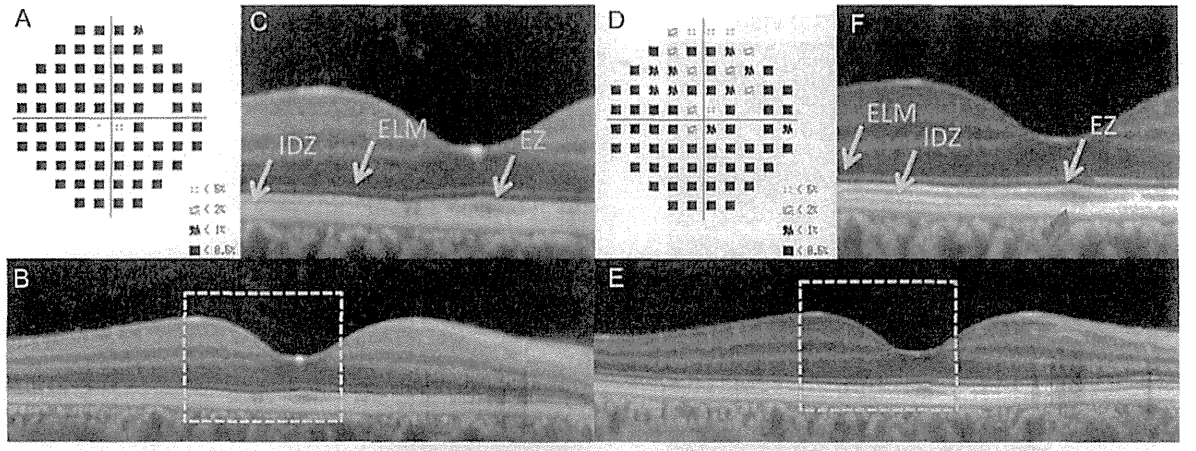

Figure 3. Static visual field and spectral-domain optical coherence tomographic (SD-OCT) results in the right eye of Case 2 at the initial visit (A–C) and six months after the initial visit (D–F). A: Deviation plot obtained with the Humphrey 30-2 program at the initial visit. B: Horizontal SD-OCT image through the fovea at the initial visit. C: Magnified view of the area outlined by dashed yellow line box in the image of B. D: Deviation plot obtained by the Humphrey 30-2 program at six months after the initial visit. E: Horizontal SD-OCT image through the fovea at six months after the initial visit. F: Magnified view of the area outlined by dashed yellow line box in the image of B. The COST line is still blurred near the fovea (red arrow). ELM, external limiting membrane. EZ, ellipsoid zone. IDZ, interdigitation zone.
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Based on these findings, he was diagnosed with AZOOR. He was treated with intravenous drip methylprednisolone.

His SD-OCT findings at the initial visit are shown in Figures 3B and 3C. The ELM and EZ were judged to be "continuous", but the IDZ was judged to be "discontinuous" (Fig. 3B & 3C).

Six months later, he reported some improvements of his visual symptoms, and his visual field showed recovery at several points (Fig. 3D). At this time, the ELM and EZ were judged to be "continuous", but the IDZ was judged to be "discontinuous", because it was still blurred near the fovea (yellow arrow, Fig. 3F, red arrow). The ONL was intact both at the initial visit and at 6 months.

To evaluate the changes in outer retinal high-reflective bands more quantitatively, a longitudinal reflectivity profile (LRP) was created in the retina of Case 2 (Fig. 4). One vertical straight line was drawn at 0.5 mm temporal retina from the foveola (red dotted lines of Fig. 4). We found that that IDZ was undetectable at the initial visit, but it became detectable as a third highly reflective band six month later (Figs. 4C and 4E).

Case 3: AZOOR with Worsening of Visual Fields. A 34-year-old myopic woman reported experiencing photophobia and vision reduction of one month duration in her right eye. She had a history of Basedow disease for eight years. Her decimal BCVA was 0.5 OD. Fundus examination and fluorescein angiography were normal, but Humphrey visual field tests revealed a temporal scotoma extending into the fixation point in the right eye (Fig. 5A). The multifocal ERGs were reduced in the centro-temporal field. Based on these clinical findings, she was diagnosed with AZOOR. She was treated with intravenous drip methylprednisolone.

Her SD-OCT findings of the right eye at the initial visit are shown in Figures 5B and 5C. Her ELM and EZ were judged to be "discontinuous", and the IDZ was judged to be "absent". Despite intravenous drip methylprednisolone and following orally administrated predonisolone for six months, she felt that there was a gradual worsening of her visual decrease, and visual field tests showed an enlargement of the temporal scotoma (Fig. 5D). At this time, the ELM and EZ were judged to be "discontinuous", and the IDZ still remained "absent" (Figs. 5E and 5F). The ONL thickness was also reduced at the areas of the visual field defects.

Summary of SD-OCT findings

The changes in the ELM, EZ, and IDZ at the initial visit and six months later are summarized in Figure 6 (see also Table 1). The status of the three highly reflective bands were evaluated at the retinal areas with visual field defects. In this evaluation, we excluded the retinal areas of undetectable ONL, because we noted that the retinal areas with loss of ONL at the initial visit did not show any improvement both in the SD-OCT findings and the visual field defects. Therefore in Figure 6, the results are shown only at the retinal areas of intact ONL.

We found that the IDZ was most vulnerable among the three outer retinal bands at the initial visit. There were no AZOOR-complex patients who had a continuous IDZ at the area of visual field defect at the initial visit. The IDZ was also most slow to recover at six months after the initial visit. Even at six months, the IDZ was continuous only in three eyes (18%), discontinuous in six eyes (35%), and still absent in eight eyes (47%).

When compared to the IDZ, the ELM and EZ were relatively well preserved at six months. The ELM was continuous in 14 of 17 eyes (82%) at six months. Similarly, the EZ was continuous in 15 of 17 eyes (88%) at six months (Fig. 5).

We also found that 15 of 17 eyes (88.3%) had a recovery of at least one of the three bands during six months (Table 1) if the ONL was intact, and these 15 eyes also showed an improvement of the visual field defects.

Discussion

We investigated the changes in the outer retinal microstructures at the initial visit and six months later in 17 eyes with AZOOR-complex using the SD-OCT. There have been many case series which described the change of OCT findings with time in AZOOR-complex patients [12,15–24], but the best of our knowledge, this is the first systematic report focusing on the

