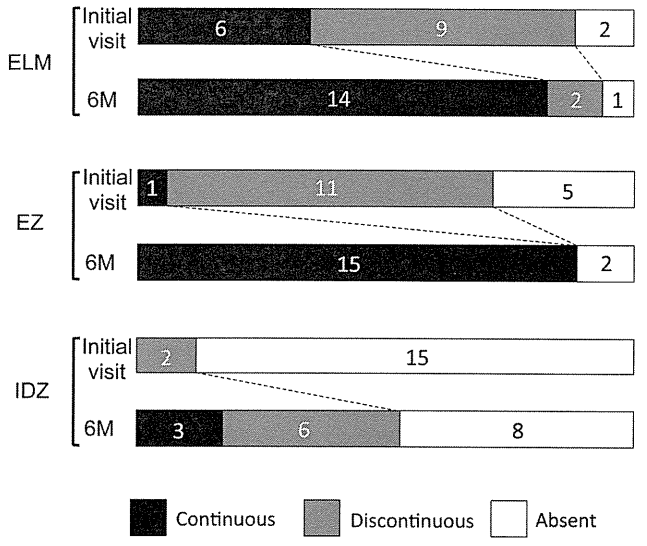

Figure 6. Summary of findings of the three highly reflective bands at the outer retina obtained by SD-OCT at the initial visit and six months later. These three lines were divided into three types; "continuous", "discontinuous" and "absent". ELM, external limiting membrane. EZ, ellipsoid zone. IDZ, interdigitation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

RIGHTSLINK

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

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

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)					
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

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															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*)

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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

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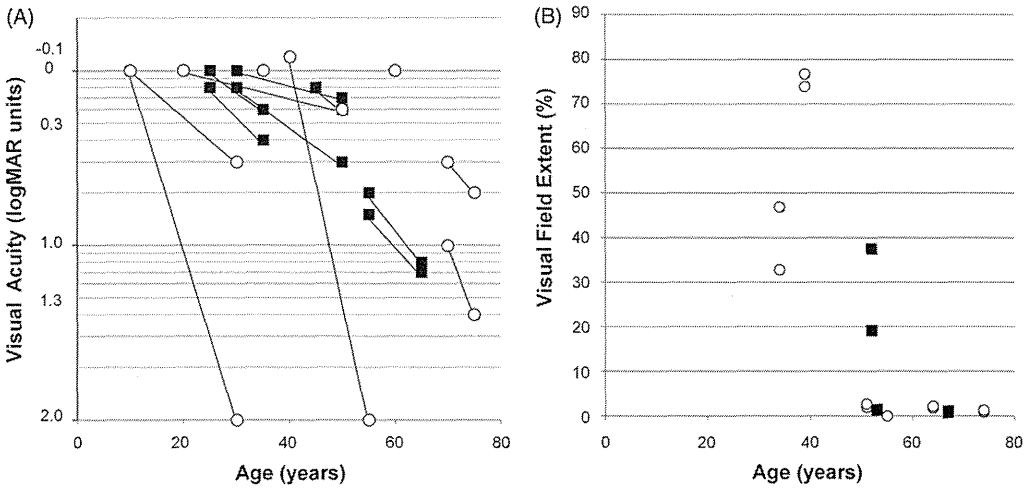

FIGURE 1. (A) Visual acuity was expressed in logMAR units, as a function of age of the subjects. The graph shows a decrease in visual acuity (y-axis) with age in years (x-axis). Symbols indicate the time points of assessment. ■, subjects with homozygous c.4957_4958insA mutation; ○, other subjects. The 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.

