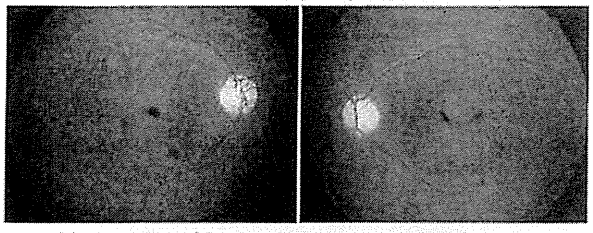
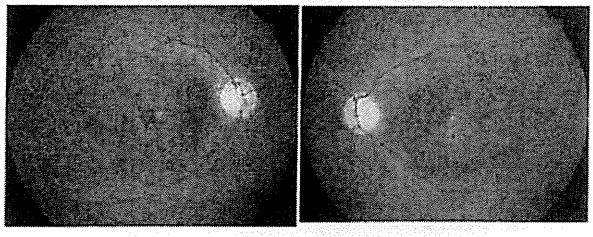
OD OS

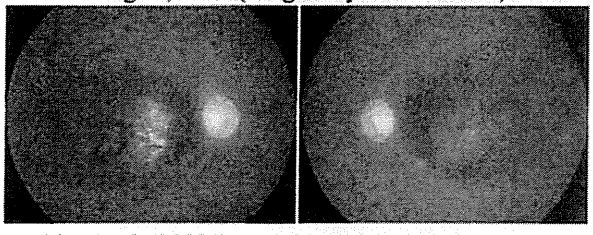
31st October, 1986 (at age 4 years 8 months)



16th August, 1991 (at age 9 years 5 months)



12th August, 1999 (at age 17 years 5 months)



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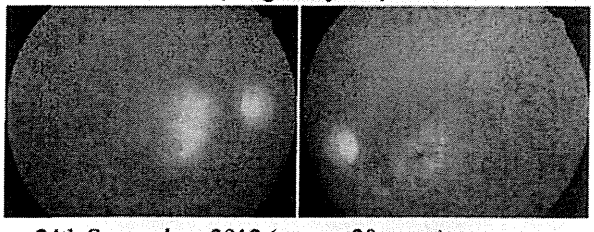
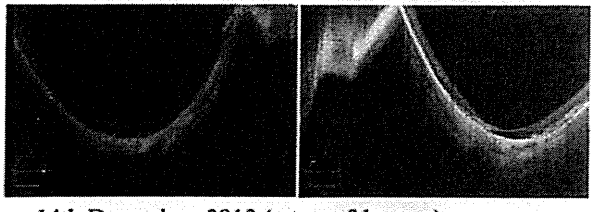
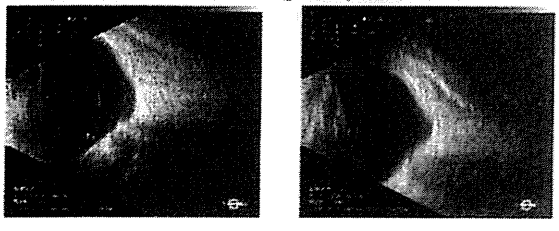


24th September, 2012 (at age 30 years)

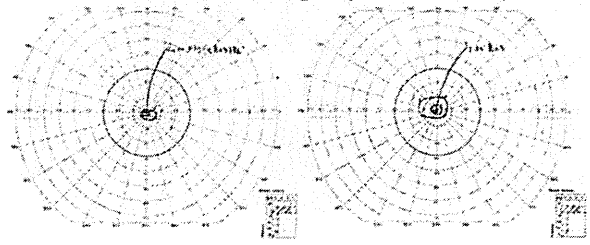


14th December, 2013 (at age 31 years)

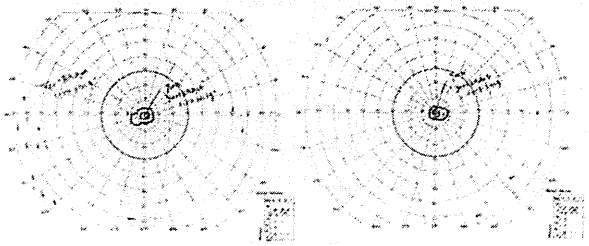


OS OD

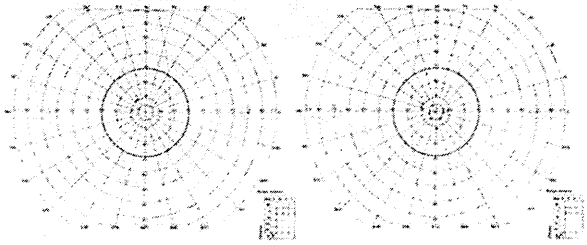
31st October, 1986 (at age 4 years 8 months)



7th January, 1992 (at age 9 years 10 months)



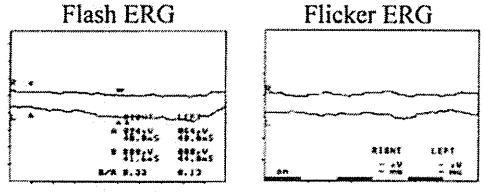
12th August, 1999 (at age 17 years 5 months)



16th August, 1991 (at age 9 years 5 months)

Flash ERG Flicker ERG

OD
OS



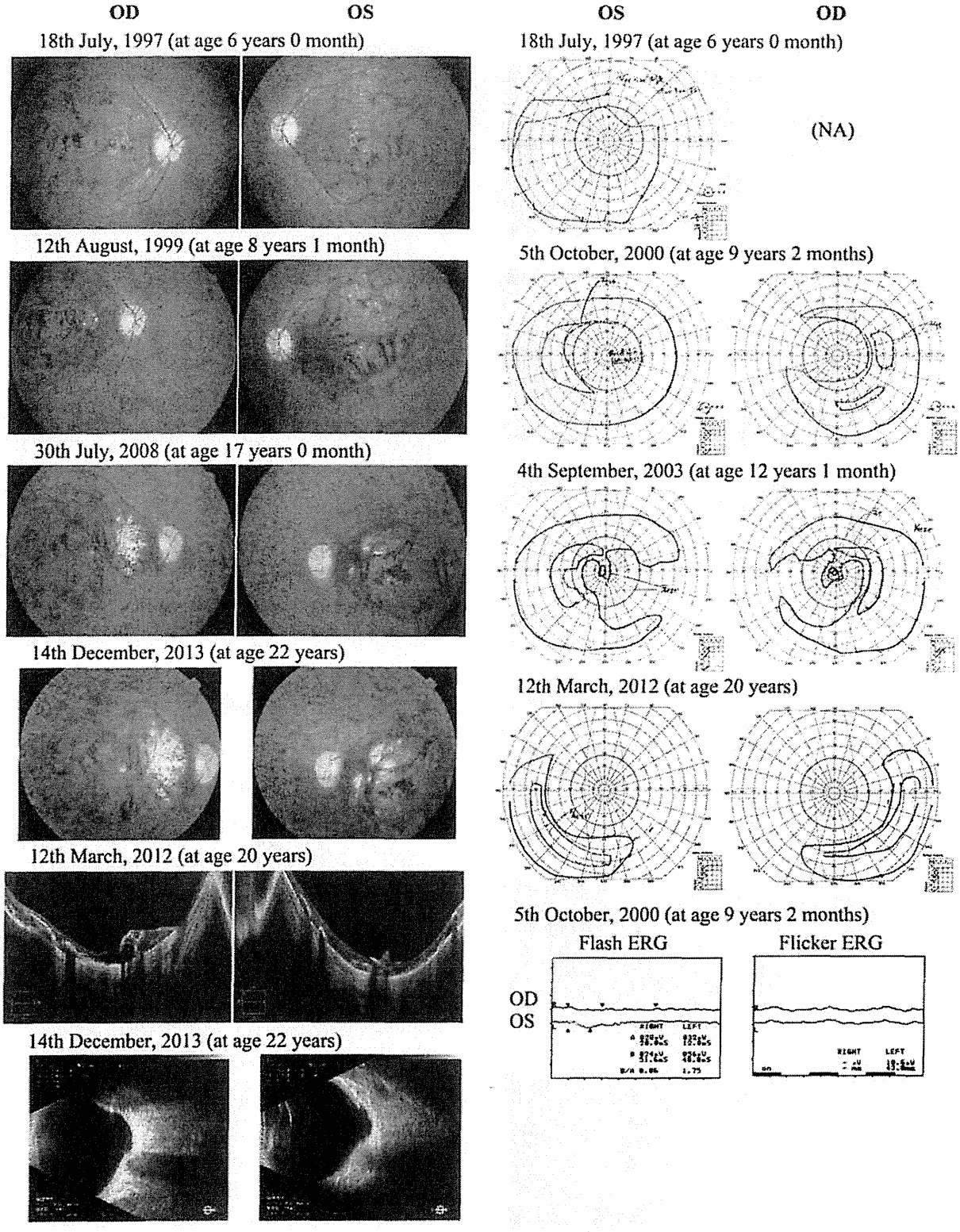


Fig. 4 Fundus photographs (*left*), OCT and ultrasonographic images (*lower left*), Goldmann kinetic visual fields (*right*), and full-field ERGs (*bottom right*) of Patient 2. Patient 2 was younger brother of Patient 1 (Figs. 1, 3). *NA* not available