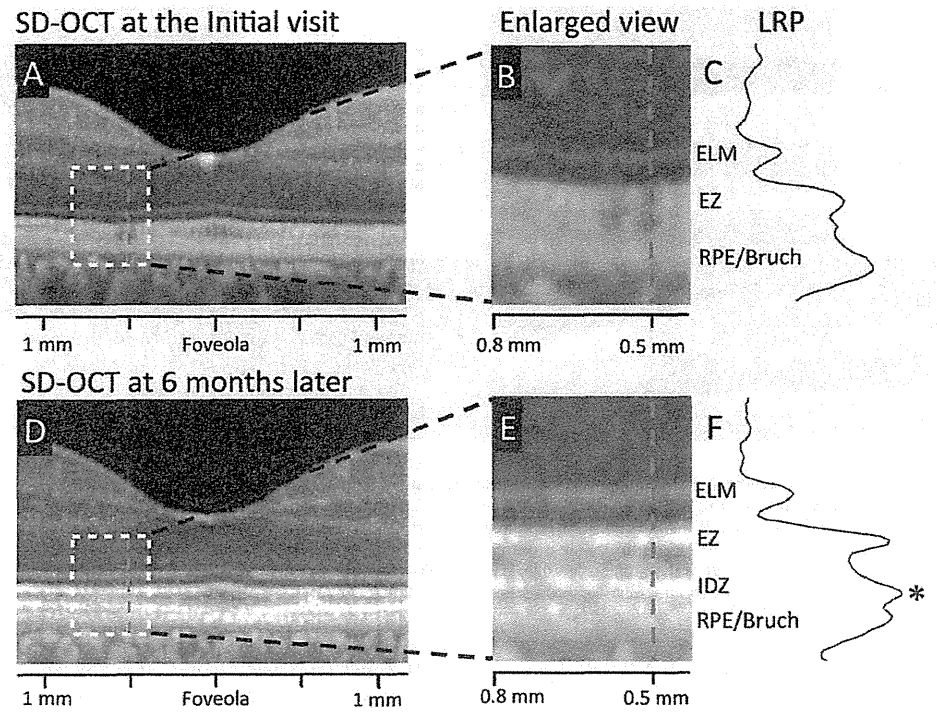

Figure 4. Results of longitudinal reflectivity profile (LRP) in the retina of Case 2. A. Horizontal SD-OCT scan through the fovea at the initial visit. B. Magnified view of the area outlined by dashed white line box in the image of A. C. Longitudinal reflectivity profile (LRP) along the vertical line at 0.5 mm temporal from the foveola (red dotted line) at the initial visit. D. Horizontal SD-OCT scan through the fovea at six months after the initial visit. E. Magnified view of the area outlined by dashed white line box in the image of D at six months after the initial visit. F. Longitudinal reflectivity profile (LRP) along the vertical line at 0.5 mm temporal from the foveola (red dotted line) at six months after the initial visit. At the retinal area of 0.5 mm temporal from the foveola, IDZ was nearly undetectable at the initial visit, but the peak of IDZ was clearly detectable as a third highly reflective band at six month later (asterisk). ELM, external limiting membrane. EZ, ellipsoid zone. IDZ, interdigitation zone. RPE/Bruch, retinal pigment epithelium/Bruch's membrane complex. LRP, longitudinal reflectivity profile.
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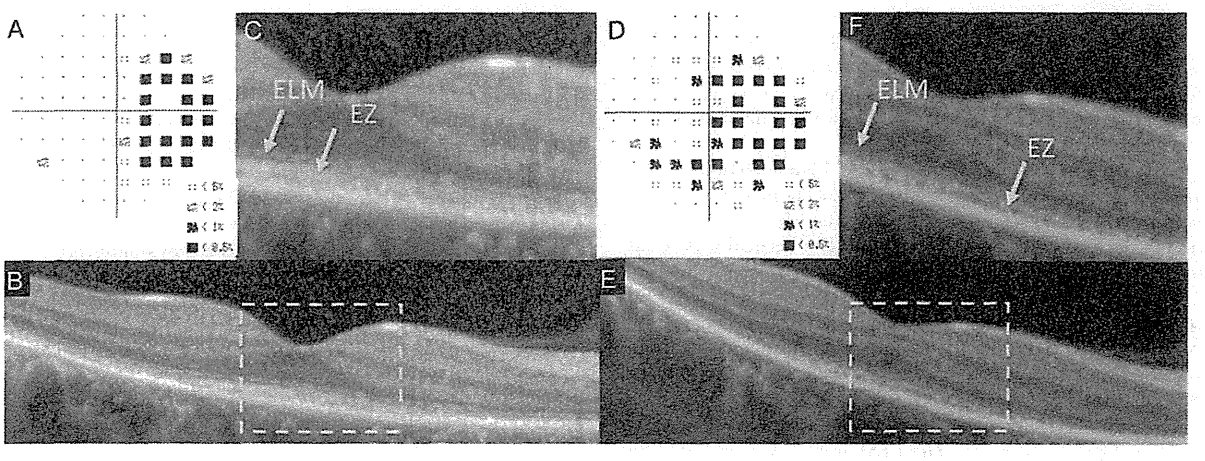

Figure 5. Static visual field and spectral-domain optical coherence tomographic (SD-OCT) results in the right eye of Case 3 at the initial visit (A–C) and six months after the initial visit (D–F). A: Deviation plot obtained by the Humphrey 30-2 program at the initial visit. B: Horizontal SD-OCT image through the fovea at the initial visit. C: Magnified view of the area outlined by dashed yellow line box in the image of B. D: Deviation plot obtained by the Humphrey 30-2 program at six months after the initial visit. E: Horizontal SD-OCT image through the fovea at six months after the initial visit. F: Magnified view of the area outlined by dashed yellow line box in the image of E. ELM, external limiting membrane. EZ, ellipsoid zone. IDZ, interdigitation zone.
doi:10.1371/journal.pone.0110592.g005

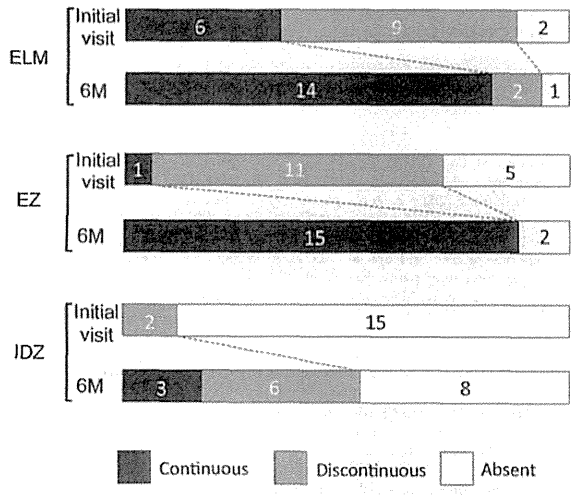

Figure 6. Summary of findings of the three highly reflective bands at the outer retina obtained by SD-OCT at the initial visit and six months later. These three lines were divided into three types; "continuous", "discontinuous" and "absent". ELM, external limiting membrane. EZ, ellipsoid zone. IDZ, interdigitation zone.
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changes in the three outer retinal bands of SD-OCT during a fixed time period.

We found that the IDZ was most vulnerable among the three outer retinal bands in the retina of AZOOR-complex. At the initial visit, the IDZ was abnormal, i.e., discontinuous or absent, in all 17 eyes (100%), while the ELM and EZ were abnormal in 11 (65%) and 16 eyes (94%), respectively (Fig. 6). Similarly, at six months after the initial visit, the IDZ was still abnormal in 14 eyes (82%), while the ELM and EZ were abnormal in only three (18%) and two eyes (12%), respectively. These results indicate that the IDZ is the most vulnerable microstructure and can be used to detect and follow the alterations of the outer retina in eyes with AZOOR-complex.

The origin of IDZ has not been established, and is currently thought to correspond to the junction [14] or contact cylinder [13] between the RPE apical processes and the external portion of the cones. Thus, this band is thought to be a useful indicator of the integrity of outer segments of the photoreceptors. Recent studies have reported that the integrity of the IDZ was significantly correlated with visual function in different retinal diseases including occult macular dystrophy [27,28], epiretinal membrane [29–31], and central serous chorioretinopathy [32]. On the other hand, it is also known that the IDZ cannot be identified clearly even in some normal subjects [33,34]. Thus, Rii et al. reported that the incidence of eyes with an intact foveal IDZ was about 95% in normal individuals [33]. Taken together, we now interpret our findings by concluding that the IDZ is the most vulnerable microstructure in eyes with our AZOOR-complex patients. This is because the visibility of the IDZ is most easily affected when the photoreceptors are damaged and not necessarily because this region is the primary site of this disorder.

There are several reports suggesting that the abnormality of EZ was present in the region of the visual field defects in AZOOR-complex [12,15–24]. Our results agree with this because most of our patients (16 of 17 eyes, 94%) had abnormal EZ at the region of the visual field defect at the initial visit. However, we also noted that one of our patients had a "continuous" EZ even at the retinal area of visual field defect at the initial visit (Case 2). In this area, only the IDZ was "discontinuous" (Fig. 3C). Tsunoda et al. [23] recently described two AZOOR patients whose EZ was normal, but the IDZ was not present or indistinct at the retinal area of visual field defect. So et al. [22] also reported an AZOOR patient whose EZ recovered, but the IDZ was still absent at the one month follow-up examination. We recommend focusing on not only the EZ but also the IDZ to enhance the detection of abnormal retinal microstructures in eyes with the AZOOR-complex [22,23].

Spaide et al. [18] reported that there was no visual field or anatomic improvements in the retinal regions where there was outer nuclear loss and that the improvement of scotoma and restoration of EZ were only seen in areas that had no loss of outer nuclear layers. Our results agree with their findings. We noted that three of 17 eyes (18%, Case 5, 11, and 16) that had retinal regions with a loss of the ONL at the initial visit did not show any recovery both in the retinal microstructures or the visual fields at these regions during six months (red squares of Fig. 1). These results support the idea that the photoreceptor outer segments can recover by the process of renewal only when the photoreceptor cell bodies are intact [18,20], and also suggests that the OCT findings of ONL can be useful in predicting whether the visual field defect can recover in eyes with AZOOR-complex.