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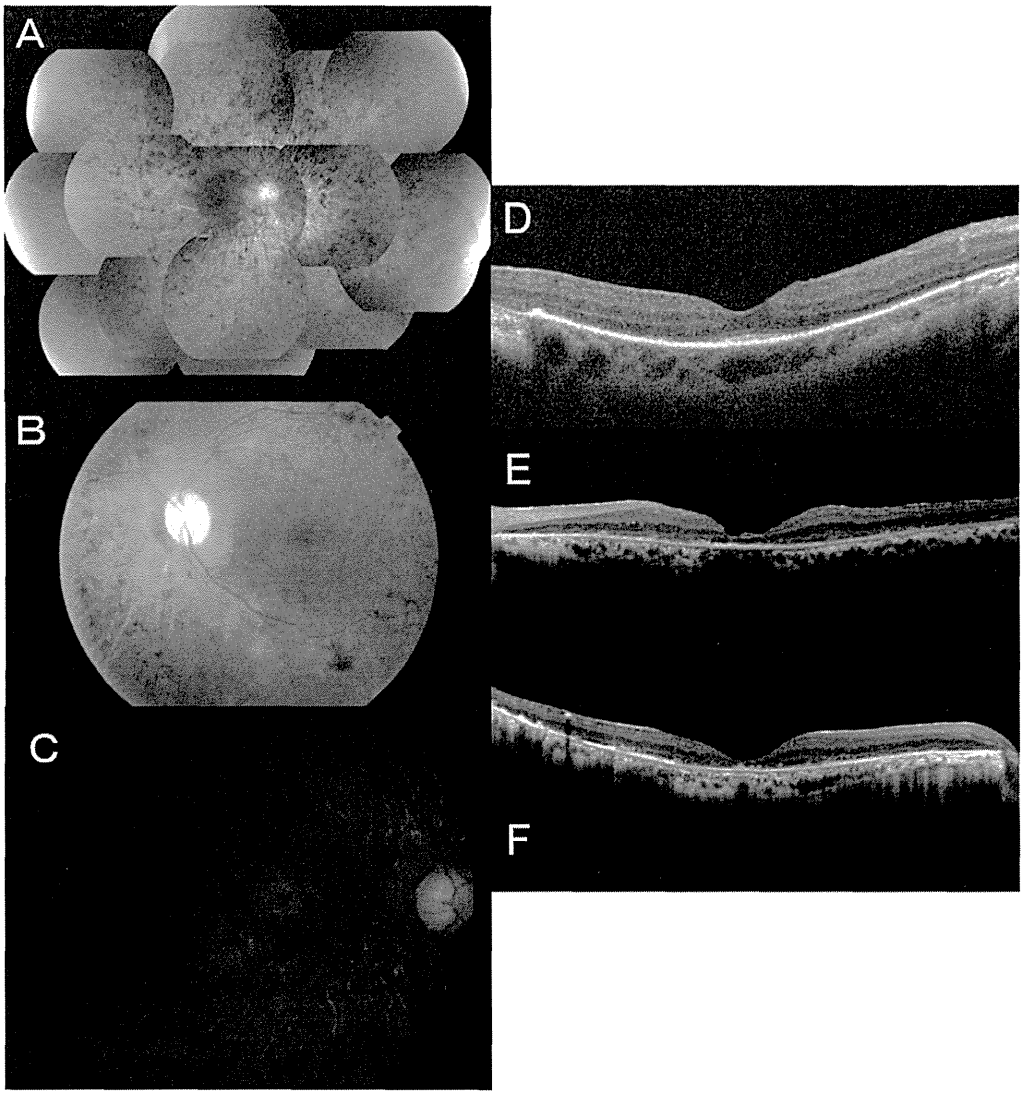

FIGURE 2. Fundus photographs of patients carrying mutations in the *EYS* gene. The entire retina of the right eye of patient RP21H at age 55 years showed extensive bone-spicule pigmentation throughout the fundus (A). The central retina of the left eye of patient RP54K, aged 51 years, showed a waxy optic 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];

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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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Changes in angle of optic nerve and angle of ocular orbit with increasing age in Japanese children

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ABSTRACT

Purpose To study changes in the opening angle of the optic nerve and the angle of the ocular orbit with increasing age in normal Japanese children.

Methods We studied 147 normal children (aged 6 months to 18 years) who had undergone CT as a diagnostic procedure. Measurements were performed on axial CT images that included the entire optic nerve of both eyes. The opening angle of the optic nerve was defined as the angle formed by the intersection of a line running through the left optic nerve and a vertical line passing through the centre of the nose. The opening angle of the orbit was defined as the angle formed by the intersection of a line running tangentially along the deep lateral wall of the left orbit and a vertical line passing through the centre of the nose. The relationship between age and these opening angles was analysed by regression analysis.

Results The correlation between age and opening angle of the optic nerve was not significant. In contrast, the opening angle of the orbit decreased relatively rapidly until about 2–3 years of age, and then it stabilised. The decrease in the opening angle of the orbit with increasing age was significant ($p<0.001$). The relationship between these two parameters was best fitted by a logarithmic regression curve.