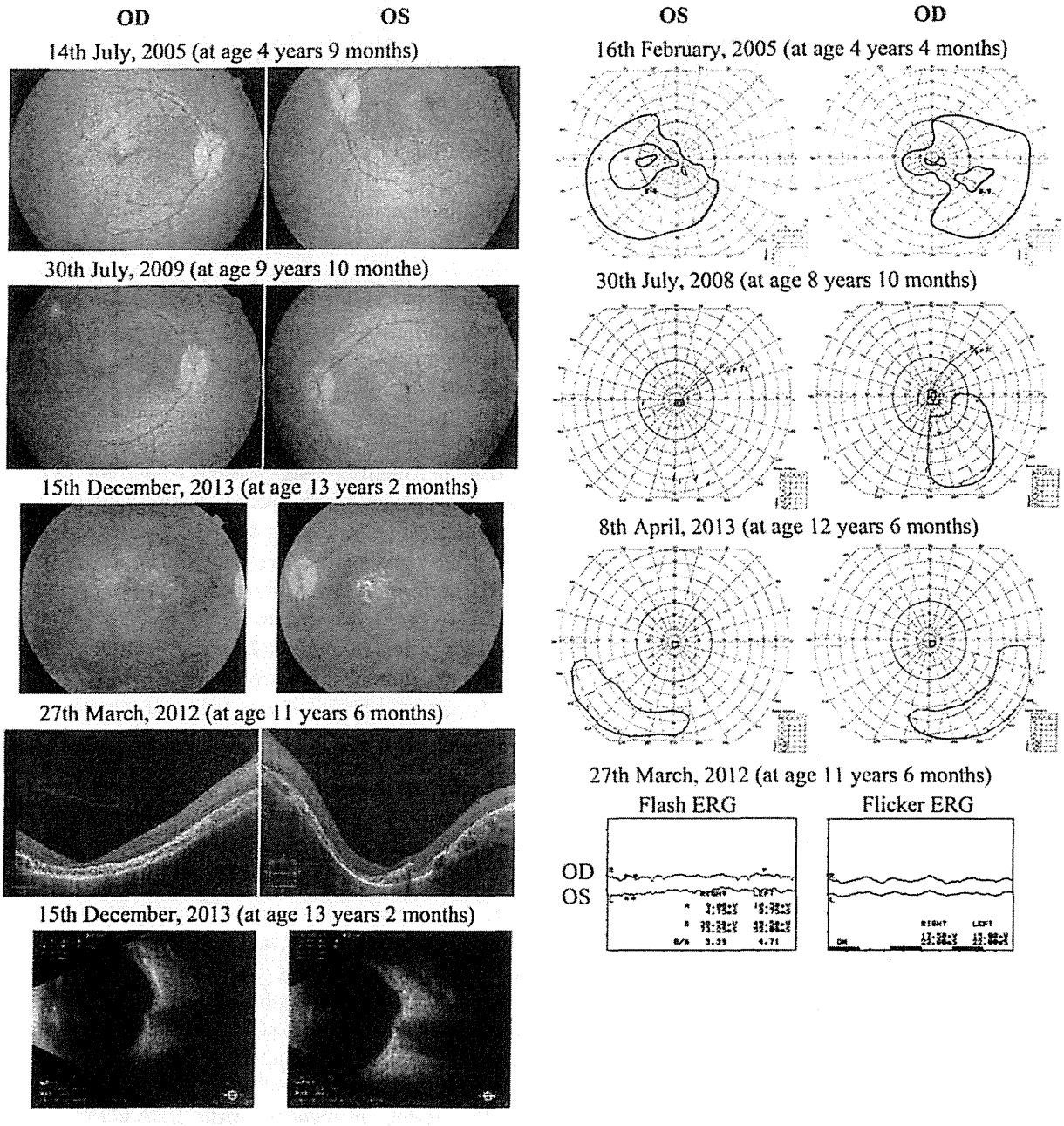


Fig. 5 Fundus photographs (*left*), OCT and ultrasonographic images (*lower left*), Goldmann kinetic visual fields (*right*), and full-field ERGs (*bottom right*) of Patient 3. Patient 3 was from unrelated family to that of Patients 1 and 2 (Fig. 1)

disease-causing gene. Then, genetic analysis revealed a homozygous c.377C>T transition in exon 4 resulting in an alanine126 to valine substitution (A126V) in the *RDH12* gene. Genetic analyses of her non-symptomatic parents (kinki-1046 and 1047, Fig. 1) revealed a heterozygous A126V substitution in the *RDH12* gene.

Patient 2 (Fig. 4, kinki-1045 in Fig. 1): Patient 2 was a boy who was 6-year old when we first examined him in 1997. He was the younger brother of Patient 1 (Fig. 1). He visited our clinic because his parents noticed he was having visual difficulties since age 5 years. His decimal visual acuity was 0.07 in his right eye. The vision was uncorrectable, and his left BCVA was 0.4 with 0 DS and −1.5 DC ax 160°. Ophthalmoscopy showed diffuse retinal degeneration, but it was especially severe in the macula which was similar

to that of his older sister, Patient 1 (Fig. 3, 4). The fundi appeared reticulated before the age 10 years. The macular degeneration gradually spread, and a posterior staphyloma developed and progressed in both eyes (Fig. 4). His central vision decreased to hand motion in his late teens (Fig. 2). He is now 22-year old, and he still has some peripheral vision but no cataracts in both eyes.

The full-field ERGs, OCT, and ultrasonographic findings were similar to those of his older sister (Patient 1), namely, non-recordable single-bright flash ERGs, barely recordable flicker ERGs, and deep excavation and thin retina at the posterior pole of both eyes (Fig. 4). The axial length at age 22 years was 23.82 ± 0.05 mm in the right eye and 24.06 ± 0.02 mm in the left eye.

Genetic analysis revealed a homozygous A126V substitution in *RDH12* gene, the same as his sister (Patient 1).

Patient 3 (Fig. 5, kinki-1076 in Fig. 1): Patient 3 was a girl who was 3-year old when we first examined her in 2004. She was a member of a family (kinki-F33) unrelated to that of Patients 1 and 2 (Fig. 1). She was brought to our clinic because of esotropia and nystagmus. Her decimal BCVA was 0.07 with +6.0 DS and −1.0 DC ax 115° in the right eye and 0.07 with +5.5 DS and −1.5 DC ax 175° in the left eye. Ophthalmoscopy showed diffuse retinal degeneration with pigmentation in the macular area (Fig. 5). Her fundi appeared reticulated before the age 10 years. She was followed until the age of 13 years, and her vision gradually decreased to light perception in both eyes (Fig. 2).

Single-bright flash full-field ERGs were non-recordable, and flicker ERGs were barely recordable at age 11 years (Fig. 5). OCT and ultrasonography performed at 11 and 13 years of age revealed excavation of the posterior pole of both eyes (Fig. 5). The axial length at age 13 years was 20.92 ± 0.37 mm in the right eye and 21.22 ± 0.93 mm in the left eye.

