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Five major sequence variants and copy number variants in the *EYS* gene account for one-third of Japanese patients with autosomal recessive and simplex retinitis pigmentosa

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Purpose: To elucidate the variant spectrum of the *EYS* gene in a large cohort of Japanese patients with autosomal recessive and simplex retinitis pigmentosa (arRP and sRP).
Methods: We performed a direct sequencing analysis of 44 exons of the *EYS* gene in 469 patients with RP (including 144 arRP, 288 sRP, and 17 autosomal dominant RP (adRP) cases) in eastern and western regions of Japan and a multiplex ligation-dependent probe amplification (MLPA) of patients who had a single heterozygous pathogenic variant.
Results: We identified six pathogenic and 16 likely pathogenic variants from a total of 186 nucleotide sequence variants, of which five variants, c.2528G>A (p.(Gly843Glu)), c.4957dupA (p.(Ser1653Lysfs*2)), c.6557G>A (p.(Gly2186Glu)), c.6563T>C (p.(Ile2188Thr)), and c.8868C>A (p.(Tyr2956*)), were prevalent in patients with arRP and sRP. The homozygous and heterozygous combinations of these five variants accounted for 32.4% (140/432) of Japanese patients with arRP and sRP. Five patients with adRP also had these variants. These five variants segregated with the phenotype in 15 families with RP. MLPA revealed seven copy number variations (CNVs) of the *EYS* exon(s).
Conclusions: This study showed that five major sequence variants and CNVs in the *EYS* gene account for one-third of Japanese patients with arRP and sRP, and these variants are also responsible for RP showing an autosomal dominant inheritance pattern. This is the first report showing the pathogenicity of three missense variants (p.(Gly843Glu), p.(Gly2186Glu), and p.(Ile2188Thr)) and the presence of CNVs in the *EYS* gene of Japanese patients with arRP and sRP.

Retinitis pigmentosa (RP) is an inherited retinal dystrophy characterized by progressive degeneration of photoreceptors, which causes night blindness and constriction of the visual fields followed by impairment of central and color vision. The prevalence of RP is 1 in 3,000 to 5,000 individuals worldwide [1]. RP can be inherited as an autosomal dominant (ad), autosomal recessive (ar), or X-linked (xl) trait. The major inheritance pattern of RP is autosomal recessive if assuming that all of isolated cases are autosomal recessive [2]. To date, 63 genes have been identified for the autosomal recessive form of RP (arRP; RetNet). Among them, *EYS* (OMIM 612424) has been reported as the most prevalent gene responsible for arRP cases in different populations: 11% in British and Chinese [3], 12% in French [4], 5% in Dutch [5], 7% in Israeli [6], 15.9% in Spanish [7], and 15.0% to 32.8% in Japanese [8-11]. Recently, Messchaert et al. collected variants in *EYS* in patients with RP and uploaded them in the Leiden Open Variation Database (LOVD) [12].

EYS was first identified as a commonly mutated gene in arRP [13,14]. The *EYS* gene is located at chromosome 6q12, and spans 44 exons and more than 2 Mb of genomic DNA. The longest transcript isoform is composed of 10,475 nucleotides and encodes a large protein of 3,165 amino acids that contains a signal peptide, 28 EGF-like domains, and five laminin A G-like domains [14]. The human EYS protein is a homolog of the *Drosophila* eyes shut (eys) protein that plays a role in the modeling of retinal architecture as an extracellular matrix [15,16]. EYS homologs have been noted in vertebrates (for example, zebrafish, chicken, and dog), but they are absent in rodents, such as the mouse and rat. Recently, a zebrafish retina, which is morphologically similar to that of a human retina [17], was shown to express the *eys* gene, and *eys*-deficient zebrafish exhibited progressive loss of cone and rod photoreceptors [18-20].

In a previous study on patients of the National Rehabilitation Center for Persons with Disabilities (NRCD), we found that one-third of 64 Japanese patients with

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nonsyndromic autosomal recessive and simplex RP (arRP and sRP) carried probable pathogenic variants in the *EYS* gene, in which a frameshift variant c.4957dupA (p.(Ser1653Lysfs*2), hereafter denoted as JV1) and a nonsense variant c.8868C>A (p.(Tyr2956*), denoted as JV2) were major variants [8]. Furthermore, we noticed that there were significantly more patients with arRP and sRP carrying a missense variant c.2528G>A (p.(Gly843Glu), denoted as JV3) compared with controls, suggesting that this variant is possibly pathogenic. Thereafter, a study on patients at Kyoto University was performed with next-generation sequencing and showed that variants in *EYS* accounted for 15.0% of the patients with arRP and sRP [10]. Three reports on the *EYS* variants of Japanese patients with arRP showed that JV1 and JV2 are prevalent variants, but JV3 is regarded as a benign variant [9-11], mainly because the minor allele frequency (MAF) of JV3 is relatively high (about 0.02) in the Japanese population. Although copy number variations (CNVs) in *EYS* have also been shown to be the candidate for the second pathogenic variant in a patient with arRP with a single heterozygous variant [21], there has been no report of CNVs in *EYS* in Japanese patients with arRP.

In this study, we performed a direct sequencing analysis of 44 exons and exon-intron boundaries of the *EYS* gene using a large cohort of patients with arRP and sRP in eastern and western regions of Japan, to elucidate the variant spectrum of the *EYS* gene of Japanese patients with arRP and sRP, and performed segregation analysis to obtain evidence of the pathogenicity of major likely pathogenic variants, including JV3. Furthermore, the presence of CNVs was examined in patients with a single heterozygous pathogenic variant.