Conclusions Because the opening angle of the orbit decreased significantly with increasing age, this factor must be considered when diagnosing and treating strabismus in children.

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INTRODUCTION

The morphology of the orbit is known to change during normal development. The angle of the orbit is wide during early fetal development and gradually becomes narrower as gestation progresses.[1] Examining normal developmental changes in orbital morphology after birth should provide important information on the management of strabismus in children with and without skull or orbital abnormalities.

There have been a number of studies on changes in the volume of the orbit and the diameter of the orbital opening during development.[2–6] However, there are only three studies on the opening angle of the optic nerve or the orbit.[7–9] Therefore, the purpose of this study was to determine changes in the opening angle of the optic nerve and orbit with increasing age using axial CT images in normal Japanese children.

SUBJECTS AND METHODS

Subjects

We studied 147 children (aged 6 months to 18 years) who had been examined at the Osaka Medical Center and Research Institute for Maternal and Child Health from 2008 to 2011. These children had undergone head CT because they had suffered a blow to the head or had headaches of unknown origin. The attending doctors needed to rule out intracranial injuries or diseases. Children found not to have head or orbital abnormalities served as subjects. Children whose height or weight fell outside the mean±2SD for their age group were excluded. In addition, children with conditions potentially affecting the normal formation of the skull or facial bones, such as hydrocephalus or craniosynostosis, and also children with apparent eye position abnormalities were excluded. The procedures used conformed to the tenets of the World Medical Association's Declaration of Helsinki. The institutional ethics review board approved this retrospective study of the patients' medical records (approved No 675).

The children were divided into year age groups; to minimise age variations in each age group due to the date of birth, subjects were selected by the following method. Ten infants who underwent CT at 6 ±1 months of age were placed in the 6-month age group. For the 1–8-year groups, 10 children who had undergone CT on their date of birth ±1 month were selected. Similarly, of the children who underwent a CT on their date of birth ±2 months from ages 9–12 and ages 15–17 years, 10 of each age were selected. Seven children who underwent a CT at age 18 years ±2 months were also selected. In total, 147 children were studied. A histogram of the age and sex of these children is shown in figure 1.

CT and measurement of opening angles

A CT system (Aquilion16; Toshiba, Japan) was used to obtain tomographic images of the head including both orbits with the optic nerves and the surrounding orbital walls (figure 2). The CT scan was performed with the subject in the supine position with their face directed upward, and measurements were made on the CT image in which the face position was straight upward. Imaging conditions were: tube voltage, 120 kV; tube current, 200 mA; slice thickness, 0.5 mm; 16 data acquisition system channels; helical scan with a pitch of 0.67. The slice thickness for images used for the measurements was 1 mm. Opening angles of the optic nerve and orbit were measured in the axial CT images.

The opening angle of the optic nerve was defined as the angle formed by the intersection of a

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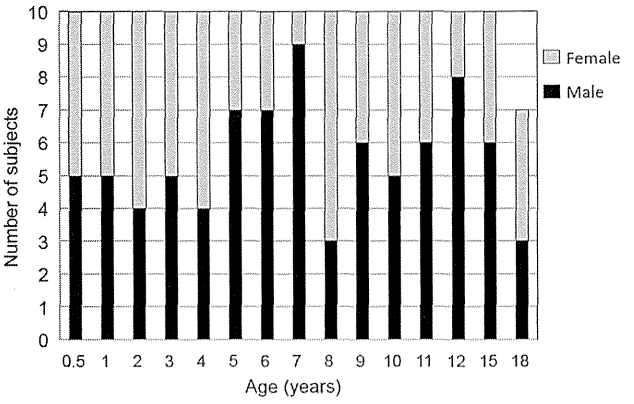

Figure 1 Histogram showing the age and sex of the 147 children who were subjects of this study.

line running through the left optic nerve and a vertical line passing through the centre of the nose (figure 2, left panel). The opening angle of the orbit was defined as the angle formed by the intersection of a line running tangentially along the deep lateral wall of the left orbit and a vertical line passing through the centre of the nose (figure 2, right panel). We measured these angles when the optic nerve appeared in one CT slice. An automatic measuring tool, which was included in the electronic medical charts, was used to measure these angles. To reduce interobserver variation, all measurements were performed by two independent observers (HT and TM), who were blinded to the age of the subject. The mean values of the two observers were used for the statistical analyses.