When the sequence of her whole exome was compared with the reference human genome (hs37d5), 1,488,313 mutations were found. After excluding common mutations, 406 mutations remained. We filtered the remaining mutations by the pattern of inheritance with her parents and found 16 genes as causal candidates. Finally, they were compared to that of Patients 1 and 2, and only *RDH12* was shared between three patients. As a result, genetic analysis showed a homozygous c.377C>T transition in exon 4 resulting in alanine126 to valine substitution (A126V) in the *RDH12* gene. Genetic analyses on her non-symptomatic parents (kinki-1077 & 1078, Fig. 1) showed heterozygous A126V substitution in the *RDH12* gene.

Discussion

ERG findings in carrier relatives

The *RDH12* gene is located at 14q 24.1 and encodes a photoreceptor cell retinol dehydrogenase. Mutation of the *RDH12* gene is estimated to account for <4 % of all autosomal recessive LCA/EORD patients [5, 8]. To date, 16 different mutations have been reported in this gene [6]; however, the homozygous substitution of A126V in the *RDH12* gene has never been reported except in a highly consanguineous Arabic family [13] and our patients. In the Arabic family, a non-symptomatic relative who was a heterozygous carrier of A126V had markedly reduced rod ERGs, and the cone ERGs were at the lower limits of normal [13]. Another study reported that heterozygous mutations in the *RDH12* gene can cause a late-onset, relatively mild autosomal dominant retinitis pigmentosa [24].

The parents of our patients were non-symptomatic, and their fundi were normal. The rod and cone ERGs performed on three of them (kinki-1047, kinki-1077, and kinki-1078 in Fig. 1) were normal.

Clinical course of visual acuity

The initial visual disturbance in our patients was noticed at age 2–5 years, and there was a progressive decrease thereafter (Fig. 2). Their central vision decreased to light perception in the teens. Patients 2 and 3 maintained some peripheral vision at age 22 and 13 years although Patient 1 lost vision in the entire visual field at age 17 years (Figs. 3, 4, 5).

The vision in patients with LCA/EORD was investigated by Fulton et al. [25] and Walia et al. [26]. Walia et al. [26] related the vision of patients with LCA/EORD to their causative genes and reported that LCA/EORD caused by *RPE65* (LCA2), *CRB1* (LCA8), and *RDH12* (LCA13) mutations led to a wide variations in visual disturbances, whereas LCA/EORD caused by *GUCY2D* (LCA1), *AIPL1* (LCA4), *RPGRIP1* (LCA6), and *CRX* (LCA7) gene mutations had severe visual disturbances which began in the first year of life. Other studies on LCA/EORD associated

with *RDH12* mutations reported an initial vision reduction occurring between birth to 20 years with most of them at age 3–7 years [7–16].

These results are consistent with our patients who had decreased vision at age 2–5 years and loss of their central vision in their teens (Fig. 2).

Coloboma/posterior staphyloma and LCA/EORD

The fundus of our three patients appeared similar; namely, they showed diffuse retinal degeneration and macular atrophy (Figs. 3, 4, 5). The fundi also had a reticulated appearance (Figs. 3, 4, 5). These findings are similar to the phenotype reported for *RDH12*-associated LCA/EORD [7–16].

In our patients, the macular degeneration progressed to atrophic macula with the formation of a posterior staphyloma which resembled a coloboma (Figs. 3, 4, 5). The relationships between LCA and macular coloboma have been discussed in several papers [27–29], before the causative genes for LCA/EORD were discovered. Recently, a macular coloboma/posterior staphyloma was reported in patients with *LCA5* (LCA5) [30], *CRX* (LCA7) [31], *CRB1* (LCA8) [32], *NMNAT1* (LCA9) [33], and *RDH12* (LCA13) mutations [7, 9–11, 14, 16]. A relationship between LCA/EORD and the macular coloboma/posterior staphyloma is still unknown. Single-gene mutation cannot explain the formation of a macular coloboma/posterior staphyloma because they are present in cases of LCA/EORD associated with several different causative genes.

In our patients, the reticulated appearance of the fundus was present in early childhood, and it became less apparent after the formation of the posterior staphyloma. Whether the reticulated appearance was related to the development of the staphyloma was not determined.

One limitation of this study is the small number of the patients. In addition, a more detailed screened investigation of the phenotypes and genotypes of patients with LCA/EORD is needed to confirm our results.

In conclusion, we report the longitudinal clinical course of three patients in two families with LCA/EORD who had homozygous A126V substitution in the *RDH12* gene. All of the patients had a progressive retinal degeneration and posterior staphyloma, and impairment of the central vision. This is the first report of Japanese patients with LCA/EORD which was caused by *RDH12* gene mutation.

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Conflict of interest All authors have no commercial interests related to this research.

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CLINICAL CASE REPORT

Autosomal recessive cone–rod dystrophy associated with compound heterozygous mutations in the *EYS* gene

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Abstract
Background *EYS* mutations have been identified only in patients with autosomal recessive retinitis pigmentosa (arRP). This study was conducted to describe clinical and genetic features of a Japanese patient with autosomal recessive cone–rod dystrophy (arCRD) and *EYS* mutations.
Methods We performed complete ophthalmic examinations including full-field electroretinography (ERG). Genetic analysis using whole-exome sequencing and Sanger sequencing was performed to identify the disease-causing mutation in a 31-year-old male patient.
Results At the initial visit, the patient's decimal best-corrected visual acuity (BCVA) was 0.9 and 0.6 in his right and left eyes, respectively. Funduscopy indicated retinal degenerations were predominantly affected within the vascular arcades and preserved retinal vessels in the mid-periphery in both eyes. Visual field testing showed there were relative central scotomas and preserved peripheral visual fields in both eyes. ERG indicated there was a decreased pattern for both the rod and cone responses. At the age of 36 years, his BCVA decreased to 0.2 in both eyes. Optical coherence tomography showed marked retinal thinning of the macular regions in both eyes. Genetic analysis identified compound heterozygous truncating mutations (p.Y2935X and p.S1653KfsX2) in the *EYS* gene. His unaffected parents were heterozygous for each mutation.
Conclusions Our results demonstrated that *EYS* mutations can be the cause of not only arRP but also arCRD. Our findings extend the phenotypic spectrum of patients with *EYS* mutations.

Keywords *EYS* gene · Whole-exome sequencing · Genetics · Retinitis pigmentosa · Cone–rod dystrophy

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Introduction

The eyes shut homolog (*EYS*) gene (Online Mendelian Inheritance in Man: *612424), largest gene known to be expressed in the human eye, spanning more than 2 Mb within the *RP25* locus (6q12). The human EYS protein is a homolog of the *Drosophila* eyes shut/spacemaker (eys) protein, which is an extracellular matrix protein essential for photoreceptor development and morphology of the insect eye.

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In 2008, mutations in the *EYS* gene were reported in patients with autosomal recessive retinitis pigmentosa (arRP) [1, 2]. The *EYS* mutations, which have been shown to be predominantly truncating mutations, have been described in patients with different ethnic origins and account for 5–16 % of arRP [3–7]. Thus, disruption of the EYS function has been identified as a frequent cause of arRP worldwide. With the exception for arRP, to date there have been no *EYS* mutations reported for any other phenotype.

Here, we report clinical and genetic features of a patient with autosomal recessive cone–rod dystrophy (arCRD) associated with compound heterozygous *EYS* mutations.