Many of our patients with AZOOR-complex had myopia, and the average spherical equivalent refractive error in our 17 eyes was -4.4 D. This is consistent with past reports showing a high prevalence of myopia in eyes with AZOOR-complex [4,35]. In this study, we also noted that the eyes with more severe myopia of ≥ -5.0 D tended to have worse outcomes with abnormal EZ or IDZ at 6 months (Table 1). It should be interesting to study the correlation between the degree of myopia and the severity of outer retinal damage in more patients with AZOOR-complex.

There are two major limitations in this study. The first limitation is the small number of patients who were studied retrospectively. Because the AZOOR-complex is a very rare disease, we could not collect many patients from only two institutions. In addition, some patients were excluded because of insufficient SD-OCT or clinical data. Longer prospective studies with a larger number of patients may clarify more detailed information on the structural and functional changes with time in AZOOR-complex.

The second limitation is that we have combined the three different subtypes of the AZOOR-complex, MEWDS, AZOOR, and AIBSE. Although Gass et al. [6] suggested that these diseases may be part of a spectrum of a single disease, the prognosis is clearly different among the different types of AZOOR-complex [36]. Therefore, subgroup analysis for each type of disease may add more useful information.

Despite these limitations, our results demonstrated that 15 of 17 eyes (88%) with AZOOR-complex have some recovery of the retinal microstructures during six months if the ONL is intact. We also showed that the IDZ was the most vulnerable at the initial visit, and difficult to recover in this disorder. The SD-OCT was very useful for monitoring the changes of the outer retinal microstructure in eyes with the AZOOR-complex.

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Author Contributions

Conceived and designed the experiments: YM HM SU YI HT MK. Performed the experiments: YM HM MK. Analyzed the data: YM HM SU YI HT MK. Contributed reagents/materials/analysis tools: YM HM SU YI HT MK. Wrote the paper: YM MK.

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RESEARCH REPORT

Clinical Phenotype in Ten Unrelated Japanese Patients with Mutations in the *EYS* Gene

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ABSTRACT

Background: To characterize the clinical phenotypes associated with previously-reported mutations of the eyes shut homolog (*EYS*) gene, including a truncating mutation, c.4957_4958insA, which is a major causative mutation for retinitis pigmentosa (RP) in Japan.

Materials and Methods: The study population comprised ten unrelated RP subjects with very likely pathogenic mutations in both alleles, four of them with a homozygous c.4957_4958insA mutation. The phenotype analysis was based on ophthalmic examination, Goldmann perimetry, and digital fundus photography.

Results: The study population included six men and four women aged 34–74 years. The average age at first visit was 31 years (range, 14–44 years), and the patients typically presented with night blindness as the initial symptom and subsequently developed progressive constriction of the visual field. Myopia was noted in 9/20 affected eyes. For most patients, central visual acuity was preserved relatively well up to their thirties, after which it deteriorated rapidly over the next two decades. The visual acuity of patients homozygous for the c.4957_4958insA mutation was uniform. Visual fields were constricted symmetrically, and the extent of constriction seemed to be better correlated with age than visual acuity. The fundus displayed bone spicules, which increased in density with age, and attenuated retinal vessels.

Conclusions: Although additional studies with more patients with mutations of the *EYS* gene are required, it appears that patients share a relatively uniform phenotype with near-normal central visual function up to their twenties. The patients homozygous for the c.4957_4958insA mutation showed a uniform course of visual acuity changes.

Keywords: Autosomal recessive, eyes shut homolog (*EYS*) gene, founder effect, Japanese patient, retinitis pigmentosa

INTRODUCTION

Retinitis pigmentosa (RP [MIM 268000]) is a genetically highly heterogeneous retinal degeneration characterized by night blindness and visual field constriction, which eventually lead to severe visual impairment. The disease can be inherited via an autosomal recessive (ar), autosomal dominant (ad), or X-linked recessive mode or may occur in isolation; more than half the cases in Japan are isolated.[1]

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Rod dysfunction precedes cone dysfunction; this results in the typical symptoms of night blindness, which is followed by the loss of the peripheral visual field in most cases. Subsequently, the cones in the central retina may also be affected, causing loss of visual acuity in the later stages of the disease. Ophthalmoscopic abnormalities include a waxy pallor of the optic disc, attenuation of retinal vessels, and peripheral bone spicule pigmentations as well as atrophy of the retinal pigment epithelium (RPE).

To date, 55 causative genes and eight loci have been found to be associated with RP (http://www.sph.uth.tmc.edu/Retnet/; accessed May 20, 2012). The eyes shut homolog (*EYS*) gene encodes an ortholog of *Drosophila* spacemaker (spam) and a protein essential for maintaining the photoreceptor morphology. *EYS* spans over 2 Mb, making it one of the largest genes known to be expressed in the human eye.[2,3] *EYS* gene mutations, which include primarily truncating and some missense mutations, have been detected in arRP-affected families of different ancestral origin and are reported to account for 5–16% of arRP cases.[4–7] Recently, we screened all *EYS* gene exons in 100 unrelated Japanese RP patients and, found *EYS* gene mutations in at least 20% of the arRP patients (see the Supplementary Table in the Supplementary Material – available online).[8] In the current study, we examined the clinical features of ten unrelated Japanese patients with RP caused by the *EYS* gene mutation and compared the phenotype of four patients with the homozygous c.4957_4958insA (p.S1653KfsX2) mutation, which is a major causative mutation of RP in Japan, to that of the other RP patients.

MATERIALS AND METHODS

Ethics Statements

This study was approved by the Institutional Review Board for Human Genetic and Genome Research at the three participating institutions (Hamamatsu University School of Medicine, RIKEN Center for Developmental Biology, and Nagoya University Graduate School of Medicine), and its procedures conformed to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants before molecular genetic studies. Ten RP patients who carried homozygous or compound heterozygous mutations in the *EYS* gene were clinically re-evaluated at either the Department of Ophthalmology, Hamamatsu University Hospital in Hamamatsu (by YH); the Department of Ophthalmology, Kobe City Medical Center General Hospital in Kobe (by MT); or the Department of Ophthalmology, Nagoya University Hospital in Nagoya (by MK and SU).

Patients and Clinical Evaluation

The study subjects were ten unrelated Japanese RP patients residing in various geographical regions, ranging from Tokyo to Osaka. The cohort comprised nine unrelated patients with previously-reported homozygous or compound heterozygous *EYS* mutations[8] and one patient with a homozygous c.4957_4958insA mutation (RP115N). The doctors were asked to inquire about the family history of patients in as much detail as possible, and they confirmed that the parents of the patients with homozygous mutations were not consanguineous. The complete history and medical records of all the patients were reviewed. In addition, the patients were also clinically evaluated by the measurement of the best-corrected visual acuity, slit-lamp biomicroscopy, and ophthalmoscopy after pupillary dilatation. Refraction was determined using an autorefractometer. Additional examinations included fundus photography and Goldmann kinetic perimetry (targets, V-4e, III-4e, and I-4e to I-1e) to assess the size and extent of the visual field and spectral-domain optical coherence tomography (OCT; Spectralis, Heidelberg Engineering, Heidelberg, Germany or Cirrus, Carl Zeiss Meditec Inc., Dublin, CA, USA), to visualize the *in vivo* retinal architecture. Electroretinograms (ERGs) were recorded according to the protocol set by the International Society for Clinical Electrophysiology of Vision.[9]

Goldmann visual fields were scanned with a Canon or Epson scanner and analyzed using the ImageJ software (available at http://rsbweb.nih.gov/ij/) in the following manner: transparent layers were added to each field, and the isopters of the visual fields were manually traced onto these layers. The areas of fields that were circular or elliptical were calculated using the appropriate equations, while those with other irregular forms were calculated using ImageJ. Further, the area of the fields for the V-4e and I-4e targets were measured and compared with the normal area.[10]

Mutation Analyses

Genomic DNA of one proband, RP115N, was extracted from the peripheral lymphocytes by using standard procedures. All 44 exons of *EYS* and their flanking sequences were studied initially. DNA was amplified by PCR. The PCR and sequencing procedures used have been described previously.[8]

P21H was homozygous for a deletion in exon 32 of the *EYS* gene, which is an in-frame deletion that results in the replacement of amino acids from D2142 to S2191 with G2142 (p.D2142_S2191delinsG).[8] To precisely determine the deletion breakpoints, PCR amplification was performed using a

specially-designed primer pair: forward primer 5′-ATGGCTGTAGGAAACAATACAATGA-3′, located in intron 31, and reverse primer 5′-TTACTTCCAA ATTTCATGGTCATCT-3′, in intron 32 (see the Supplementary Figure – available online). Direct sequencing analysis was performed using the following primers: forward primer, 5′-ATAGATTC AATGCCATCCCCATCAAGCT-3′ and reverse primer, 5′-TGAGAAGTGTCTGTTCATATCCTTCA-3′ (Supplementary Figure). The amplification conditions were as follows: PCR was performed using the KOD FX PCR kit (TOYOBO, Japan) for 35 cycles at 98 °C for 10 s, 60 °C for 30 s, and 68 °C for 18 min in an automated thermal cycler.