Statistical analyses

Mann–Whitney U tests were used to compare opening angles between two groups of different ages. In addition, the following three types of regression analysis were performed to examine the relationship between age and opening angle of the optic nerve or opening angle of the orbit: linear regression analysis using the actual values, linear regression analysis in which the relationship between angle and age was approximated with an exponential function, and linear regression analysis in which the relationship between angle and age was approximated with a logarithmic function. The coefficient of determination (r^2) and p values were determined on the basis of these regression analyses. $p<0.05$ was considered significant.

RESULTS

The opening angles of the optic nerve in each age group are shown in figure 2. The mean opening angle of the optic nerve was 23.9±4.1° (mean±SD) in the 10 infants in the 6-month age group and 21.7±1.7° in the 10 in the 3-year age group (figure 3). The difference between these two groups was not significant ($p=0.82$; Mann–Whitney U test). The relationship between age and opening angle of the optic nerve was analysed using three regression analyses (table 1). We found that there was no significant correlation between age and angle of the optic nerve ($p>0.40$), and the coefficient of determination (r^2) of age to the opening angle of the optic nerve was less than 0.01 (1%) for all three regression analyses (table 1).

The opening angles of the orbit in each age group are plotted in figure 4. The mean opening angle of the orbit was 50.8±4.1° in 10 infants who were 6 months old, and this was significantly larger than that (45.2±1.9°) in 10 infants who were 3 years of age ($p<0.001$; Mann–Whitney U test). In addition, the data show that the angle decreased asymptotically up until the age of 2–3 years.

The relationship between age and opening angle of the orbit was analysed using three regression analyses. We found that there were significant correlations between age and angle of orbit for all regression analyses ($p<0.001$, table 1). The coefficient of determination (r^2) of age to the opening angle of the orbit was about 0.13 (13%) and 0.12 (12%) according to linear and exponential regression analyses, respectively, and 0.19 (19%) in the logarithmic regression analysis (table 1).

DISCUSSION

Body parts change considerably during the fetal period and early childhood. A number of studies have examined morphological changes in the orbit with increasing age,[1–9] but only a few studies have measured changes in the opening angle of the optic nerve and orbit after birth in normal subjects.[7–9] We

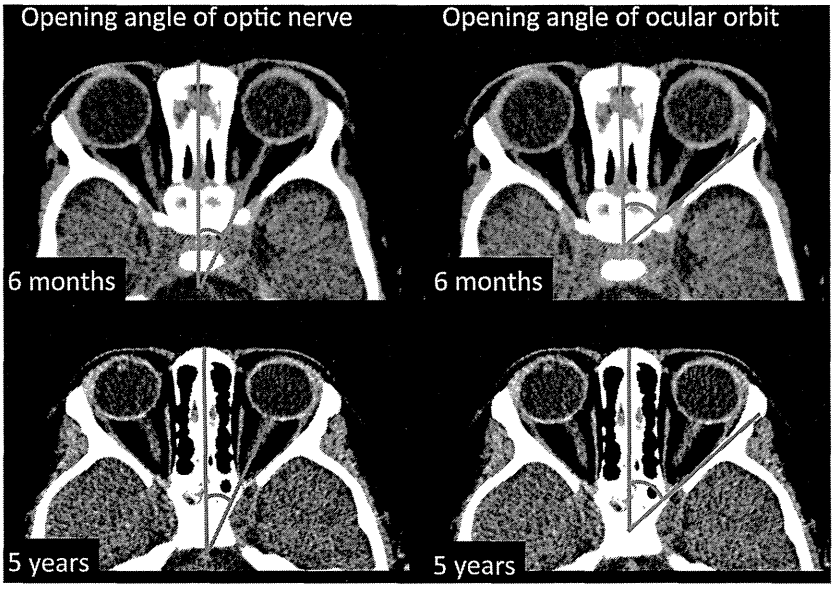

Figure 2 Opening angle of the optic nerve and opening angle of the orbit for representative typical infants measured on the axial CT image at 6 months and 5 years of age. The opening angle of the optic nerve was defined as the angle formed by the intersection of a line running through the left optic nerve and a vertical line passing through the centre of the nose (left panel). The opening angle of the orbit was defined as the angle formed by the intersection of a line running tangentially along the deep lateral wall of the left orbit and a vertical line passing through the centre of the nose (right panel). There were no major differences in the opening angle of the optic nerve (left panel) in infants at 6 months and 5 years of age, but the opening angle of the orbit (right panel) was less in the 5-year-old infant.