Case report

A 31-year-old male patient (JU#0659) was referred to our hospital with a complaint of loss of visual acuity. He first reported a decreased visual acuity at the age of 29 years. Family history indicated that his parents had no previous reports of any ocular symptoms (Fig. 1). At his initial examination, decimal best-corrected visual acuity (BCVA) was 0.9 [with −6.00 diopter (dpt), cylinder (cyl) −1.25 dpt axis (Ax) 130°] in his right eye and 0.6 (with −4.50 dpt, cyl −0.75 dpt Ax 180°) in his left eye. Anterior segment examination showed no remarkable findings. Intraocular pressures were 16 mmHg in the right and 15 mmHg in the left eye. Funduscopy showed retinal degenerations within the vascular arcade in both eyes (Fig. 2a). Neither retinal degeneration nor attenuation of retinal vessels

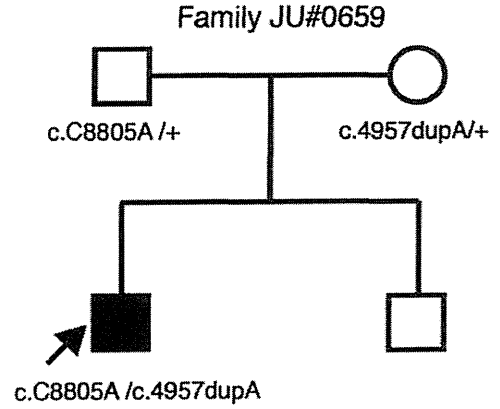

Fig. 1 Pedigree of a Japanese family. Unaffected family members (males, *open squares*; females, *open circles*) and an affected proband (male, *solid square*) are shown

was observed in the periphery. Fluorescein angiography (VISUCAM NM/FA; Carl Zeiss Meditec AG, Dublin, CA, USA) showed a hyperfluorescence pattern due to a window defect within the vascular arcades of both eyes (Fig. 2b). At the age of 32 years, visual field testing using Goldmann kinetic perimetry (GP; Haag-Streit, Bern, Switzerland) showed bilateral central scotomas of the I-3e and I-4e isopters with relative sparing of the center, but preserved peripheral visual fields of the V-4e and I-4e isopters in both eyes (Fig. 3). Full-field electroretinography (ERG) was performed according to the protocols of the International Society for Clinical Electrophysiology of Vision. The procedure and conditions for ERG recording have been reported previously [8]. The ERG showed the rod, standard combined, cone, and 30-Hz flicker responses were bilaterally reduced to about one-third of those in a control, but the peak implicit time of each response was not delayed (Fig. 4).

At the age of 36 years, his BCVA decreased to 0.2 in both eyes. Funduscopic images using the Optos 200Tx imaging system (Optos PLC, Dunfermline, United Kingdom) showed retinal degenerations within the vascular arcades in both eyes, but relatively preserved mid-peripheral to peripheral retinal findings with no apparent attenuation of the retinal vessels (Fig. 5a). Fundus autofluorescence imaging (FAI) (Optos PLC) showed decreased autofluorescence within the vascular arcades but increased autofluorescence of the foveal area, and increased autofluorescence outside the vascular arcades in both eyes (Fig. 5b). Optical coherence tomography (OCT) (Cirrus HD-OCT; Carl Zeiss Meditec AG) showed retinal thinning with a visible foveal external limiting membrane line (Fig. 5c), and entire macular thinning in both eyes (Fig. 5d).

To identify disease-causing gene mutations, we performed whole-exome sequencing analysis as per a previously described method [9]. The obtained sequence data in the patient were compared with reference human genome sequences. Initially, we focused on only variants that could change the amino acid sequence. Subsequently, we filtered the remaining variants based on the criteria that a frequency of mutation was less than 1 % in the 1000-genome database (http://www.1000genomes.org). Finally, we screened variants residing within 207 retinal disease-associated genes published in the November 15, 2013

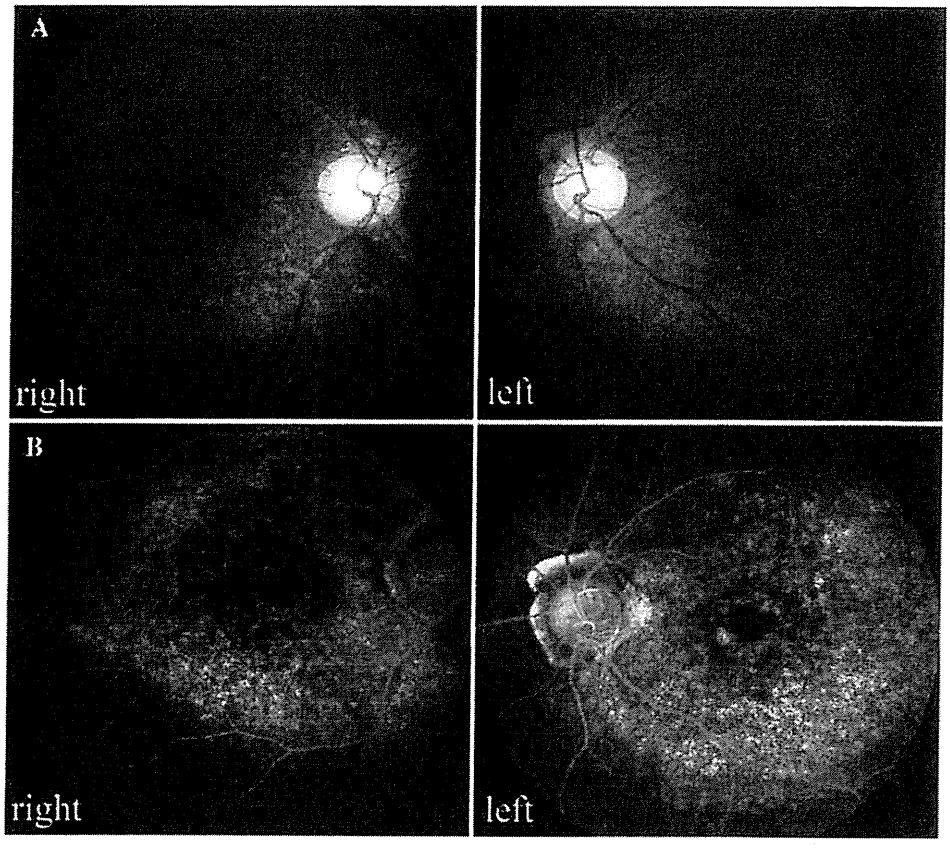

Fig. 2 Fundus photographs and fluorescein angiography images (FA) of the patient at the age of 31 years. **a** Fundus photographs show retinal degenerations within the vascular arcades in both eyes. **b** FA shows a hyperfluorescence pattern due to a window defect within the vascular arcades of both eyes

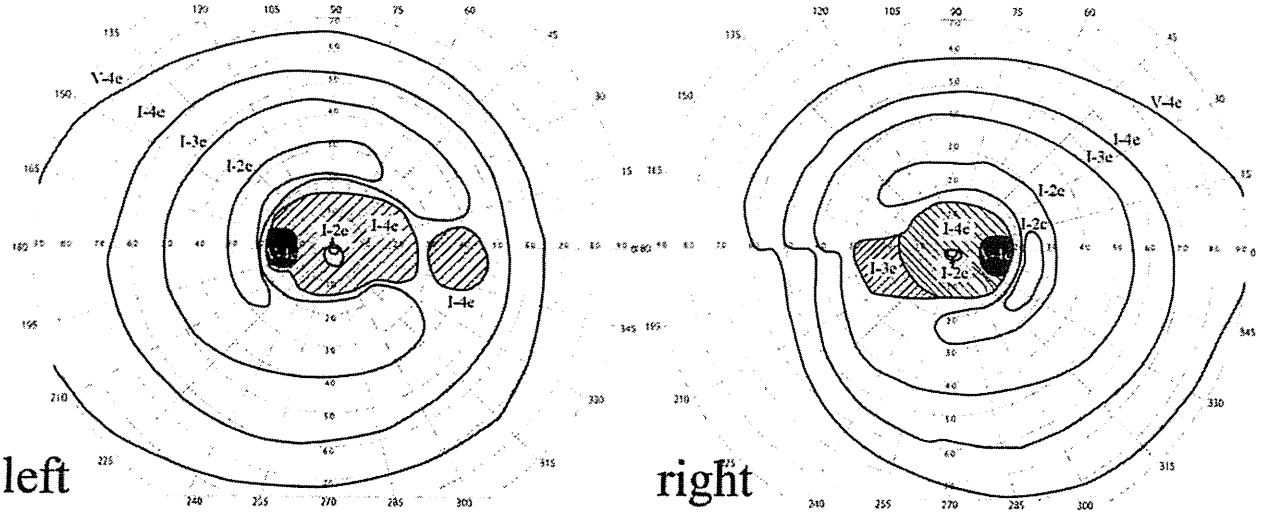

Fig. 3 Visual field testing using Goldmann kinetic perimetry (GP) at the age of 32 years. GP shows bilateral central scotomas of the I-3e and I-4e isopters with relative sparing of the center, but with preserved peripheral visual fields of the V-4e and I-4e isopters in both eyes