RESULTS

Clinical and functional findings are summarized in Table 1. The patients' ages ranged from 14–37 years at the time of initial diagnosis (average, 31 years), while their ages at the time of initial examination for this study ranged from 34–74 years (average, 53 years). The patients were from diverse geographical regions, ranging from Tokyo to Osaka in Japan. Six patients' pedigrees were compatible with a recessive mode of inheritance, while the remaining four were considered isolated cases (data not shown). All ten patients had night blindness, with age at onset ranging from childhood to age 50 years (median, 17 years).

Mutation Analysis

A p.D2142_S2191delinsG mutation was detected by PCR by using a specially designed primer in the severest case (patient RP21H). In brief, after failing to amplify exon 32 in this case, we hypothesized that the patient may have homozygous deletion of a long genomic region, including exon 32. We successfully obtained an amplified product by using a primer pair, of which one (forward) was in intron 31 and the other (reverse) was in intron 32 (Supplementary Figure). Sequence analyses showed that the amplified DNA contained truncated intron 31 and truncated intron 32. The boundary between truncated intron 31 and 32 had a 58-nucleotide sequence string, GGGCAA...ATTGAC. We could not determine the precise breakpoints in both introns because the exact sequence identity around possible breakpoints could not be delineated; however, the deletion size was elucidated to be 12197 nucleotides, irrespective of the position of the break. We denoted the deletion as c.6425-?_6571+?del, as per the nomenclature guidelines provided by the Human Genome Variation Society (http://www.hgvs.org/mutnomen/).

Visual Acuity

Clinical examination revealed that corrected visual acuity varied considerably and ranged from 0 logMAR unit to light perception; the differences were attributable to the different stages of macular involvement (Table 1 and Figure 1). From the findings, a common pattern emerged: relatively well-preserved visual acuity up to the 3rd or 4th decade, with subsequent rapid deterioration to less than 1.0 logMAR unit at approximately 60 years of age. This pattern was noted in all cases, except one eye (left) of the youngest patient (patient RP44K; Table 1) who had visual acuity of counting fingers at the age of 34 years. Patient RP21H exhibited complete deletion of exon 32, which was the severest phenotype with vision being limited to light perception. The visual acuity of patients homozygous for the c.4957_4958insA mutation was of a uniform phenotype.

Refractive Error

Twelve eyes were ametropic; three exhibited slight hyperopia, and nine had various degrees of myopia ranging from −1 D to −6 D. The remaining eight eyes were emmetropic. Patients with severe forms of myopia (over −8 D), hypermetropia (over +4 D), or astigmatism (over 3 D) were not found in this study.

Visual Fields

Constriction of the visual fields was symmetric, except for one patient who had no testable visual field in the central area or any targets in one eye (left eye of patient RP44K; Table). The extent of vi constriction correlated with age (Table 1 and Figure 1B). The visual field at the time of the study ranged from constriction to 10–12° for the V-4 target in a 63-year-old man (RP87N) to no light perception for the V-4 target in a 55-year-old man (RP21H). Figure 1B shows that the disease progressed relatively rapidly over the 3rd and 4th decades.

Cataracts and Anterior Segment Abnormalities

Cataract was observed in seven of the ten affected subjects, including four individuals who had pseudophakia (seven eyes).

Retinal and Macular Findings

Retinal changes were relatively uniform among the subjects. The optic disc appeared to be relatively

TABLE 1. Genotypes and phenotypes in Japanese patients with mutations in the *EYS* gene.

										Visual Acuity		Refraction					
Patient	Age at onset (years)	Age at first visit (years)	Current age (years)	Follow-up duration (years)	Gender	Origin	Family history	Type of change	Nucleotide change	Right Eye	Left Eye	Right Eye (D)	Left Eye (D)	Lens Status	Goldmann Perimetry (V-4e)	Fundoscopy Results	OCT Results	ERG Results
RP3H	14	35	67	12	Female	Tochigi	ar	Homozygous	c.4957_4958insA[8,12]/ c.4957_4958insA [8,12]	0.07	0.08	+1	+1.25	Bilateral pseudophakia	OD: 8°; OS: 7°	Waxy optic disc, attenuated retinal vasculature, RPE changes in the periphery, extensive bone spicules throughout periphery	No data	Extinguished
RP21H	20	35	55	17	Male	Hamamatsu	ar	Homozygous	c.6425-?_6571+?del[8]/ c.6425-?_6571+?del[8]	LP	LP	−3.0	−3.0	Bilateral pseudophakia	Unmeasurable	Waxy optic disc, attenuated retinal vasculature, RPE changes in the periphery and posterior pole, extensive bone spicules throughout periphery	Relatively preserved foveal lamination	Extinguished
RP35K	20	30 s	39	3	Male	Toyooka	iso	Homozygous	c.8868C>A[8,12]/ c.8868C>A[8,12]	1	1	−6.0	−5.25	Clear	OD, OS: concentric constriction with remaining central island; remaining peripheral island. P0	Normal optic disc, slightly attenuated vessels, RPE changes in the periphery, bone spicules sprinkled throughout periphery	No data	No data
RP44K	14	14	34	4	Female	Tokyo	ar	Heterozygous/ Heterozygous	c.4957_4958insA[8,12]/ c.6557G>A[4,7,8]	0.4	CF	+0.5	0	Bilateral cataract	OD: concentric constriction with remaining central island. OS: concentric constriction with remaining peripheral island	Normal optic disc, attenuated retinal vasculature, RPE changes in the periphery and posterior pole, extensive bone spicules	No data	Extinguished

																throughout periphery		
RP48K	13	26	36	2	Male	Osaka	iso	Homozygous	c.4957_4958insA[8,12]/ c.4957_4958insA [8,12]	0.6	0.6	−1.25	−1.0	Clear	OD: 6°; OS: 7°	Normal optic disc, slightly attenuated vessels, RPE changes in the periphery and posterior pole, extensive bone spicules throughout periphery	No data	No data
RP54K	20 s	30	53	2	Male	Himeji	ar	Homozygous	c.4957_4958insA[8,12]/ c.4957_4958insA [8,12]	0.7	0.3	−2.25	−1.5	Right cataract Left pseudophakia	OD: 8°; OS: 8°	Waxy optic disc, attenuated retinal vasculature, RPE changes in the periphery and posterior pole, extensive bone spicules throughout periphery	Foveal thinning	No data
RP56K	20	37	74	5	Male	Tokyo	ar	Compound Heterozygous	c.4957_4958insA[8,12]/ c.8351T>G[8]	0.04	0.2	−1.5	−1.5	Bilateral pseudophakia	OD: 8°; OS: 9°	Waxy optic disc, attenuated retinal vasculature, RPE changes in the periphery and posterior pole, extensive bone spicules throughout periphery	Foveal thinning	No data
RP81K	Childhood	20	51	2	Female	Higashi-Osaka	ar	Compound Heterozygous	c.2522_2523insA[8]/ c.6557G>A[4,7,8]	0.6	0.6	0	+1.5	Bilateral cataract	OD: 11°; OS: 11°	Waxy optic disc, attenuated retinal vasculature, RPE changes in the periphery and posterior pole, some drusen-like deposits	No data	No data
7N	50*	44*	64	8	Male	Nagoya	iso	Heterozygous/ Heterozygous	c.4957_4958insA[8,12]/ c.7793G>A[8]	1	0.8	0	−1.0	Bilateral cataract	OD: 10°; OS: 10°	Normal optic disc, attenuated retinal vasculature, RPE changes in the	No data	No data

(*continued*)

TABLE 1. Continued.