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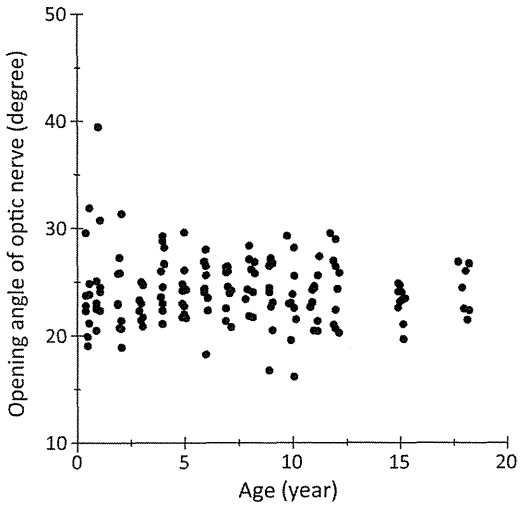

Figure 3 Changes in the opening angle of the optic nerve during development. The results of regression analyses showed no significant changes in the opening angle of the optic nerve with increasing age.

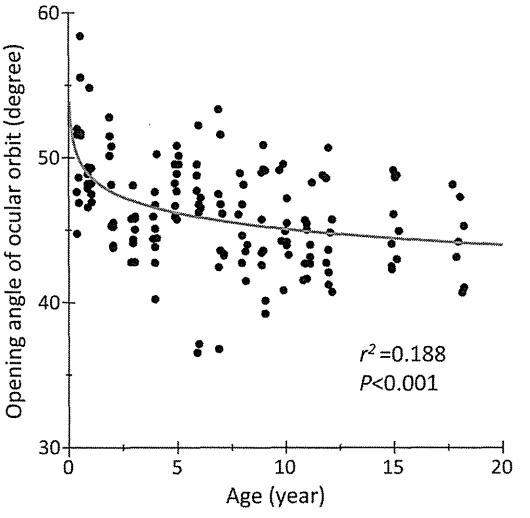

Figure 4 Changes in the opening angle of the orbit during development. The opening angle of the orbit was found to decrease significantly during development. The red line is the logarithmic curve generated by regression analysis (r^2=0.188, p<0.001). Equation: *y* (angle of ocular orbit, degrees)=48.7–3.7 log *x* age (years).

measured the opening angle of the optic nerve and orbit using axial CT images in 147 normal Japanese children aged from 6 months to 18 years.

In an autopsy study, Zimmermann *et al*[7] measured the angle formed by two lines running from the optic chiasm to the site where the optic nerves are attached to each eye. They reported that the opening angle of the optic nerve measured by this method decreased slightly during the fetal period, and that the angle was ~71.5° at birth and ~68° in adults. This early study involved measurements on autopsied heads, and the method used to measure the opening angle of the optic nerve differed from that used in our study, so a direct comparison may not be meaningful. However, they found that the opening angle of the optic nerve changed very little during development, which is consistent with our results.

Escaravage *et al*[8] measured the angle formed by the central axis of the two orbits. They reported that the angle formed by the central axes of the two orbits decreased until about 1 year of age, but changed little thereafter. Although their measurement method was similar to our method for the angle of optic nerves, it was not identical. In addition, Escaravage *et al* obtained data from many infants whose ages ranged from birth to 6 months, whereas our study included infants ≥6 months of age. Their findings clearly detected changes from birth to the age of 1 year.

There were considerable variations in the opening angle of the optic nerve, particularly in infants younger than 1 year (figure 3). One possible reason for this is that there is a variation in the position of the optic nerve on the CT images of infants younger than 1 year. Another possibility is that the opening angle of the optic nerve was affected by the direction of the eyes during the CT scans, especially in infants younger than 1 year. Alternatively, this variation in the opening angle of the optic nerve may simply be caused by a large variation in the growth of the head and orbit.

There have been studies on changes in the opening angle of the orbit during development. Lemke and Lucarelli[9] used CT images to measure the opening angle of both orbits, and they reported that the opening angle of the orbits was ~90° in adults. This angle is similar to that of the teenage group in our study whose ages ranged from 15 to 18 years (unilateral orbital angle, 45.5±3.5°, n=18).