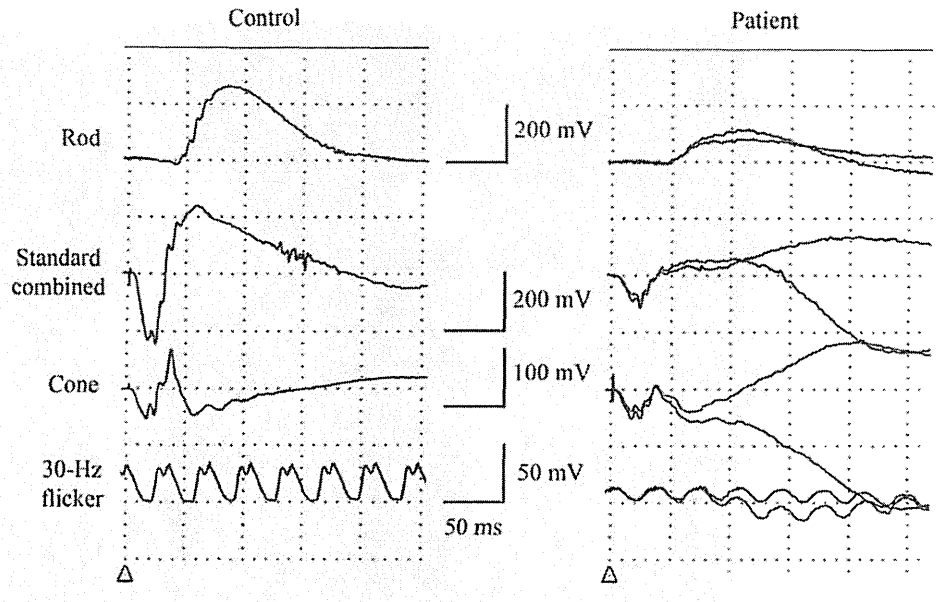

Fig. 4 Full-field electroretinography (ERG) at the age of 32 years. ERG shows that the rod, standard combined, cone, and 30-Hz flicker responses are bilaterally reduced to about one-third of those in a control, but the peak implicit time of each response is not delayed

RetNet database (https://sph.uth.edu/retnet/). Based on the obtained data, known *EYS* mutations were identified in a compound heterozygous state as disease-causing mutations. In the other 206 genes, there were no mutations found in compound heterozygous or homozygous states. The identified *EYS* gene mutations were c.C8805A and c.4957dupA, which result in the truncating mutations p.Y2935X and p.S1653KfsX2, respectively. The findings were confirmed by Sanger sequencing. The patient's unaffected parents were heterozygous for each mutation. The compound heterozygous mutations (p.Y2935X and p.S1653KfsX2) have been previously reported as a cause of arRP [7]. The accession number of the *EYS* mRNA reference sequence we used was NM_001142800.1 from the National Center for Biotechnology Information.

Discussion

In this report, whole-exome sequencing analysis identified *EYS* mutations in a Japanese patient with arCRD. *EYS* mutations have previously only been reported in patients with arRP [1, 2]. This is the first report that describes arCRD associated with compound heterozygous mutations in the *EYS* gene.

Previous studies have revealed that the EYS protein is expressed specifically in the human retina and is localized in the outer segment of the photoreceptor layers of the porcine retina [1, 2]. Although the function and structure of human EYS protein remain unclear, it has been suggested to be essential for photoreceptor morphogenesis [2]. In fact, *EYS* mutations give rise to RP phenotypes with thinning of the outer retinal layers [5, 7], which results from the degeneration of both the rod and cone photoreceptors. Clinical features of patients with *EYS* mutations include a typical form of RP that is characterized by a progressive constricted visual field, bone pigmentations and attenuation of the retinal vessels [3–5]. The pattern of the ERG in RP patients shows nonrecordable or markedly decreased responses [3–5]. On the other hand, our patient exhibited retinal degenerations that were predominantly seen within the vascular arcades (Fig. 5a, b), central scotomas and preserved peripheral visual fields (Fig. 3), and decreased responses in the both rod and cone ERG (Fig. 4) in both eyes. Generally, CRD exhibits several features such as decreased central vision, a predominant degeneration of the macular region as compared with the mid-peripheral region, and decreased amplitudes in the cone ERG that are equal to or worse than the decreased rod ERG amplitudes [10, 11]. These characteristics of CRD were clearly consistent with the phenotype of our patient. Taken together, these findings indicated that the patient diagnosis was arCRD and not arRP.

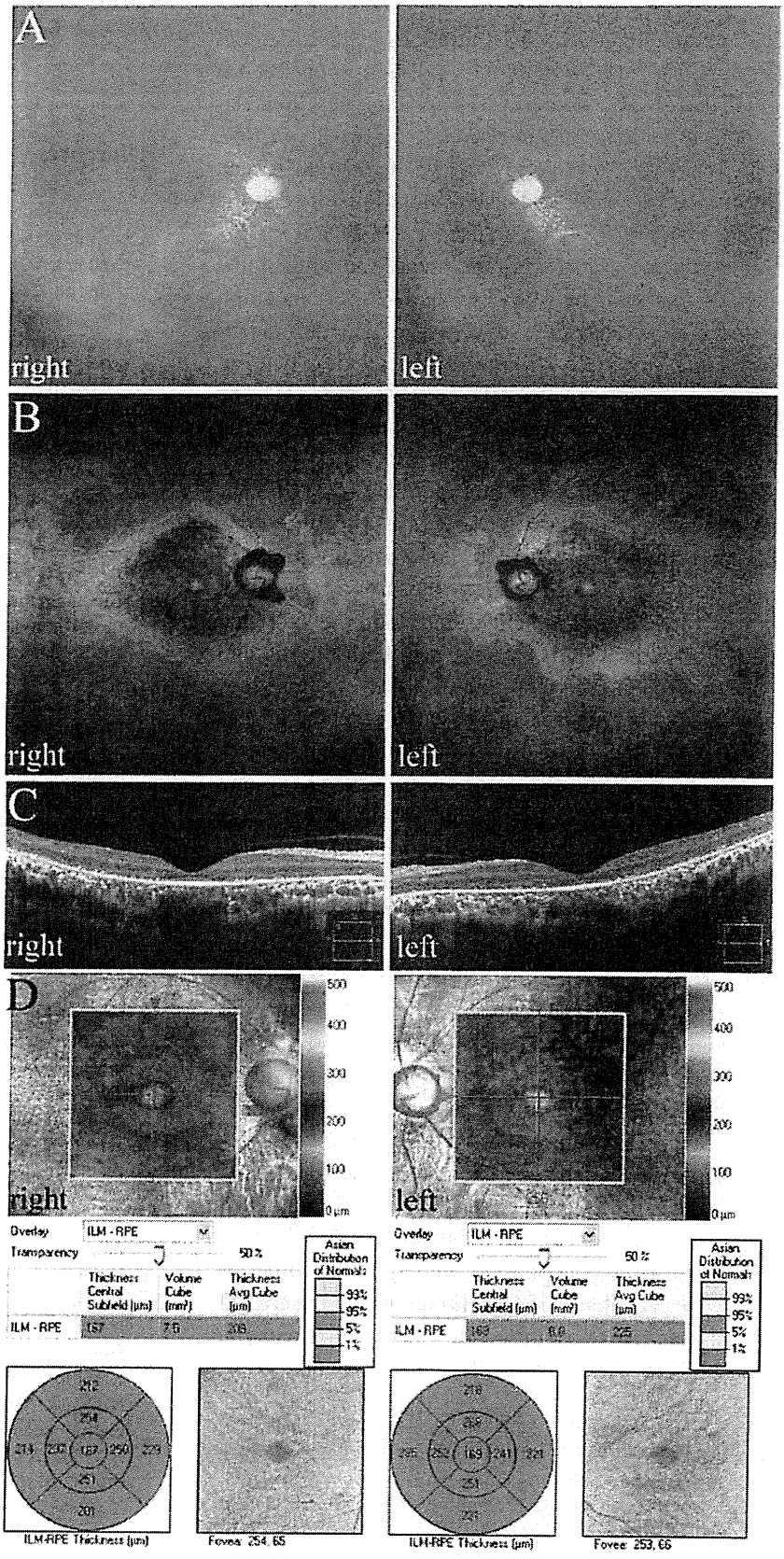

Fig. 5 Fundus photographs, fundus autofluorescence images (FAI), and optic coherence tomography images (OCT) at the age of 36 years. **a** Fundus photographs show retinal degenerations within the vascular arcades in both eyes, but relatively preserved mid-peripheral to peripheral retinal findings with no apparent attenuation of the retinal vessels. **b** FAI shows decreased autofluorescence within the vascular arcades but increased autofluorescence of the foveal area, and increased autofluorescence outside the vascular arcades in both eyes. **c** OCT (HD 5-line raster scan) shows retinal thinning with a visible foveal external limiting membrane line in both eyes. **d** OCT (Macular cube scan) shows entire macular thinning in both eyes