METHODS

Patients: We studied 261 unrelated patients with RP, including 68 patients reported in the previous paper [8]), who visited the low-vision clinic at NRCD and 38 family members of 15 pedigrees, and 208 unrelated patients with RP who visited the Department of Ophthalmology and Visual Science, Kyoto University Graduate School of Medicine. The NRCD patients included 73 arRP and 160 sRP cases, and the Kyoto patients included 71 arRP and 128 sRP cases. The total number of patients with arRP and sRP was 432. All patients underwent visual acuity measurements, slit-lamp biomicroscopy, ophthalmoscopy, optical coherence tomography, Goldman visual field testing, and electroretinography. A pedigree was constructed based on a patient interview, from which an inheritance pattern was inferred. Control subjects were recruited from the staff of the NRCD, all of whom declared having neither a personal history nor a family history of night blindness or unexplained visual loss. The study was approved by the institutional review boards of the NRCD and Kyoto University Graduate School of Medicine, and was conducted in accordance with the Declaration of Helsinki. All participants were fully informed of the purpose and procedures of this study, and written consent was obtained from each participant.

Sequencing analysis: Genomic DNA was isolated from venous blood using a DNA purification kit (Puregene; Gentra Systems, Minneapolis, MN) for the subjects of NRCD and a DNA extraction kit (Quick-Gene-610L; Fujifilm, Tokyo, Japan) for the patients of Kyoto University. Genomic DNA of family members was isolated from their saliva using a DNA self-collection kit (Oragene®-DNA OG-500; DNA Genotek, Ottawa, Canada). The primer sequences used for PCR to amplify each exon of the *EYS* gene and sequencing primers were shown in previous papers [8,14]. Two hundred nanograms of genomic DNA were amplified with Taq polymerase (TaKaRa PrimeSTAR; Takara Bio Inc., Shiga, Japan), and a sequence variant analysis was performed with direct sequencing of the purified PCR products (BigDye Terminator Cycle Sequencing Kit; Applied Biosystems, Foster City, CA). Sequencing reaction products were run on an automated capillary sequencer (3130xl Genetic Analyzer; Applied Biosystems).

Sequence data analysis: The genomic sequence of the *EYS* locus (NC_000006.12) and the mRNA sequence (NM_001292009.1) were retrieved from the National Center for Biotechnology Information, and were used as reference sequences. Nucleotide A of the initiation codon of *EYS* was defined as position 1. The sequence variants were designated in accordance with the Human Genome Variation Society recommendations (HGVS). The following reference databases on nucleotide sequence variants were used: dbSNP, 1000Genome, ExAC, Genome Aggregation Database, Human Genetic Variation Database (HGVD), and Integrative Japanese Genome Variation Database (iJGVD, 3.5KJPN). HGVD and iJGVD are the genetic variant databases of individuals in the Japanese population. To assess the pathogenicity of missense variants, seven types of prediction scores for amino acid substitutions were used: PolyPhen2, SIFT, PMut, SNAP2, Mutation Tester2, Mutation Assessor, and Combined Annotation-Dependent Depletion (CADD) [22]. Variants interpreted as putative pathogenic were classified according to standards and guidelines from the American College of Medical Genetics and Genomics (ACMG) [23].

Copy number variation analysis: Multiplex ligation-dependent probe amplification (MLPA) was performed

by FALCO Biosystems Ltd. (Kyoto, Japan) using a Salsa® MLPA® Probemix P328-A1 EYS kit (MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer's instructions. This kit is designed to detect 44 exons of the *EYS* gene.

RESULTS

Nucleotide sequence variants: We identified a total of 186 nucleotide sequence variants in the *EYS* genes of 469 patients with RP (including 144 patients with arRP, 288 patients with sRP, 17 patients with adRP, three patients with xlRP, three patients with Leber congenital amaurosis (LCA), seven patients with Usher syndrome, and seven uncertain patients with RP) and 105 controls. The nucleotide change, the predicted protein change, and the MAF values of each variant are shown in Appendix 1. Four variants were found in a promoter region, 78 were in exons, and 104 were in introns. There was no variant at the exon-intron junction. The 78 variants in the exons consisted of 65 single nucleotide substitutions, seven deletions, four insertions, and two deletion-insertions. Sixty-two single nucleotide substitutions were found in the coding region of exons; they consisted of 43 missense variants, six nonsense variants, and 13 silent variants. Eleven deletion and insertion variants found in exons caused a frameshift followed by a premature termination codon. One hundred variants had an MAF of >0.01 and had already been registered in dbSNP, except c.*183A>C that was linked to JV2. Thirty-two variants with an MAF of <0.01 were novel, of which 15 were found in exons. Twenty-three variants with an MAF of <0.01 were found in one subject for each variant.