Patient	Age at onset (years)	Age at first visit (years)	Current age (years)	Follow-up duration (years)	Gender	Origin	Family history	Type of change	Nucleotide change	Visual Acuity		Refraction		Lens Status	Goldmann Perimetry (V-4e)	Fundoscopy Results	OCT Results	ERG Results
										Right Eye	Left Eye	Right Eye (D)	Left Eye (D)					
																periphery, extensive bone spicules throughout periphery		
RP115N	6–12 years	30 s	52	7	Female	Aichi	iso	Homozygous	c.4957_4958insA[8,12]/ c.4957_4958insA [8,12]	0.6	0.6	0	0	Clear	OD, OS: concentric constriction with remaining central island; remaining peripheral island	Waxy optic disc, attenuated retinal vasculature, RPE changes in the periphery, extensive bone spicules throughout periphery	No data	No data

* Subject RP87N was diagnosed with retinitis pigmentosa by fundoscopy at the age of 44 years, when he was asymptomatic; thereafter, he developed night blindness at the age of 50 years.
"Age at Onset" was based on history and "Age at First Visit" was based on medical records.
Tochigi, Tokyo, Osaka, and Aichi are prefectures and Hamamatsu, Toyooka, Himeji, Higashi-Osaka, and Nagoya are cities.
References for previously-reported mutations are indicated in the column labeled "Nucleotide Change".
All clinical data were obtained from the latest examinations, but the refraction of the eyes that underwent cataract surgery was assessed on the basis of the latest phakic data.
ar, autosomal recessive; iso, isolated case; D, diopter; LP, light perception; CF, counting fingers; OD, oculis dextra (right eye); OS, oculis sinistra (left eye); OCT, optical coherence tomography; ERG: electroretinogram

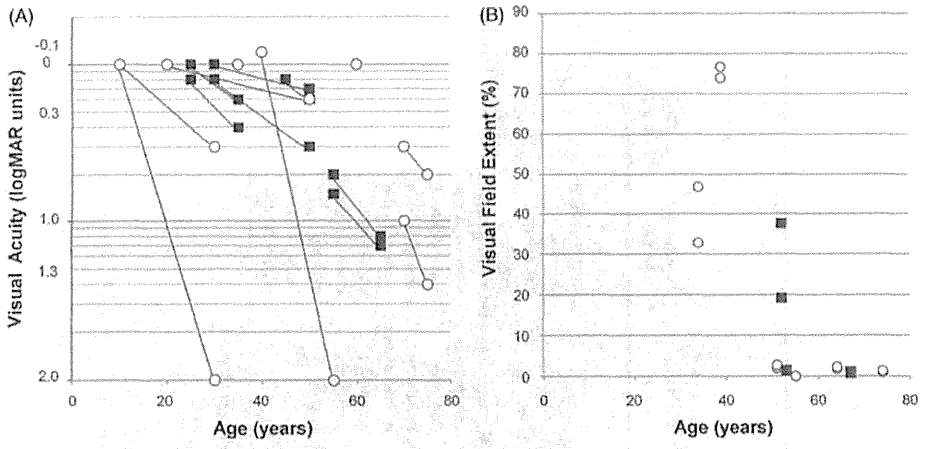

FIGURE 1. (A) Visual acuity was expressed in logMAR units, as a function of age of the subjects. The graph shows a decrease in visual acuity (y-axis) with age in years (x-axis). Symbols indicate the time points of assessment. ■, subjects with homozygous c.4957_4958insA mutation; ○, other subjects. The visual acuity was preserved well into the thirties or forties, after which it declined. Visual acuity of subjects with the homozygous c.4957_4958insA mutation was uniform. (B) The extent of the visual field (kinetic perimetry with V-4e test target) loss was expressed as a percentage of the normal mean and plotted as a function of age in the ten subjects. ■, subjects with the homozygous c.4957_4958insA *EYS* mutation; ○, other subjects. The extent of visual constriction correlated with age.

well-preserved, and compared to the venules, the arterioles showed mild to moderate attenuation. Profound atrophy of the retinal pigment epithelium (RPE), choriocapillaris, and outer segment at the mid-peripheral retina were observed in all patients. In the later stages of the disease, the macular region and, sometimes, the fovea were abnormal. We also observed varying amounts of bone spicule-like pigmentation dispersed in the posterior pole, mid-periphery, and anterior portions of the fundus; the deposits were more prominent in the older patients, but were detected in all the cases in mid-peripheral retina (Figure 2A, B, C). Only one patient had a history of cystoid macular edema (CME) (10%) at 66 years of age; this frequency is less than that reported-previously.[13]

OCT Images

High-resolution OCT images showed a marked reduction of retinal thickness resulting from the loss of photoreceptor layers (Figure 2D, E, F). The photoreceptor inner segment/outer segment junction (IS/OS line) was either completely absent or only detectable at the fovea in four subjects. Although patient RP21H, with complete deletion of exon 32, had light perception in both eyes, the OCT image demonstrated relatively preserved foveal structures, including the IS/OS line (Figure 2D).

ERG Recordings

ISCEV-standard full-field ERGs were recorded for three patients [patients RP3H (at the age of 59 years), RP21H (at the age of 41 years), and RP44K (at the age of 27 years)] and were nearly undetectable for all patients in both rod and cone components.

DISCUSSION

In this report, we describe the phenotype of ten unrelated Japanese patients affected with arRP caused by *EYS* gene mutations. Our previous study on 100 Japanese arRP patients indicated very likely pathogenic mutations and possible pathogenic mutations in 18% (18/100) and 8% (8/100), respectively, of the study population; these values are higher than those previously reported.[4–7] Our previous study has shown that 16% of Japanese patients with arRP displayed either the c.4957_4958insA or the c.8868C>A mutation, which accounted for 57% (15+5/35) of the mutated alleles and seem to be frequent among Japanese patients with arRP.[8] However, a detailed haplotype analysis of the *EYS* gene has not been performed, and therefore, currently, we cannot verify whether each mutation occurred in an ancient common ancestor. This high prevalence of *EYS* gene mutations, including two frequent mutations, has recently been confirmed by another study in the Japanese population.[12] Together, these findings strongly suggest that *EYS* gene mutations play a major role in the pathogenesis of arRP affecting the Japanese population. In this study, we only recruited patients with very likely pathogenic mutations and involvement of both alleles because a second mutant allele could not be detected by direct sequencing in 17/26 patients in the previous study, and the genotype of such patients could not be determined.