Whole-exome sequencing analysis disclosed that our patient had the *EYS* mutations, which demonstrates that the *EYS* mutations can be responsible for both the arCRD and the arRP phenotypes. Interestingly, mutations in the *ABCA4* [12–14], *CERKL* [15–18], and *C8orf37* [19, 20] genes have also been reported to be disease-causing mutations of both the arCRD and arRP phenotypes. With regard to the *ABCA4* gene mutations, the degree of functional damage caused by the various *ABCA4* mutation types can underlie the different degeneration patterns, for example, Stargardt disease (a type of macular dystrophy), arCRD or arRP [12–14]. The majority of patients with *CERKL* mutations exhibit arCRD [17, 18] and less frequently arRP [15, 16]. This is consistent with the fact that the CERKL protein is predominantly expressed in the cone photoreceptors [21]. In addition, different *C8orf37* mutations can cause either the arCRD or arRP phenotypes, which is consistent with the fact that the C8orf37 protein is expressed in both the rod and cone photoreceptors [19, 20]. However, this does not explain the pattern of the photoreceptor degeneration. On the other hand, the compound heterozygous *EYS* mutations (p.Y2935X and p.S1653KfsX2) that were found in our patient have also been reported in an arRP patient [7]. Although it is not understood why the same compound heterozygous mutations would underlie either the arCRD or arRP phenotypes, this finding suggests there is the presence of different modifier alleles between the arCRD or arRP patients with the compound heterozygous *EYS* mutations. Even so, our whole-exome sequencing analysis did not demonstrate any compound heterozygous or homozygous mutations in other 206 retinal disease-associated genes published in the RetNet database.

In conclusion, we demonstrated that *EYS* mutations are the cause of not only arRP but also arCRD. Further investigations will need to be undertaken in order to clarify the prevalence of *EYS* mutations among arCRD patients, and to determine the genotype–phenotype correlations between the arCRD and *EYS* mutations.

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Conflict of interest The authors declare there are no conflicts of interest for this study.

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Clinical Ophthalmology Dove

ORIGINAL RESEARCH

Retinal angiomatous proliferation associated with risk alleles of *ARMS2/HTRA1* gene polymorphisms in Japanese patients

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Background: The purpose of this study was to investigate the association between *ARMS2/HTRA1*, *CFH*, and *C3* gene polymorphisms and retinal angiomatous proliferation (RAP), an infrequent and severe form of exudative age-related macular degeneration, which is characterized by intraretinal neovascularization.

Methods: Diagnosis of RAP was based on fundus photographs, images of fluorescein and indocyanine green angiographies, and optical coherence tomography findings. Six single nucleotide polymorphisms (SNPs), A69S (rs10490924) in *ARMS2*, rs11200638 in *HTRA1*, I62V (rs800292) in *CFH*, Y402H (rs1061170) in *CFH*, R80G (rs2230199) in *C3*, and rs2241394 in *C3*, were genotyped in eight Japanese patients with RAP.

Results: The two SNPs in the *ARMS2/HTRA1* were in complete linkage disequilibrium. The frequency of the risk T allele in *ARMS2* (the risk A allele in *HTRA1*) was 93.8% in the RAP patients. The frequency of homozygosity for the risk genotype TT of *ARMS2* (the risk genotype AA of *HTRA1*) was 87.5%. The frequency of the non-risk allele (A) of I62V was 100%. The frequencies of risk alleles of Y402H, R80G, and rs2241394 were 12.5%, 0%, and 18.8%, respectively.

Conclusion: Our results suggest that the risk alleles of the *ARMS2/HTRA1* SNPs may be associated with development of RAP and play a major role in the pathogenesis of intraretinal angiogenesis.

Keywords: age-related macular degeneration, retinal angiomatous proliferation, single nucleotide polymorphisms, *ARMS2/HTRA1* genes, components of the complement system

Introduction

Age-related macular degeneration (AMD) is the most common cause of legal blindness in the elderly, affecting more than 50 million people worldwide.[1] In Japan, the prevalence of AMD has risen from 0.87% in 1988 to 1.4% in 2007.[2,3] Maruko et al have classified exudative AMD patients into three subtypes, namely typical wet-type AMD, polypoidal choroidal vasculopathy (PCV), and retinal angiomatous proliferation (RAP).[4]

AMD is a multifactorial disease with genetic, behavioral, and environmental factors.[5] Recently, genetic association studies have revealed that single nucleotide polymorphisms (SNPs) in *CFH* (1q32), *ARMS2/HTRA1* (10q26), and *C3* (19p13) have been identified as major contributors to the pathogenesis of AMD.[6–17] Among various SNPs of those genes, the Y402H (rs1061170) and I62V (rs800292) variants in the *CFH* gene and the A69S (rs10490924) variant in the *ARMS2* gene have been investigated in detail.[6–15,18–27] The differences in genotypes associated with AMD

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have been investigated among various ethnic groups and by subtypes of exudative AMD,[16,18–27] showing that the I62V and A69S variants are associated with AMD in both Caucasian and Asian subjects.[18–27] The Y402H and R80G (in the *C3* gene) variants have been associated with AMD in Caucasians[6–15] but not in Asians.[18,19,21–25,28] The C allele of the Y402H variant and the G allele of the R80G variant are infrequent in Asians.

The term RAP was first coined by Yannuzzi et al in 2001.[29] They suggested the retinal origin of this neovascularization, which proceeds posteriorly and finally forms a retinal-choroidal anastomosis. RAP is sometimes called type 3 neovascularization to distinguish it from type 1 neovascularization (choroidal neovascularization under the retinal pigment epithelium) and type 2 neovascularization (choroidal neovascularization that penetrates the retinal pigment epithelium).[30,31] RAP accounts for 4.5% of all exudative AMD in Japanese patients[4] and 15% of exudative AMD in Caucasian patients.[32] RAP is characterized by bilateral, multiple soft drusen, intraretinal hemorrhages, and intraretinal edema. The natural history of RAP is characterized by a rapid loss of vision.[33] RAP resists various treatments and recurs persistently.[34–40]

The phenotypic diversity of AMD is thought to be related to differences in genetic backgrounds.[20,24–27] Various reports have examined genetic backgrounds in PCV. Lee et al reported that the I62V and A69S variants, but not the Y402H variant, were related to PCV in Chinese patients.[23] Hayashi et al reported that all three of these SNPs (I62V, Y402H, and A69S) were related to PCV in Japanese patients.[26] Goto et al reported that rs2241394 in the *C3* gene was associated with PCV.[25]

Wegscheider et al reported that the Y402H polymorphism was associated with RAP in Caucasians.[20] However, the genetic association with RAP has not been evaluated sufficiently because of the rarity of RAP in Japan. There are only a few reports about associations between A69S and RAP in the Japanese population.[26,27] The purpose of the current study was to investigate the involvement of genetic factors in not only the *ARMS2/HTRA1* but also the *CFH* and *C3* genes in Japanese patients with RAP.