Nucleotide deletion and insertion variants and nonsense variants: Table 1 shows 11 frameshift and two non-frameshift variants caused by deletion or insertion of nucleotides, and six nonsense variants. All variants except a non-frameshift variant c.7028_7029delinsATCGT (p.(Leu2343delinsHisArg)) were classified as pathogenic or likely pathogenic according to the ACMG guideline. Three frameshift variants, c.942delT (p.(Ala315Leufs*24)), c.7616delG (p.(Gly2539Glufs*14)), and c.8331delC (p.(Val2778Phefs*14)), were novel. The most frequent variant was JV1 in exon 26, which was carried by 75 of 469 patients with RP (16.0%). The second most frequent variant was JV2 in exon 44, which was carried by 42 of 469 patients (9.0%). According to HGVD, the MAFs of JV1 and JV2 in the Japanese population were 0.0021 (5/2404) and 0.0029 (7/2420), respectively. There are no data for JV1 and JV2 in 1000Genome, ExAC, and iJGVD. Interestingly, gnomAD contains JV1 (1/151914) and JV2 (7/188242); both were found in the Korean population, and the MAFs of JV1 and JV2 were 0.00053 (1/1870) and 0.0016 (3/1870), respectively.

Two frameshift variants, two nonsense variants, and one non-frameshift variant were found in more than two patients. p.(Asn404Lysfs*3) was carried by three patients, and has been reported in one Moroccan Jewish patient with RP [6] and one Chinese patient with RP [24]. This variant would not be a founder variant because the variant was on the different haplotype (data not shown). p.(Trp2640*) was carried by four patients. This variant has been reported as disease causing in two Spanish families with arRP [7,13], one Chinese family with arRP [25], and one Dutch patient with RP [26]. Therefore, these variants seem to be recurring. The non-frameshift variant c.525_527delGGA (p.(Glu176del)) was found in five patients (MAF=0.0053), of whom four patients carried JV1, JV2, or JV3 as a compound heterozygous variant. This variant has also been reported in one Japanese patient and one control [9]. The MAF of this variant in the databases was 0.0008 (2/2420, HGVD), 0.000115 (14/121330, ExAC), and 0.00096 (27/282652, gnomAD). The MAF of this variant in the Korean population was 0.0066 (25/3792, gnomAD). c.5202_5203delGT (p.(Phe1735Glnfs*6)) and c.8012T>A (p.(Leu2671*)) have been reported to segregate with the phenotype [11,27].

Missense variants: The total number of missense variants was 43, from which 13 candidates for arRP-causing variants were selected and shown in Table 2. Twelve variants were found only in patients with RP and not in controls, HGVD, and iJGVD, four of which were novel: c.7713T>G (p.(Asn2571Lys)), c.8696C>G (p.(Ala2899Gly)), c.8759G>C (p.(Cys2920Ser)), and c.9094A>G (p.(Ile3032Val)). JV3 was found in controls, but statistically significantly more frequently in patients with RP (chi-square test, p=0.0056). Although the ratio of a patient's MAF to a control's MAF on missense variants with an MAF of >0.01 ranged from 0.34 to 2.22, the MAF ratio for JV3 was 3.64 (Appendix 1). c.6557G>A (p.(Gly2186Glu), denoted as JV4) was found in four patients with arRP and five patients with sRP. c.6563T>C (p.(Ile2188Thr), denoted as JV5) was found in one patient with arRP, one patient with adRP, and four patients with sRP. The remaining variants were carried by fewer than four patients with RP.

To assess the pathogenicity of each variant, we adopted the ACMG standards and guidelines [23]. PP3, one of the supporting criteria for a pathogenic variant, was assessed with the use of seven in silico methods that were developed to predict the functional effect of the amino acid change: PolyPhen2, SIFT, PMut, SNAP2, Mutation Taster2, Mutation Assessor and CADD. Appendix 2 shows the prediction results

Table 1. Nucleotide deletion and insertion variants and nonsense variants.

ID	Variant	No. of subjects			MAF	MAF (Database)	Reference	ACMG evidence	ACMG class
		RP	KRP	CT	RP+KRP				
Ex4–3	c.525_527delGGA p.(Glu176del)	2	3	0	0.0053	0.0008 (HGVD) 0.000115 (ExAC) 0.00096 (gnomAD)	9	PS4, PM4	LP
Ex6–1	c.942delT p.(Ala315Leufs*24)	1	0	0	0.0015	-	-	PVS1, PM2	LP
Ex8–1	c.1211dupA p.(Asn404Lysfs*3)	3	0	0	0.0032	0.000008 (ExAC) 0.000016 (gnomAD)	6, 8, 24	PVS1, PS4	P
Ex10–1	c.1485_1493delinsCGAAAAG p.(Val496Glufs*13)	1	0	0	0.0015	-	8	PVS1, PM2	LP
Ex11–2	c.1750G>T p.(Glu584*)	0	2	0	0.0021	-	10, 32	PVS1, PM2	LP
Ex26–8	c.4387delA p.(Arg1463Glyfs*15)	0	1	0	0.0011	-	10	PVS1, PM2	LP
Ex26–9	c.4395_4402dupTCAAGAGG p.(Asp1468Valfs*13)	0	1	0	0.0011	-	10	PVS1, PM2	LP
Ex26–16 (JV1)	c.4957dupA p.(Ser1653Lysfs*2)	45	30	0	0.097	0.000007 (gnomAD) 0.0021 (HGVD)	8–11, 29, 31, 32	PVS1, PS4, PM3, PP1	P
Ex26–17	c.5014C>T p.(Gln1672*)	0	1	0	0.0011	-	10	PVS1, PM2	LP
Ex26–18	c.5202_5203delGT p.(Phe1735Glnfs*6)	0	1	0	0.0011	-	10, 11	PVS1, PM2, PP1	P
Ex35–3	c.7028_7029delinsATCGT	1	0	0	0.0016	-	8	PM2, PM4	VUS