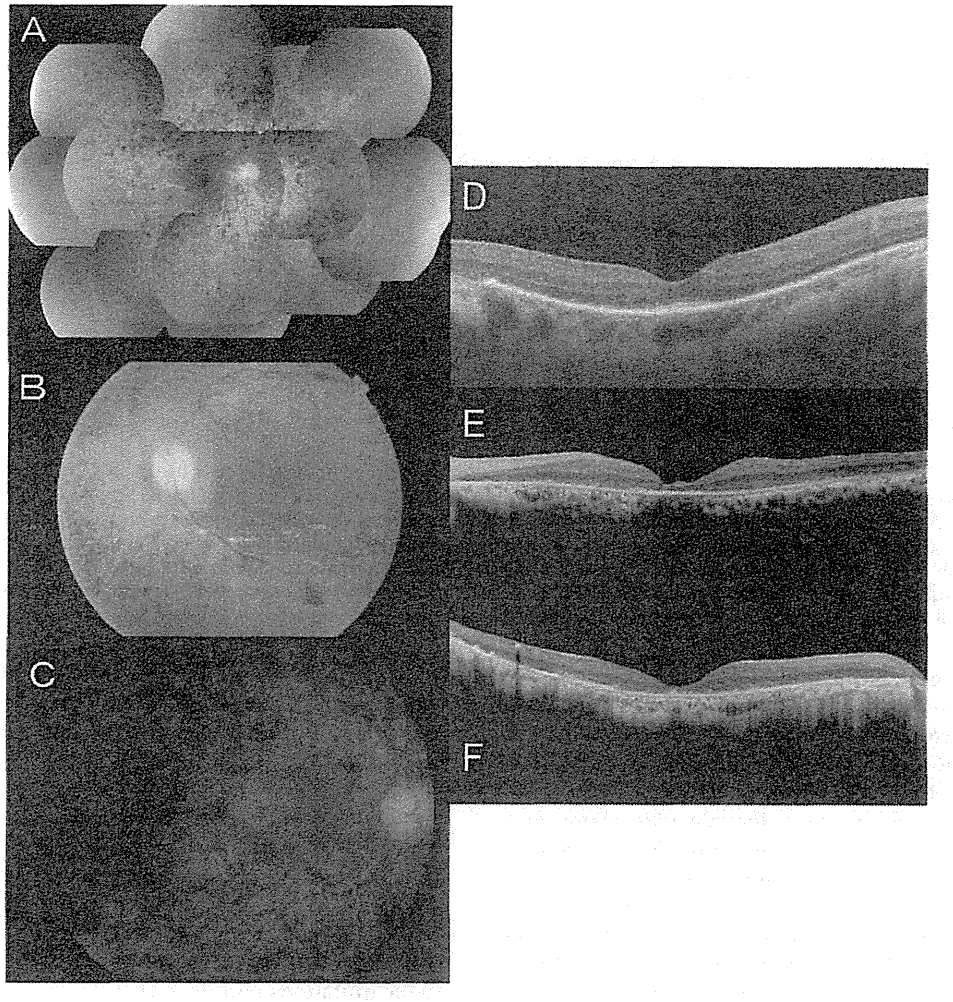

FIGURE 2. Fundus photographs of patients carrying mutations in the *EYS* gene. The entire retina of the right eye of patient RP21H at age 55 years showed extensive bone-spicule pigmentation throughout the fundus (A). The central retina of the left eye of patient RP54K, aged 51 years, showed a waxy optic disc, severely attenuated retinal vasculature, and extensive chorioretinal atrophy with minimal residual retinal pigment epithelium in the macular region (B). The central retina of the right eye of patient RP56K (age, 73 years) showed the fundus appearance of the end-stage of the disease (C). Illustration of macular changes by spectral-domain optical coherence tomography showed a marked reduction of retinal thickness. Relatively preserved foveal lamination was observed in the right eye of patient RP21H at the age of 55 years (D). Marked foveal thinning was observed in the left eye of patient RP54K at the age of 51 years (E) and in the right eye of patient RP56K at the age of 73 years (F).

The genotype includes truncated mutations and few missense mutations (Supplementary Table). No patient with missense mutations in both alleles was included. We recruited four patients with the homozygous c.4957_4958insA mutation and one patient with the homozygous c.8868C>A mutation.

Overall, most patients showed relatively well-preserved visual acuity into their thirties, after which rapid deterioration was observed in their forties or fifties. The constriction of the visual fields was symmetric, although the extent seemed to correlate better with age than visual acuity. Cataract was frequently observed among patients in their thirties. In one case (RP21H), visual acuity improved noticeably from 0.5 logMAR units to −0.1 logMAR units in both eyes after cataract extraction by the age of 37 years, but gradually progressed to light perception by the age of 40 years. In all cases, the fundus displayed typical changes of retinitis pigmentosa (RP), including attenuated retinal vessels and bone-spicule deposits over 360° of the fundus, all of which increased in density with age. Electroretinographic (ERG) responses were consistent with severe generalized rod–cone dysfunction.

Reports have been published on the phenotype of RP caused by *EYS* gene mutations in Indonesian, Pakistani, Chinese, Israeli, Spanish, French, British, Dutch, and Palestinian patient populations[4–7,13–16],

these reports contain only a brief description of the clinical features. The subjects in this study shared a relatively uniform phenotype, characterized by a symptom-free interval in the first two decades of life (median age at onset, 23 years) followed by a rapid decline in visual function. The patient RP87N was diagnosed with RP by funduscopy at the age of 44 years, when he was subjectively asymptomatic; later, he developed night blindness at the age of 50 years. The clinical features reported in this study are consistent with those reported previously. For instance, the visual acuities were relatively well-preserved up to the 3rd or 4th decades, as reported in a study of a population of European ancestry.[7] The visual acuities were relatively better and photophobia less frequent than that in seven Spanish arRP subjects carrying mutations of the ceramide kinase-like (*CERKL*) gene, which is involved in sphingolipid-mediated apoptosis in the retina.[17] The visual field loss noted among our study population was less than that reported for patients with mutations of the dehydrodolichyl diphosphate synthase (*DHDDS*) gene, which encodes an enzyme required for dolichol-pyrophosphate synthesis.[18]

To date, extensive studies focusing on the clinical features of a large number of RP patients with *EYS* gene mutations have been limited to Dutch and French arRP cohorts.[5,7] However, these studies included only a few non-European patients and showed a change in visual acuity with age in patients carrying *EYS* gene mutations; this was similar to the pattern noted in our Japanese subjects.

X-linked RP has been associated with myopia of 2 diopters or more, whereas dominant inheritance is associated with hyperopia.[19–21] In this study, 12 of the investigated eyes were ametropic (three slightly hyperopic and nine with various degrees of myopia) and eight were emmetropic. Myopia has a greater prevalence in Asian countries, including Japan, compared to Western populations.[22] Thus, our data together with those of a previous French study[5] suggest that most patients with arRP due to *EYS* mutations may have no or mild (mostly myopic) refractive error.

Three severe cases with retinal atrophy involving the posterior pole have been described previously (Family A: II-3[3], CIC01223[5] and CIC00492[5]);[3,5] however, most patients in this study presented with typical signs of progressive rod–cone dystrophy with relatively well-preserved central vision until late in the course of the disorder. It is plausible that a larger cohort of Japanese patients with *EYS* mutation may yield patients with greater involvement of the cones than rods. Two cases of sector RP with distinct fundus abnormalities with predominance of pigmentary changes in the inferior part of both retinas have been reported previously (CIC01222[5] and MOL0640 III:1[13]).[5,13] Although none of our patients had sector RP at the time of the study, we cannot rule out its presence in the earlier stages of the disease, before the patients were enrolled in this study. It is difficult to establish a clear genotype–phenotype association since we could only investigate ten patients.

We recruited six homozygous patients for this clinical and molecular study. Among them, four patients (RP3H, RP48K, RP54K, and RP115N) were homozygous for the c.4957_4958insA mutation (p.S1653KfsX2; truncating mutation in exon 26), which, as shown in our previous report, is a major causative mutation of RP in Japan. The course of visual acuity changes in homozygous patients with the c.4957_4958insA mutation was uniform. However, no remarkable clinical pattern emerged among the ten patients with variable genotypes, including the c.4957_4958insA mutation, in this study. The patients had near-normal visual function up to their twenties; this implies that slowing the progression of degeneration may be a possible therapeutic approach for preventing blindness in RP patients.

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DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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CLINICAL CASE REPORT

Case of paraneoplastic retinopathy with retinal ON-bipolar cell dysfunction and subsequent resolution of ERGs

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Abstract
Purpose To report a patient with cancer-associated retinopathy and retinal ON-bipolar cell dysfunction who had a resolution of the electroretinograms (ERGs) after a resection of an ovarian cancer and chemotherapy.
Case report A 71-year-old Japanese female patient visited us complaining of night blindness and photopsia in both eyes for 6 months. Her visual acuity was 20/20 in both eyes, and fundus examination, fluorescence angiography, and optical coherence tomography showed no abnormalities in both eyes. The rod responses of the ERGs were absent and bright-flash ERGs were electronegative. The ON responses of the focal macular ERGs and full-field long-flash ERGs were absent. These ERG findings indicate an ON-bipolar cell dysfunction. A general physical examination revealed the presence of ovarian cancer. After resection of the ovarian cancer and adjuvant chemotherapy, the ERGs of the left eye completely recovered within 2 years and those of right eye recovered subsequently. The autoantibody against transient receptor potential melastatin 1 (TRPM1) was not detected in the serum.
Conclusion Our case demonstrates that retinal ON-bipolar dysfunction can be caused by ovarian cancer. Our case indicates that some autoantibodies against other than TRPM1 might cause transient dysfunction of retinal ON-bipolar cells.

Keywords Paraneoplastic retinopathy · Retinal ON-bipolar cell · Melanoma-associated retinopathy · ERG · Transient receptor potential melastatin 1 (TRPM1) · Cancer-associated retinopathy

Introduction

Paraneoplastic retinopathy (PR) is a progressive disorder of the retina caused by the effects of remote neoplasms. Many of these retinopathies are associated with circulating anti-retinal autoimmune antibodies. PRs are characterized by a sudden and progressive decrease in the function of the retina. One subtype of PRs has been reported to be caused by an autoantibody against a protein expressed by retinal ON-bipolar cells. This PR is caused by a melanoma in most cases and is called melanoma-associated retinopathy (MAR). MAR patients often complain of a subacute onset of night blindness and photopsia in both eyes [1].