Materials and methods

The study was approved by the institutional review board of The Jikei Medical University, and all procedures were conducted in accordance with the principles of the Declaration of Helsinki. Eight unrelated Japanese patients with RAP were recruited from the Department of Ophthalmology at The Jikei Medical University and the National Hospital Organization Tokyo Medical Center. Informed consent was obtained from all subjects.

All patients with RAP underwent a full ophthalmic examination, including slit-lamp biomicroscopy, funduscopy, optical coherence tomography, and fluorescein and indocyanine green fundus angiographies. The diagnosis of RAP was based on the criteria of Yannuzzi et al[29] and was classified as a defined anastomosis connecting the retinal circulation to a vascular complex within the retina, usually with surrounding intraretinal blood and intraretinal or cystoid macular edema.

Genomic DNA was extracted from the peripheral blood of each individual. A total of six SNPs consisting of A69S (rs10490924) in *ARMS2*, rs11200638 in *HTRA1*, Y402H (rs1061170) in *CFH*, I62V (rs800292) in *CFH*, R80G (rs2230199) in *C3*, and rs2241394 in *C3* were genotyped. Polymerase chain reaction amplification was performed using LA Taq polymerase (Takara Bio Inc, Ohtsu, Japan) and primers for *ARMS2* (forward primer: 5′-GCCTATACCCAGGACCGATG-3′, reverse primer: 5′-CATGTTCTCAGCATCTCCAAGTC-3′), *HTRA1* (forward primer: 5′-TCTCTGCGAATACGGACACG-3′, reverse primer: 5′-ACT GTG TCCATT CAG CTC CTA A-3′), *CFH* Y402H (forward primer: 5′-CAGAAATAGGGCCAAGAAAAGAGT-3′, reverse primer: 5′-ATGTAACTGTGGTCTGCGC-3′), *CFH* I62V (forward primer: 5′-GATTGCAATGAACTTCCTCCAAG-3′, reverse primer:5′-GGATTAAGAGCAACC CATTCTCC-3′), *C3* R80G (forward primer: 5′-CCTCGCACCTCCTTCACA-3′, reverse primer: 5′-TCTGGCTGGCACCTCAAT-3′), and *C3* rs2241394 (forward primer: 5′-GGCTGGGTGACTGTACCTCTTC-3′, reverse primer: 5′-CATGTTCTCAGCATCTCCAAGTC-3′) to amplify these regions. Polymerase chain reaction products were used as the templates for direct DNA sequencing kits (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (3730xl DNA analyzer; Applied Biosystems).

Results

Genetic analysis

Five men and three women were analyzed in the study. The mean patient age was 82.6±4.6 years (range 76–91 years). Both eyes were affected in four patients (50.0%). All SNPs were successfully genotyped in all patients (Table 1). The two SNPs in the *ARMS2/HTRA1* were in complete linkage disequilibrium. The frequency of the risk T allele in

Table 1 Polymorphisms in *ARMS2/HTRA1/CFH/C3* genes: genotypes in Japanese patients with retinal angiomatous proliferation

Patient number	Age	Sex	Affected eye	*ARMS2*	*HTRA1*	*CFH*		*C3*	
				rs10490924 (A69S)	rs1120638	rs800292 (I62V)	rs1061170 (Y402H)	rs2230199 (R80G)	rs2241394
1	83	F	Bilateral	**TT**	**AA**	AA	TT	CC	**CG**
2	79	M	Unilateral	**TT**	**AA**	AA	**CT**	CC	CC
3	84	M	Unilateral	**TT**	**AA**	AA	TT	CC	CC
4	87	F	Unilateral	**TT**	**AA**	AA	**CT**	CC	**CG**
5	82	M	Bilateral	**TT**	**AA**	AA	TT	CC	CC
6	78	M	Unilateral	**TT**	**AA**	AA	TT	CC	CC
7	76	F	Bilateral	**TT**	**AA**	AA	TT	CC	**CG**
8	91	M	Bilateral	**TG**	**AG**	AA	TT	CC	CC

Note: Risk alleles are shown in bold.
Abbreviations: M, male; F, female.

the *ARMS2* gene (the risk A allele in the *HTRA1* gene) was 93.8%. The frequency of homozygosity for the risk genotype (TT) of the *ARMS2* gene was 87.5%. The frequency of the non-risk allele (A) of I62V was 100%. The frequencies of risk alleles of Y402H in the *CFH* gene, rs2230199 (R80G) and rs2241394 in the *C3* gene were 12.5%, 0%, and 18.8%, respectively.

Representative case (patient 1)

An 83-year-old woman with homozygosity for the risk genotype (TT) of the *ARMS2* gene presented with bilateral RAP (Figure 1). She had undergone cataract surgery in both eyes prior to diagnosis of RAP. Her decimal best-corrected visual acuity was 0.3 in the right eye and 0.07 in the left eye. Both eyes were treated by standard-fluence photodynamic therapy with verteporfin (Visudyne®, Novartis Pharma AG, Basel, Switzerland) in combination with 1.25 mg (0.05 mL) of intravitreal bevacizumab (Avastin®, Genentech, San Francisco, CA, USA), and her vision improved to 0.5 in the right eye and 0.15 in the left eye, with a rapid resolution of intraretinal edema. There was no recurrence of intraretinal edema or hemorrhages, and her vision remained stable for 2 years following the combination therapy.

Discussion

In this study we genotyped six SNPs in RAP patients that were highly representative of the common genetic variations of exudative AMD. Our results raise the possibility of an association between *ARMS2* (A69S)/*HTRA1* (rs1120638) variants and RAP, but a weak association for the other SNPs. Hayashi et al recently demonstrated that the A69S, Y402H, and I62V variants are associated with RAP and that the A69S variant has the strongest association for RAP among the three exudative AMD subtypes.[26] Tanaka et al also reported that A69S might serve as strong genetic markers of RAP.[27] Our findings are consistent with their findings[26,27] regarding the A69S variant, but we had negative results for the I62V (*CFH*) variant (Table 2).

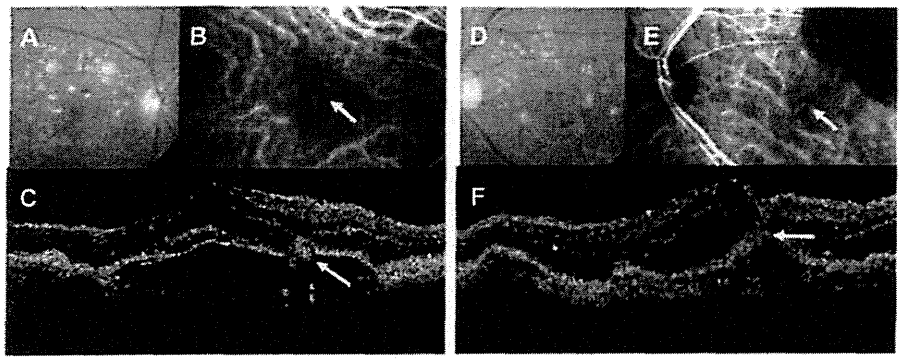

Figure 1 Color fundus photographs (**A** and **D**), indocyanine green fundus angiographies (**B** and **E**), and optical coherence tomography images (**C** and **F**) from the right eye (**A–C**) and the left eye (**D–F**) of an 83-year-old woman (patient 1).
Notes: We diagnosed her right eye with stage II retinal angiomatous proliferation and her left eye with stage III retinal angiomatous proliferation. (**A** and **D**) Fundus image shows intraretinal hemorrhages with a large number of soft drusen and pigment epithelial detachment. (**B**) Indocyanine green fundus angiographies shows some hotspots. One of them connects retinal vessels (arrow), corresponding to the intraretinal neovascularization. (**C**) A vertical optical coherence tomography image shows a pigment epithelial detachment, cystoid macular edema, and retinal angiomatous proliferation lesion (arrow). (**E**) Indocyanine green fundus angiographies shows choroidal neovascularization (arrow) that connects retinal vessels, corresponding to retinal-choroidal anastomosis. (**F**) A vertical optical coherence tomography image shows a pigment epithelial detachment, cystoid macular edema, and a retinal pigment epithelium line that has ruptured (arrow).