ID	Variant	No. of subjects			MAF	MAF (Database)	Reference	ACMG evidence	ACMG class
		RP	KRP	CT	RP+KRP				
	p.(Leu2343delinsHisArg)								
Ex37–1	c.7283C>A	1	0	0	0.0011	-	8	PVS1, PM2	LP
	p.(Ser2428*)								
Ex39–2	c.7616delG	1	0	0	0.0011	-	-	PVS1, PM2	LP
	p.(Gly2539Glufs*14)								
Ex39–3	c.7665_7666delCA	1	0	1	0.0011	-	8	PVS1, PM2	LP
	p.(Tyr2555*)								
Ex41–1	c.7919G>A	1	3	0	0.0064	0.000026 (gnomAD)	7, 10,	PVS1, PS4,	P
	p.(Trp2640*)						13, 25, 26	PP1	
Ex41–2	c.8012T>A	0	1	0	0.0011	0.000013 (gnomAD)	10, 27	PVS1, PM2,	P
	p.(Leu2671*)							PP1	
Ex44–1	c.8331delC	0	1	0	0.0011	-	-	PVS1, PM2	LP
	p.(Val2778Phefs*14)								
Ex44–2	c.8439_8442dupTGCA	0	1	0	0.0011	-	10,11	PVS1, PM2	LP
	p.(Glu2815Cysfs*19)								
Ex44–5	c.8868C>A	21	21	0	0.048	0.000037 (gnomAD)	8–11, 29	PVS1, PS4,	P
(JV2)	p.(Tyr2956*)					0.0029 (HGVD)		PP1	

MAF, minor allele frequency; LP, likely pathogenic; P, pathogenic; VUS, a variant of uncertain significance

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Table 2. Putative pathogenic missense variants.

ID	Variant	No. of subjects			MAF	MAF (Database)	Reference	ACMG evidence	ACMG class
		RP	KRP	CT	RP+KRP				
Ex4-4	c.632G>A p.(Cys211Tyr)	2	1	0	0.0032	-	11	PS4, PP3	VUS
Ex16-2 (JV3)	c.2528G>A p.(Gly843Glu)	34	24	5	0.0693	0.0014 (1000genome) 0.00002 (gnomAD) 0.022 (HGVD) 0.017 (iJGVD)	8, 9	PS4, PM3, PP1, PP3	LP
Ex23-1	c.3454G>A p.(Gly1152Arg)	1	0	0	0.0015	0.00011 (gnomAD)	-	PM2, PP3	VUS
Ex32-2 (JV4)	c.6557G>A p.(Gly2186Glu)	6	3	0	0.0117	0.00004 (gnomAD)	3, 5, 9-11, 31, 32	PS4, PM3, PP1, PP3	LP
Ex32-3 (JV5)	c.6563T>C p.(Ile2188Thr)	2	4	0	0.0064	-	11	PS4, PM3, PP1	LP
Ex35-1	c.6844G>A p.(Glu2282Lys)	1	0	0	0.0015	0.0002 (1000genome) 0.000006 (gnomAD)	-	PM2, PP3	VUS
Ex39-5	c.7713T>G p.(Asn2571Lys)	1	0	0	0.0011	-	-	PM2, PP3	VUS
Ex40-1	c.7793G>A p.(Gly2598Asp)	1	1	0	0.0021	-	9, 10	PM2, PP3	VUS
Ex44-3	c.8696C>G p.(Ala2899Gly)	0	1	0	0.0011	-	-	PM2, BP4	VUS
Ex44-4	c.8759G>C p.(Cys2920Ser)	1	0	0	0.0011	-	-	PM2, PP3	VUS
Ex44-6	c.9082G>T p.(Asp3028Tyr)	1	0	0	0.0011	0.00001 (gnomAD)	12, 28	PM2, PM3, PP1, PP3	LP
Ex44-7	c.9094A>G p.(Ile3032Val)	1	0	0	0.0011	-	-	PM2, BP4	VUS
Ex44-8	c.9164A>G p.(Tyr3055Cys)	0	1	0	0.0011	0.00005 (ExAC) 0.00001 (gnomAD)	-	PM2, PP3	VUS

MAF, minor allele frequency; LP, likely pathogenic; P, pathogenic; VUS, a variant of uncertain significance.

Table 3. Major likely pathogenic variants carried by Japanese patients with arRP and sRP.

Name	Variant	NRCD (n=233)			Kyoto (n=199)			Total (n=432)		
		Patients	%	Alleles	Patients	%	Alleles	Patients	%	Alleles
JV1	c.4957dupA p.(Ser1653Lysfs*2)	42	18	53	30	15.1	34	72	16.7	87
JV2	c.8868C>A p.(Tyr2956*)	20	8.6	23	21	10.6	21	41	9.5	44
JV3	c.2528G>A p.(Gly843Glu)	32	13.7	36	24	12.1	26	56	13	62
JV4	c.6557G>A p.(Gly2186Glu)	7	3	8	3	1.5	4	10	2.3	12
JV5	c.6563T>C p.(Ile2188Thr)	1	0.4	1	4	2	4	5	1.2	5

for each variant. As a result, four variants, JV3, JV4, JV5, and p.(Asp3028Tyr), were classified as likely pathogenic, and the others were classified as variants of uncertain significance. Two variants have been reported to be likely pathogenic: p.(Gly2598Asp) was carried together with JV1 as a compound heterozygous variant by one Japanese patient with arRP [9]; p.(Asp3028Tyr) was carried as a homozygous variant by one Indonesian family [28].