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The visual acuity at presentation is relatively good in most cases, and ophthalmoscopy shows optic nerve pallor and retinal arteriolar attenuation, but generally the fundus appears normal [2].

The electroretinographic (ERGs) findings are essential for a diagnosis of MAR [3–5]. The scotopic ERGs elicited by a bright flash have a selective reduction in the b-waves with normal a-waves. These alterations result in a waveform called a negative-type ERG, which is one of the characteristics of retinal ON-bipolar dysfunction. The symptoms and ERG findings seldom recover although improvements following treatment has been reported [6–9]. The reason why this type of PR is almost exclusively associated with melanomas has not been determined, and there have been only three English publications which reported that cancers other than melanomas were associated with the retinal ON-bipolar cell dysfunction [6, 10, 11].

We report a patient whose ERG findings were suggestive of MAR, but the underlying neoplasm was ovarian cancer. After a resection of the ovarian cancer and subsequent chemotherapy, the patient's symptoms and ERGs gradually recovered in both eyes. We report the time course of ERG recovery.

The procedures used in this study were approved by the Institutional Review Board Committee of Nagoya University Graduate School of Medicine (Approval number 1131-2). All of the procedures conformed to the tenets of the Declaration of Helsinki. A written informed consent was obtained from all the patients after they were provided with information on the procedures to be used.

Case report

A 71-year-old Japanese female patient was examined at the Nagoya University Hospital in August 2011 with complaints of shimmering photopsia and night blindness of 6 months duration in both eyes. She had no past medical history except cataract surgery on both eyes. Her visual acuity was 20/20 in both eyes. Ocular examinations including slit-lamp and fundus examinations showed that the fundus appeared normal in both eyes (Fig. 1a, b). Fluorescence angiography showed no particular abnormalities (Fig. 1c, d). OCT showed a thin epiretinal membrane on the fovea of the right eye (Fig. 1e), but no distortion or thinning of any retinal layer was detected in both eyes (Fig. 1e, f).

The retinal function was assessed by ERGs. Full-field ERGs recorded according to ISCEV standard (Fig. 2), focal macular ERGs (Fig. 3) and full-field long-duration ERGs (Fig. 3) were recorded. Focal macular ERGs were elicited from stimulating a 15° area of the macula, and the location of the stimulus was monitored with an infrared fundus camera (ER-80, Kowa, Nagoya, Japan) [12–14]. The luminance of the stimulus spot was 30 phot cd/m^2, and the stimulus duration was 100 ms. The luminance of the background was fixed at 3 phot cd/m^2. Five hundred responses were averaged for each recording. For the full-field long-duration ERGs (UTAS Visual Diagnostic System, LKC Technologies, Inc, MD, USA), the luminance of the stimulus was 200 phot cd/m^2 obtained from a white LED, and the stimulus duration was 150 or 200 ms. The luminance of the background was fixed at 30 phot cd/m^2. The ERGs were picked up by a Burian-Allen bipolar contact lens electrode (Hansen Ophthalmic Development Laboratories, Iowa City, IA, USA), and 30 responses were averaged.

Full-field ERGs were recorded according to the ISCEV standard from the right eye, and focal and full-field long-flash ERG were recorded from the left eye in August 2011. The ERGs were recorded from right and left at different times to try to minimize the chances of cornea abrasions that can arise from long recording sessions.

The full-field ERGs recorded from the right eye in August 2011 are shown in Fig. 2. The scotopic ERGs (rod responses) elicited by dim flashes were extinguished. Normal a-wave and reduced b-wave, and an electronegative-type ERG were recorded by bright-flash stimuli in scotopic condition. The cone ERGs had a wide a-wave trough and slightly reduced b-wave. The focal macular ERGs and photopic long-duration ERGs recorded from the left eye are shown in Fig. 3a and b, respectively. The ON responses of both types of ERGs were severely reduced, but the OFF responses were normal. The ERG waveforms of the both eyes resembled those recorded from eyes with the complete type of congenital stationary night blindness (cCSNB) [15]. This indicated the retinal ON-bipolar cell dysfunction in both eyes. Melanoma-associated retinopathy was highly suspected despite the absence of a history for a melanoma.

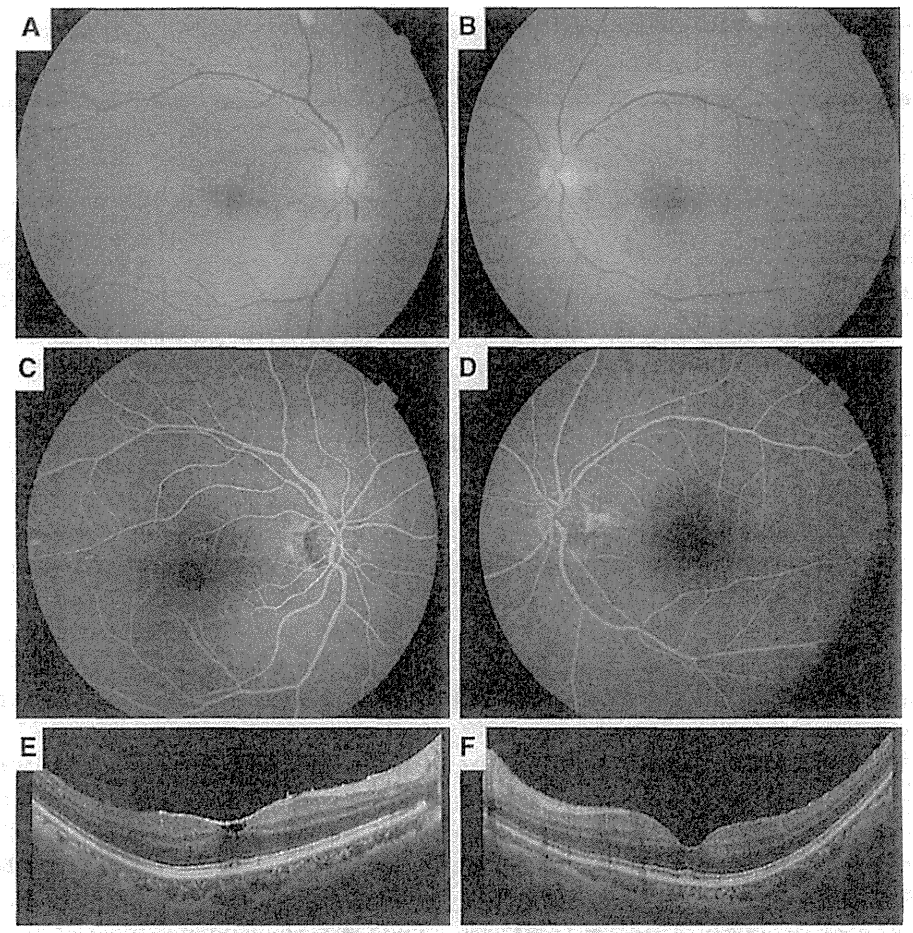

Fig. 1 Fundus photograph of right (**a**) and left (**b**) eyes. Fluorescein angiogram of right (**c**) and left (**d**) eyes. Spectral domain OCT of right (**e**) and left (**f**). No distinct abnormalities were found except an epiretinal membrane on the fovea of the right eye by OCT

A general physical examination did not find any melanoma, but positron emission tomography (PET) and computed tomography (CT) revealed ovarian cancer. In September 2011, the ovarian cancer and metastatic lymph nodes were resected. Histopathological examination showed that the tumor was a serous adenocarcinoma grade 3. After surgery, she had adjunctive chemotherapy with carboplatin and paclitaxel until December 2011.

We also examined whether autoantibody against transient receptor potential melastatin 1 (TRPM1) was present in her serum by Western blot analysis as reported [11, 16], but it was negative.