The measurements in our study indicate that the opening angle of the orbit changed substantially before the age of 3 and changed less thereafter (figure 4). In addition, the results indicate that the changes in the opening angle of the orbit can be fitted by a logarithmic curve. Escaravage *et al*[8] reported that the angle of the orbit decreased until the age of 1 year and then changed little thereafter. They measured the angle formed by a line connecting the orbital apex and the orbital process of the zygomatic bone and the medial and lateral wall of the orbit (the angle between the medial and lateral orbital wall). We measured the angle formed by the intersection of a line running tangentially along the deep lateral wall of the left orbit and a vertical line passing through the centre of the nose. Thus, a direct comparison is not possible.

We measured the angle formed by the deep lateral wall of the orbit because this wall is where the extraocular muscles are in contact with the orbital bone via a pulley system. This site is closely associated with eye movements and eye alignment.

Table 1 Coefficient of determination (r^2) and p values between age and opening angle of the optic nerve, and between age and opening angle of the ocular orbit obtained by three different regression analyses

	Age and opening angle of optic nerve		Age and opening angle of ocular orbit	
	Coefficient of determination (r^2)	p Value	Coefficient of determination (r^2)	p Value
Linear regression analysis	0.005	0.411	0.126	<0.001
Exponential regression analysis	0.003	0.517	0.121	<0.001
Logarithmic regression analysis	0.003	0.505	0.188	<0.001

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Although the relationship between the morphology of the orbit and the presence of strabismus has not been well investigated, strabismus is common in craniosynostosis, with a complication rate of 39–90.9%. Exotropia is the major type of strabismus in Crouzon syndrome and Apert syndrome.[10] Kreiborg and Cohen[11] reported that the incidence of exotropia in Crouzon syndrome was 76.6% and both the inner and the outer interorbital distances were significantly greater than that of normal orbits, resulting in an increase in the opening angle of the orbits between both lateral walls. Morax[12] studied changes in the position of the eye in patients with Crouzon syndrome after a sagittal expansion of the orbit. Eight of nine patients were exotropic before the craniofacial surgery, and the exotropia was corrected to orthophoria after the surgery without a strabismic procedure. This change in ocular alignment most likely resulted from the decrease in the opening angle of the orbits between the two lateral walls caused by the surgical procedures. In Apert syndrome, the ocular alignment changed from exotropia to esotropia after craniofacial surgery.[13 14] These findings suggest that the greater the opening angle of the orbits, the more often exotropia is present.

The results of several studies examining the relationship between exotropia and the increase in the opening angle of the orbits have been reported. It is known that ~70% of newborns have an exodeviation, but the deviation gradually disappears in most cases by 2–4 months of age.[15] The reason why this happens has not been determined, but our findings suggest that the decrease in the opening angle of the orbit may contribute to this decrease in exodeviation.

It is known that strabismus often recurs after strabismic surgery. One of the authors (TY) has reported that exotropic patients who have a recurrence soon after surgery tended to have a larger opening angle of the orbit, and that resection of the medial rectus rather than recession of the lateral rectus muscle resulted in fewer recurrences after the surgery.[16] If this is correct, then the opening angle of the orbit should be considered when the type of exotropia surgery is selected.

Intermittent exotropia is known to be more prevalent in Asians than in Caucasians.[17] One way to determine the reasons for this would be to study changes in opening angle of the orbit with increasing age in Caucasians and compare them with changes in Asians. Such research might yield interesting findings.

This study has four limitations. The first is that it did not include data from newborns to examine changes soon after birth. This is because there was a lack of data from normal newborns who had undergone CT soon after birth. The second limitation is that this study measured the angle along the optic nerve for the measurement of the opening angle of the optic nerve. However, children have difficulty controlling their eye movements, so measurements may differ from the actual opening angle of the optic nerve. The third limitation is that we did not determine the eye position and eye movements before the CT recordings. The fourth limitation is that there are no data on the changes in angles in one child at different ages—that is, a longitudinal study.

In spite of these limitations, this study revealed that the opening angle of the orbit changes logarithmically with age. The results provide important information for the management of strabismus in children with and without skull or orbital abnormalities.

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Contributors HT, YH, TY and MK planned this study. HT, YH and TM measured the angle of optic nerves and orbits. HT, YH, TM and MK analysed the data. HT, YH, TY and MK wrote the manuscript.

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Competing interests None.

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Changes in angle of optic nerve and angle of ocular orbit with increasing age in Japanese children

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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

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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

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