Twenty-two missense variants with an MAF of >0.01 found in patients were likely to be benign except JV3 (MAF=0.0693) and JV4 (0.0117), because the MAFs of these variants were similar to those of the controls (Appendix 1). Among them, p.(Cys461Tyr; MAF=0.0314, HGVD) and p.(Thr2465Ser; MAF=0.0296, HGVD) were frequently found in the Japanese population. According to gnomAD, Koreans also carry p.(Cys461Tyr; MAF=0.0153) and p.(Thr2465Ser; MAF=0.0091). Of rare missense variants with an MAF of <0.01, p.(Glu335Asp), p.(Val1270Gly), and p.(Lys1633Glu) were found in mainly Japanese and Korean populations (HGVD and gnomAD). Thus, these five variants seem to be characteristic in Japanese and Korean populations.

Variant spectrum of the EYS gene in Japanese patients with RP: Patients carrying possible pathogenic variants or rare variants with an MAF of <0.01 in the *EYS* gene are listed in Appendix 3. A total of 210 patients included 66 arRP, 133 sRP, eight adRP, one xlRP, one LCA, and one uncertain case. Unexpectedly, five patients with adRP had one of likely pathogenic variants as a homozygous or heterozygous combination: RP037 (V1/V1), RP151 (JV1/JV3), RP174 (JV1/JV2), RP233 (JV3/JV5), and RP288 (JV1/p.(Glu176del)). Three patients with adRP (RP034, RP109, RP153), one patient with xlRP (RP159), and one patient with LCA (RP294) had likely benign rare variants.

Table 3 shows the number of patients with arRP and sRP who carried at least one of five likely pathogenic variants. The most frequent variant was JV1 (16.7%, 72/432), followed in order by JV3 (13.0%, 56/432), JV2 (9.5%, 41/432), JV4 (2.3%, 10/432), and JV5 (1.2%, 5/432). Every variant was found in patients with arRP and sRP in eastern (around metropolitan Tokyo) and western (around metropolitan Kyoto) Japan. There was no statistically significant difference in the allele frequency of each variant between the eastern and western regions (chi-square test, p=0.342).

The recessive genotypes of patients with arRP and sRP were classified into homozygous (6.3%, 27/432), probable compound heterozygous (13.4%, 58/432), and single heterozygous (14.6%, 63/432), as shown in Table 4. In total, 148 out of 432 patients (34.3%) had at least one of the likely pathogenic variants in the *EYS* gene. There were all combinations among JV1, JV2, JV3, JV4, and JV5 except JV4/JV5 and JV5/JV5. The most frequent combination was JV1/JV1 (3.5%, 15/432) and JV1/JV3 (3.5%, 15/432), followed in order by JV1/JV2 (2.3%, 10/432) and JV2/JV3 (1.6%, 7/432). The homozygous and heterozygous combinations of these five variants accounted for 32.4% of Japanese patients with arRP and sRP (140/432).

MLPA: To examine the presence of CNVs, MLPA was performed on 53 patients who had a single heterozygous pathogenic variant. The presence or absence of CNVs in each patient is shown in Appendix 3. Table 5 shows six deletions and one duplication of exon(s) identified in seven patients (13.2%, 7/53), of which four cases are shown in Appendix 4. Three patients carrying JV1 (RP241, RP239, and KRP077), two patients carrying JV2 (RP177 and RP250), and one patient carrying JV4 (KRP054) had a deletion of exon(s). However, KRP190 had a duplication of exons 39–42. These

Table 4. The recessive genotypes of patients with arRP and sRP carrying likely pathogenic variants.

Variants		Type	No. of patients	Frequency (%)
Allele 1	Allele 2			
JV1	JV1	Homozygous	15	3.5
JV1	JV2	Compound heterozygous	10	2.3
JV1	JV3	Compound heterozygous	15	3.5
JV1	JV4	Compound heterozygous	3	0.7
JV1	JV5	Compound heterozygous	1	0.2
JV1	JVX	Compound heterozygous	6	1.4
JV1	NI	Single heterozygous	22	5.1
JV2	JV2	Homozygous	3	0.7
JV2	JV3	Compound heterozygous	7	1.6
JV2	JV4	Compound heterozygous	1	0.2
JV2	JV5	Compound heterozygous	1	0.2
JV2	JVX	Compound heterozygous	5	1.2
JV2	NI	Single heterozygous	14	3.2
JV3	JV3	Homozygous	5	1.2
JV3	JV4	Compound heterozygous	1	0.2
JV3	JV5	Compound heterozygous	2	0.5
JV3	JVX	Compound heterozygous	5	1.2
JV3	NI	Single heterozygous	19	4.4
JV4	JV4	Homozygous	2	0.5
JV4	NI	Single heterozygous	2	0.5
JV5	NI	Single heterozygous	1	0.2
JVX	JVX	Homozygous	2	0.5
JVX	JVY	Compound heterozygous	1	0.2
JVX	NI	Single heterozygous	5	1.2
		Total	148	34.3

JV1, c.4957dupA (p.(Ser1653Lysfs*2)); JV2, c.8868C>A (p.(Tyr2956*)); JV3, c.2528G>A (p.(Gly843Glu)); JV4, c.6557G>A (p.(Gly2186Glu)); JV5, c.6563T>C (p.(Ile2188Thr)); JVX and JVY, one of likely pathogenic variants; NI, not identified

CNVs seem to be pathogenic variants, because these nucleotide changes would cause a frameshift or a large deletion of an amino acid sequence in the EYS protein.