We followed the ERGs after surgery. Full-field ERGs recorded showed an ON-bipolar cell dysfunction in both eyes in October 2011 (Fig. 2). However, approximately 1 year after the surgery, the patient felt that the night blindness and photopsia was no longer present in her left eye. ERG tests were repeated. The ERG of the left eye had recovered to within the normal range, but the ERGs of the right eye had not changed in November 2012 (Fig. 2). The patient reported a recovery from the night blindness in the right eye at the beginning of 2013. The ERGs of the right eye were slightly improved in May 2013; rod responses were detected and the amplitude of the photopic a-wave was smaller. Nevertheless, the ERGs elicited by bright flashes still had a negative waveform and had not fully recovered until March 2014. The focal macular ERGs recorded in January 2013 were almost normal for the left eye, but the b-wave was not present in the right eye. The focal macular ERGs of the right eye also improved, and the b-wave become prominent in August 2013 (Figure 3a). Long-duration ERGs

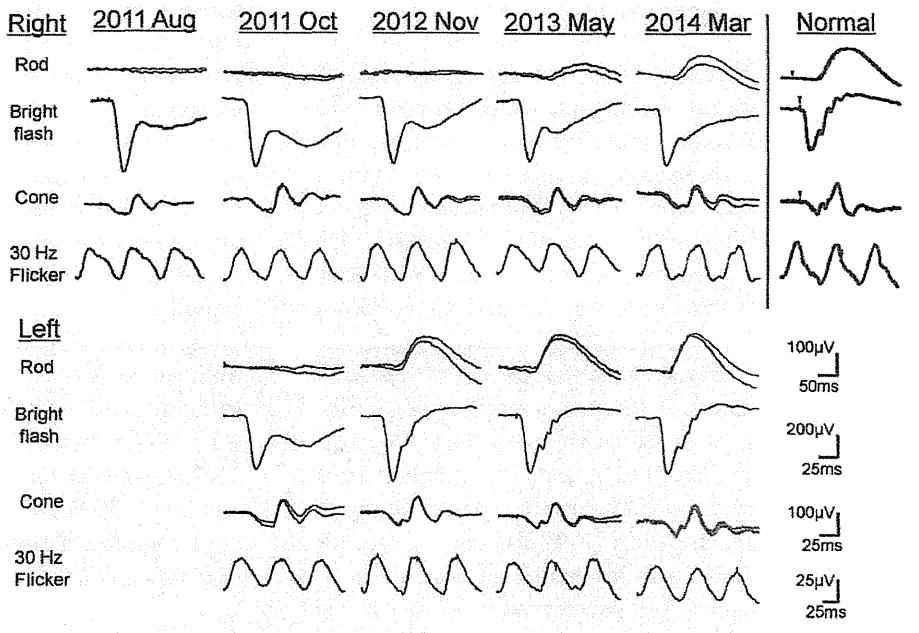

Fig. 2 Time course of full-field ERGs recorded according to the ISCEV protocol. Full-field ERGs were recorded in August 2011, October 2011, November 2012, May 2013, and March 2014. In August 2011, the ERGs of only the right eye were recorded. Data of a representative normal eye are shown in the *upper right*

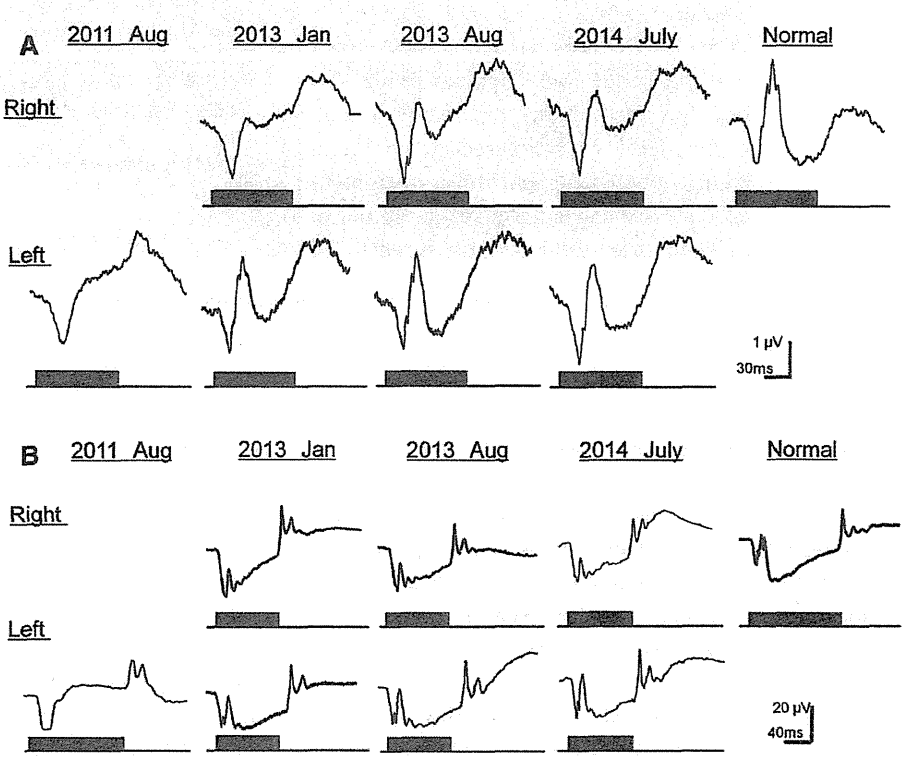

Fig. 3 Time course of focal macular ERGs (a) and full-field long-duration ERGs (b). ERGs were recorded in August 2011, January 2013, August 2013, and July 2014. In August 2011, the ERGs of only the left eye were recorded. Data of a representative normal eye are shown in the *upper right* of each panel. *Black boxes* indicate stimulus duration

recorded in January and August 2013 had distinct b-waves in both eyes, but that of left eye was much larger than that of the right eye (Figure 3b).

At present (September 2014), the patient has not had recurrences of the cancer or return of the visual symptoms.

Comments

Our case of PR had two distinct features; one is that the neoplasm was not a melanoma which has been shown to cause retinal ON-bipolar cell dysfunction. The other feature is the recovery of the ERGs after the treatment of cancer. A search of PubMed extracted one paper of a patient with oat-cell carcinoma [10], one with lung small cell carcinoma [11], and another with breast cancer [6]. These cases were reported to be PR with retinal ON-bipolar dysfunction caused by other than melanoma. Our case is the first case of PR caused by an ovarian cancer. In addition, a recovery of the ERGs in PR patient with retinal ON-biopolar cell dysfunction has been described in only four patients [6–9]. The ERGs of three MAR cases recovered slowly just as they did in our patient [7–9]. But one PR case with breast cancer [6] was different from our case in two ways; the patient had severe vasculitis and a rapid recovery of the ERG in both eyes within 4 months after chemotherapy.

Our patient had ON-biopolar cell dysfunction in both eyes, and the ERGs had a typical retinal ON-bipolar dysfunction. One interesting finding was that the ERGs of the left eye recovered to within the normal range in about 1 year, but those of right eye recovered more slowly and did not reach the normal range even 2 years after the resection surgery. The mechanism for the different speed of recovery was not determined.

PRs with retinal ON-bipolar cell dysfunction is assumed to be caused by an autoantibody against ON-bipolar cells according to the findings made in MAR patients. However, one question is why neoplasms other than melanomas cause the ON-bipolar cell dysfunction. Recently, our group and another group reported that the transient receptor potential melastatin 1 (TRPM1) protein was one of the antigens for the autoantibody against the ON-bipolar cells in PR patients [11, 17]. TRPM1 is a member of the TRPM subfamily of TRP proteins and is a calcium channel expressed on retinal ON-bipolar cells and on melanocytes. TRPM1 was originally discovered as a melanocyte-specific gene that is silenced in aggressive melanoma cells [18]. It was later found to be an ion-conducting plasma membrane channel of retinal ON-bipolar cells [19–21]. The expression of TRPM1 on both retinal ON-bipolar cells and melanocyte seemed to be suitable candidate for the antigen in MAR patients. The mechanism of retinal ON-bipolar dysfunction due to the autoantibody against TRPM1 has been shown by several groups. Thus, Xiong et al. [22] showed an uptake of TRPM1 autoantibodies by ON-bipolar cells, where they bind to an intracellular epitope of the channel which then reduces the ON-bipolar cell response to light. Our group showed that the intravitreal injection of the patient's serum including the TRPM1 antibody into mouse eyes caused a retinal ON-bipolar cell degeneration [16]. These studies indicated that retinal ON-bipolar cell dysfunction in eyes with PR is due to anti-TRPM1 antibody causing ON-bipolar degeneration which did not recover. In our case, the autoantibody against TRPM1 was not found and the symptoms and ERGs recovered. These results indicated that autoantibodies against another antigen caused the transient dysfunction of the retinal ON-bipolar cells in our patient.

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Conflict of interest None.

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