Table 2 Genotype frequency of A69S (*ARMS2*), I62V (*CFH*), and Y402H (*CFH*) polymorphisms in Japanese controls and Japanese patients with retinal angiomatous proliferation

Genotype	rs10490924 (A69S)			rs800292 (I62V)			rs1061170 (Y402H)		
	TT	TG	GG	GG	GA	AA	CC	CT	TT
Controls (n=1,351)[26]	196 (14.6%)	638 (47.8%)	502 (37.6%)	456 (34.1%)	649 (48.5%)	233 (17.4%)	8 (0.6%)	160 (11.9%)	1,174 (87.5%)
Patients (n=36)[26]	31 (86.1%)	3 (8.3%)	2 (5.6%)	20 (55.6%)	11 (30.6%)	5 (13.8%)	0 (0%)	5 (14.3%)	30 (85.7%)
Patients (n=51)[27]	39 (76.5%)	10 (19.6%)	2 (3.9%)	29 (56.9%)	20 (39.2%)	2 (3.9%)	ND	ND	ND
Patients (n=8) (in present study)	7 (87.5%)	1 (12.5%)	0 (0%)	0 (0%)	0 (0%)	8 (100%)	0 (0%)	2 (25%)	6 (75%)

Note: Risk alleles are shown in **bold**.
Abbreviation: ND, not described.

Components of the complement system have been identified in drusen, indicating a potential role of the complement system in the pathogenesis of AMD.[41,42] C3 and CFH are key components of the alternative complement pathway. C3 is the most abundant complement component and is synthesized predominantly in the liver. Cleavage of C3 into C3a and C3b is the central step in complement activation and can be initiated by the classic antibody-mediated pathway, the lectin pathway, or the alternative complement pathway. CFH is a critical negative regulator of the alternative pathway of the complement system. It binds to C3b, promotes the decay of C3 convertase, and serves as a cofactor for the factor I-mediated proteolytic inactivation of C3b, resulting in inhibition of the complement cascade.

Because there are bilateral multiple soft drusen in the presence in RAP, we suspected that RAP would be more strongly associated with genetic abnormalities in the complement system than other AMD subtypes. However, we hardly detected the risk alleles of the *CFH* and *C3* genes. One reason for these results is the infrequency of the C allele in Y402H and the G allele in R80G in Asians. Reticular pseudodrusen (RPD) are defined as "drusen that form ill-defined networks of broad interlacing ribbons" in the Wisconsin grading system for maculopathy.[43] RPD have been recognized as a distinctive morphologic feature observed in exudative AMD.[44] Recent studies have demonstrated the association between RPD and reduced macular sensitivity.[45,46] Importantly, it is reported that the prevalence of RPD was high in patients with RAP and the risk genotype (TT) in A69S,[47] and RPD usually occurs bilaterally,[48] suggesting the impact of genetic background for RPD.

It was an unexpected result that we did not detect the risk G allele in I62V (Table 2). The I62V variant has been associated with exudative AMD in both Caucasian and Asian patients.[18,19,21–25,27] In Japanese population samples, it has been demonstrated that the risk genotype (GG) in I62V is significantly associated with RAP (Table 2).[26,27] However, the risk genotype in I62V was not detected in our RAP patients (Table 2). Our findings suggest that the presence of the risk genotype (I62V) may not be necessarily associated with development of RAP. A larger sample size will be required to determine whether the risk genotype in I62V is eventually associated with RAP.

RAP is characterized by intraretinal neovascularization above the retinal pigment epithelium. Two different origins of this neovascularization have been proposed. Yannuzzi et al suggested that the neovascularization in RAP originates from the neural retina.[29] On the other hand, Freund et al proposed type 3 neovascularization that originates not only from deep retinal capillaries but also from the choroid.[31] As for the location of gene expression, the *CFH* gene is expressed primarily in the retinal pigment epithelium, drusen, and choroidal capillaries;[6] the *C3* gene is expressed in the neural retina, choroid, and retinal pigment epithelium;[41] and the *ARMS2* gene is expressed in the ellipsoid region of the photoreceptor cells.[14] Since it seems that the location of characteristic neovascularization corresponds to the location of susceptible gene expression in RAP, our results support the hypothesis by Yannuzzi et al[29] that the origin of neovascularization in RAP is in the neural retina.

In conclusion, our results suggest that the risk alleles/genotypes of the *ARMS2/HTRA1* SNPs may be strongly associated with development of RAP and that they play a major role in the pathogenesis of intraretinal angiogenesis.

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Disclosure

The authors report no conflicts of interest in this work.

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Whole-exome sequencing identifies a novel *ALMS1* mutation (p.Q2051X) in two Japanese brothers with Alström syndrome

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Purpose: No mutations associated with Alström syndrome (AS), a rare autosomal recessive disease, have been reported in the Japanese population. The purpose of this study was to investigate the genetic and clinical features of two brothers with AS in a consanguineous Japanese family.

Methods: Whole-exome sequencing analysis was performed on two brothers with AS and their unaffected parents. We performed a complete ophthalmic examination, including decimal best-corrected visual acuity, slit-lamp and funduscopic examination, visual-field and color-vision testing, full-field electroretinography, and optical coherence tomography. Fasting blood tests and systemic examinations were also performed.

Results: A novel mutation (c.6151C>T in exon 8) in the Alström syndrome 1 (*ALMS1*) gene that causes a premature termination codon at amino acid 2051 (p.Q2051X), was identified in the homozygous state in the affected brothers and in the heterozygous state in the parents. The ophthalmologic findings for both brothers revealed infantile-onset severe retinal degeneration and visual impairment, marked macular thinning, and severe cataracts. Systemic findings showed hepatic dysfunction, hyperlipidemia, hypogonadism, short stature, and wide feet in both brothers, whereas hearing loss, renal failure, abnormal digits, history of developmental delay, scoliosis, hypertension, and alopecia were not observed in either brother. The older brother exhibited type 2 diabetic mellitus and obesity, whereas the younger brother had hyperinsulinemia and subclinical hypothyroidism.

Conclusions: A novel *ALMS1* mutation was identified by using whole-exome sequencing analysis, which is useful not only to identify a disease causing mutation but also to exclude other gene mutations. Although characteristic ophthalmologic findings and most systemic findings were similar between the brothers, the brothers differed slightly in terms of glucose tolerance and thyroid function.

Alström syndrome (AS; OMIM: 203800) is a rare and autosomal recessive hereditary disease with an estimated prevalence of less than 0.001% [1,2]. AS is caused by mutations in the *ALMS1* gene, which is located on chromosome 2p13 [3,4]. ALMS1 is localized to centrosomes and ciliary basal bodies [5,6] and has been implicated in the function, formation, and maintenance of primary cilia [5,7–9]. Dysfunction of primary cilia caused by mutations in genes such as *ALMS1* leads to a multitude of human monogenic disorders known as ciliopathies [10,11]; these include plural systemic diseases, such as AS, Usher syndrome, Bardet–Biedl syndrome (BBS), Senior–Løken syndrome, Joubert syndrome, Meckel–Gruber syndrome, and orofaciodigital syndrome 1 [11,12]. The majority of *ALMS1* mutations are nonsense and frameshift variations (primarily clustered in exons 8, 10, and 16) that are predicted to cause truncated proteins [3,4,13]. In the photoreceptors, *ALMS1* mutations lead to defective function of the connecting cilium.

AS is characterized by a wide spectrum of disorders, such as early onset severe retinal degeneration, obesity from childhood, hyperinsulinemia, type 2 diabetic mellitus (T2DM), hepatic dysfunction, heart failure, sensory hearing loss, and renal failure [14]. Other manifestations include acanthosis nigricans, alopecia, hypogonadism, hypothyroidism, hyperlipidemia, short stature, and scoliosis [15,16]. In most cases of AS, cone–rod degeneration in the first decade, normal intelligence, and no polydactyly serve as a differential diagnosis of BBS, which exhibits similar clinical findings to AS [17].

Almost all patients with AS show nystagmus and severe photophobia from infancy [14,18]. Visual impairment is usually seen at an age younger than 1 year [18]. Although the rate of progression of vision loss is variable, all patients

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