Segregation analysis of families with RP: We performed segregation analysis on 15 families with RP in which each proband carried at least one of the five likely pathogenic variants. The number of analyzed families was seven for JV1, five for JV2, ten for JV3, three for JV4, and two for JV5. Figure 1A shows three families with sRP carrying homozygous variants. A deceased individual with a normal phenotype seemed to carry a single heterozygous variant indicated in the parentheses. Figure 1B shows seven families (four with sRP, two with adRP, and one with arRP) carrying a likely compound heterozygous variant in combination with JV3. Interestingly, the RP174 family carries three types of variants (JV1, JV2, and JV3). Figure 1C and Figure 1D show three families with sRP carrying JV4 and two families (one sRP and one adRP) carrying JV5, respectively. These segregation analyses showed that all of the five likely pathogenic variants segregated with the phenotype in the families with RP.

DISCUSSION

In a previous study, we showed that one-third of 64 Japanese patients with arRP and sRP carried probable pathogenic variants in the *EYS* gene, including two founder variants, c.4957dupA (JV1) and c.8868C>A (JV2). In the present study,

Table 5. Copy number variations analysis on patients carrying a single heterozygous variant.

Variant	No. of patients		Patient ID	Copy number variations	
Allele 1	Sum	with CNVs		Exon	DNA change
JV1	20	4	RP241	Exon 1-Intron 1	Deletion
			RP239	Exon 31	Deletion
			KRP077	Exon 33	Deletion
			KRP190	Exon 39-Exon 42	Duplication
JV2	12	2	RP177	Exon 1	Deletion
			RP250	Exon 33	Deletion
JV3	18	0	-	-	-
JV4	2	1	KRP054	Exon 6-Exon 8	Deletion
JV5	1	0	-	-	-
Total	53	7			

JV1, c.4957dupA (p.(Ser1653Lysfs*2)); JV2, c.8868C>A (p.(Tyr2956*)); JV3, c.2528G>A (p.(Gly843Glu)); JV4, c.6557G>A (p.(Gly2186Glu)); JV5, c.6563T>C (p.(Ile2188Thr))

to analyze the variant spectrum of the *EYS* gene in Japanese patients with RP, we performed PCR-based direct sequencing analysis of 44 *EYS* exons and exon-junction boundaries on a total of 469 patients with RP who visited two hospitals located in eastern and western regions in Japan. As a result, we identified six pathogenic and 16 likely pathogenic variants in which five variants, JV1, JV2, p.(Gly843Glu; JV3), p.(Gly2186Glu; JV4), and (p.(Ile2188Thr; JV5), were prevalent in 432 patients with arRP and sRP. As expected from our previous study, 32.4% (140/432) of patients with arRP and sRP carried the homozygous or probable compound heterozygous combination of these five variants. Furthermore, we performed an MLPA of 53 patients who had single heterozygous variants, and identified CNVs of the *EYS* exon(s) in seven patients. The present study suggested that the detection of five major variants and CNVs is effective for genetic diagnosis of RP in the Japanese population. This is the first report showing the pathogenicity of three missense variants (JV3, JV4, and JV5) and the presence of CNVs in the *EYS* gene of Japanese patients with RP.

JV1 and JV2 have been reported to be a major cause of arRP and sRP in Japanese patients by several groups [8-11,29]. The present study showed that these two variants were prevalent in eastern and western regions in Japan. As shown in a previous study, JV1 and JV2 seem to be founder variants, because each variant was present on a particular haplotype [8]. According to HGVD, the MAF in the Japanese population is 0.0021 (5/2404) for JV1 and 0.0029 (7/2420) for JV2. However, the MAF in patients with RP was 0.097 for JV1 and 0.048 for JV2. The discrepancy in the ratio of the patients' number to the carriers' number may be interpreted by the weak pathogenicity of JV2 compared with JV1. From this point of view, the fate of *EYS* transcripts in retina of the patient is interesting to understand the difference of pathogenicity. In a recent study, we produced photoreceptor-like cells from dermal fibroblasts of patients carrying JV1 and JV2 using the redirect differentiation method, and analyzed the *EYS* transcripts expressed in these cells [30]. As a result, the transcript with JV1 was partially degraded by nonsense-mediated decay (NMD) and that with JV2 escaped from NMD, suggesting that both transcripts would produce truncating proteins in different expression levels. Furthermore, the protein product predicted from the transcript with JV1 lacks all five laminin A G-like domains, but that from the transcript with JV2 lacks only one domain at the C-terminal. The expression level and the extent of the lacking portion may affect the extent of the reduction of EYS protein function in the retina, resulting in the difference in pathogenicity between JV1 and JV2.

In a previous study, we expected that JV3 could be a possible pathogenic variant. JV3 was also present in controls, but the MAF in patients with RP (0.0693) was statistically significantly higher than in controls (0.0190; chi-square test, p=0.0056). Although the high frequency of JV3 in the Japanese population suggests that JV3 is benign, we concluded that JV3 is a likely pathogenic founder variant in Japanese patients with RP based on the following evidence: (i) The allele frequency of JV3 in patients with RP was statistically

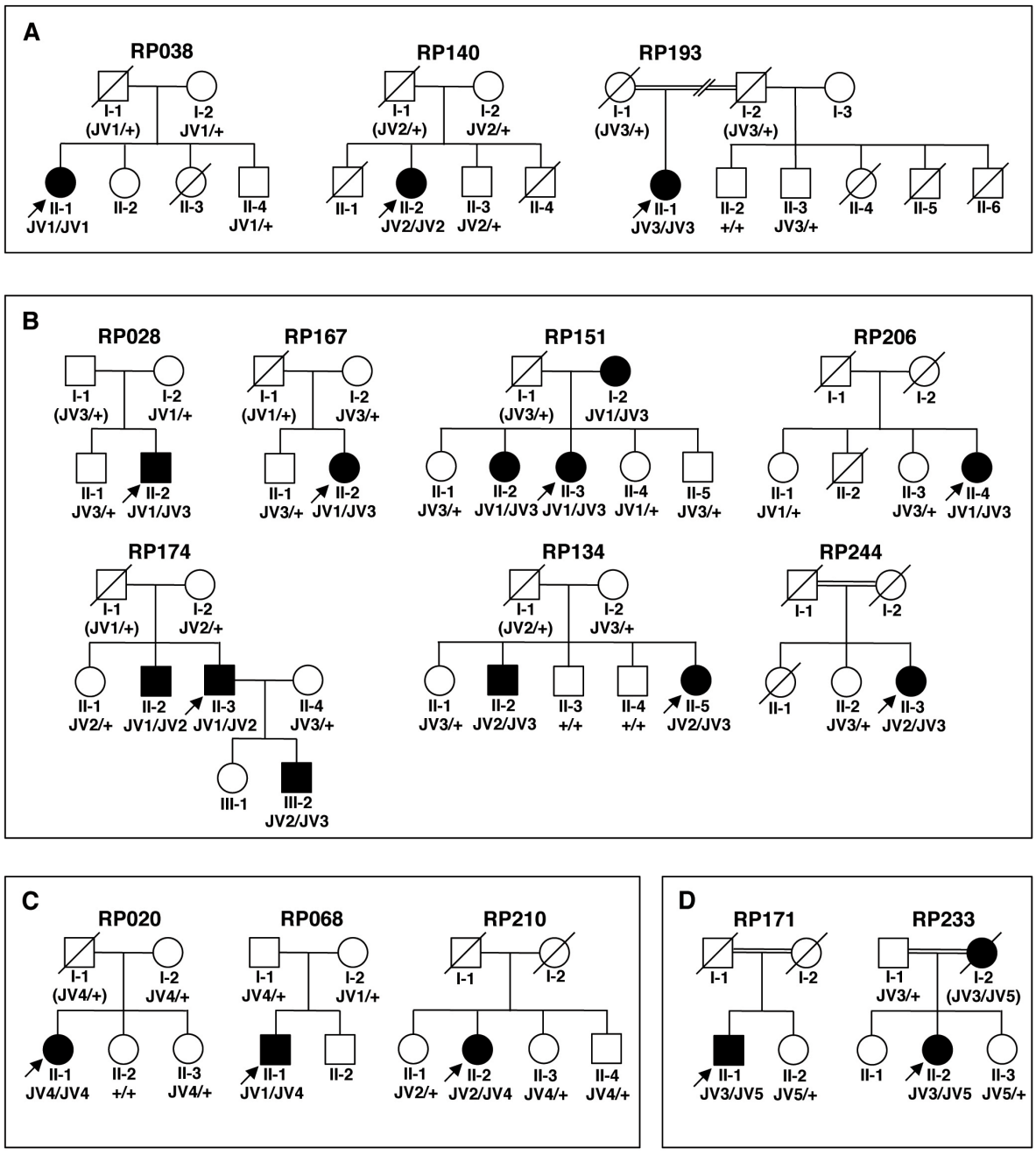

Figure 1. Segregation analysis of the families carrying any one of five likely pathogenic variants. The five variants are JV1, c.4957dupA (p.(Ser1653Lysfs*2)) ; JV2, c.8868C>A (p.(Tyr2956*)); JV3, c.2528G>A (p.(Gly843Glu)); JV4, c.6557G>A (p.(Gly2186Glu)); and JV5, c.6563T>C (p.(Ile2188Thr)). **A**: Families carrying homozygous JV1, JV2, or JV3. **B**: Families carrying compound heterozygous JV3. **C**: Families carrying JV4. **D**: Families carrying JV5. A filled symbol indicates an affected individual. An arrow indicates a proband in the family. Genotype was represented as follows: For example, JV1/JV1 indicates homozygous variants c.[4957dupA];[4957dupA]; JV1/+ indicates a single heterozygous variant c.[4957dupA];[4957=]; JV1/JV2 indicates compound heterozygous variants c.[4957dupA];[8868C>A]; +/+ indicates wild-type alleles. The probable genotype of a deceased individual is shown in parentheses.

significantly higher than that in controls (corresponding to PS4 in the ACMG classification). (ii) There were five patients carrying homozygous JV3. (iii) JV3 was detected in trans with a pathogenic variant (PM3). (iv) JV3 segregated with the disease phenotype in all ten families carrying JV3 (PP1). (v) Six in silico analyses predicted that JV3 is damaging, because this position is highly conserved among the EGF domains and evolutionary conserved among EGF homologs from various species (PP3). (vi) JV3 was present on the particular haplotype as shown in the previous paper [8]. All seven individuals carrying JV3 in 1000Genome were Japanese, but JV3 was also found in the Korean population (MAF=0.0016 (3/1870)) according to gnomAD. The MAF of JV3 in the Japanese population is 0.022 and 0.017 according to HGVD and iJGVD, respectively. The number of patients who have homozygous JV3 is low despite the high frequency of JV3 carriers. This may be explained by assuming mild symptoms or late onset of patients carrying JV3. This is partially supported by the fact that HGVD contains one homozygous JV3 carrier.

Two missense variants, JV4 and JV5, were carried by nine and six patients, respectively. JV4 and JV5 are located in the laminin A G-like domain and separated by one amino acid. JV4 has been reported to be possibly pathogenic in Chinese [3,31], Korean [5,32], and Japanese [9-11] populations. JV5 has been found in eight alleles in Japanese patients with RP [11]. The segregation analysis of three families with JV4 and two families with JV5 showed that JV4 and JV5 segregated with the RP phenotype. These results support that JV4 and JV5 are likely pathogenic variants. Interestingly, the RP233 family carrying JV5 seems to show an autosomal dominant inheritance pattern (Figure 1D). This is explained by assuming that the deceased I:2 had the compound heterozygous JV3/JV5 or homozygous JV5/JV5. Considering the consanguineous marriage in this case, the genotype of I:2 is likely JV3/JV5, because her cousin I:1 carried JV3.

Unexpectedly, five out of 17 patients with adRP had one of five likely pathogenic variants. As shown in Figure 1, the RP151 and RP174 families showed that one parent had compound heterozygous variants, and the other had a single heterozygous variant. A case similar to the RP174 family was reported by Abd El-Aziz et al. [3]. Oishi et al. also reported that three patients with RP initially diagnosed as autosomal dominant carried homozygous or heterozygous combinations of the *EYS* variants (JV1/JV1, JV1/JV2, JV1/JV4) [10]. These cases suggest that there are some families with arRP-causing variants showing an autosomal dominant inheritance pattern. Especially, it will occur when the ar-causing variant has a high MAF in the population similar to the *EYS* variants in the Japanese population. Taking into account the highly frequent variant JV3, more Japanese patients with adRP should be caused by a homozygous or compound heterozygous combination of these *EYS* variants.

There have been some reports on population-specific frequent variants in the *EYS* gene: p.(Thr135Leufs*26) in Moroccan Jewish [6] and p.(Ile1451Profs*3) in a population of western European ancestry [5,12,33,34]. Compared with the *EYS* variant spectrum of other populations, the high prevalence of five variants is characteristic of Japanese patients with RP. JV1, JV2, and JV4 have also been reported in other populations of East Asian countries: JV1 in two Koreans [9] and one Chinese [31], JV2 in one Korean [32], and JV4 in two Koreans [5,32] and three Chinese [3,31]. Furthermore, gnomAD contains nine Koreans carrying these variants: one for JV1, three for JV2, three for JV3, and two for JV4. The *EYS* gene variant spectrum in Korean and Chinese patients with RP is required to determine the distribution of these four variants in East Asian countries. At present, JV5 has been found only in Japanese patients, suggesting that this may be a Japanese-specific variant.

The patient with a single heterozygous pathogenic variant should have had a second variant on another allele. One possibility of variants is a midsized genomic rearrangement such as a large deletion or duplication of nucleotides. Pieras et al. reported that CNVs in the *EYS* gene constitute the second pathogenic variant in about 15% of the families with a single heterozygous variant [21]. We performed MLPA on 53 patients who had single heterozygous pathogenic variants. As a result, we identified six deletions and one duplication of the *EYS* exon(s) in seven patients (13.2%, 7/53), suggesting that CNVs are also one of the prevalent variants responsible for arRP and sRP in Japanese patients.

The second variants carried by the remaining patients with a single heterozygous variant are still unknown. There are several possibilities: a second pathogenic variant located in the promoter region, unknown exons, the 3′-untranslated region, or the regulatory element in the intron. To search the variant in these regions, it is necessary to sequence the whole genomic region of the *EYS* locus. Other possibility is a variant in the second gene involved in the maintenance of photoreceptor cells in association with EYS.

In a previous study, we examined the genotype–phenotype correlation using a small number of cases, and suggested that the type of variant was related to the extent of severity of the symptoms [8]. Now we have many cases with various types of variants, which are useful for examining the relation between genotype and clinical features. These are our next challenge.

In conclusion, the variant spectrum study in the *EYS* gene revealed that the homozygous and heterozygous combinations of major five variants account for 32.4% of Japanese patients with arRP and sRP, and these variants are also responsible for RP showing an autosomal dominant inheritance pattern. Thus, the screening of these five variants is the first choice for genetic diagnosis of Japanese patients with RP. Furthermore, CNVs in the *EYS* gene locus were shown to be one of the prevalent variants responsible for Japanese RP. These data will be useful for a future study to examine the relationship between genotype and clinical features.

APPENDIX 1. NUCLEOTIDE SEQUENCE VARIANTS IN THE EYS GENE IN THE JAPANESE POPULATION.

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

APPENDIX 2. IN SILICO PREDICTION OF PATHOGENICITY OF MISSENSE VARIANTS.

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

APPENDIX 3. PATIENTS CARRYING POSSIBLE PATHOGENIC VARIANTS OR RARE VARIANTS WITH AN MAF OF <0.01 IN THE EYS GENE.

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

APPENDIX 4. THE RESULTS OF A MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION ANALYSIS.

To access the data, click or select the words "Appendix 4." A vertical axis indicates a relative copy number of each exon. A red arrow indicates a deleted or duplicated exon.

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