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Next-generation sequencing-based comprehensive molecular analysis of 43 Japanese patients with cone and cone-rod dystrophies

Maho Oishi,[1] Akio Oishi,[1] Norimoto Gotoh,[2] Ken Ogino,[1] Koichiro Higasa,[2] Kei Iida,[3] Yukiko Makiyama,[1] Satoshi Morooka,[1] Fumihiko Matsuda,[2] Nagahisa Yoshimura[1]

[1]Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan; [2]Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan; [3]Medical Research Support Center, Kyoto University Graduate School of Medicine, Kyoto, Japan

Purpose: To investigate the efficacy of targeted exome sequencing for mutational screening of Japanese patients with cone dystrophy (CD) or cone-rod dystrophy (CRD).
Methods: DNA samples from 43 Japanese patients with CD or CRD were sequenced using an exome-sequencing panel targeting all 193 known inherited eye disease genes and next-generation sequencing methodologies. Subsequently, candidate variants were screened using systematic data analyses, and their potential pathogenicity was assessed using distinct filtering approaches, which included the frequency of the variants in normal populations, in silico prediction tools, and cosegregation.
Results: Causative mutations were detected in 12 patients with CD or CRD (27.9%). In total, 14 distinct mutations were identified in the genes *ABCA4*, *CDHR1*, *CRB1*, *CRX*, *GUCY2D*, *KCNV2*, *PROM1*, *PRPH2*, and *RDH5*, including four novel mutations, c.3050+1G>A in *ABCA4*, c.386A>G in *CDHR1*, c.652+1_652+4del in *CRB1*, and c.454G>A in *KCNV2*. Moreover, a putative pathogenic mutation was identified in *RGS9BP*, a gene recognized as the source of bradyopsia.
Conclusions: Targeted exome sequencing effectively identified causative mutations in Japanese patients with CD or CRD. The results confirmed the heterogeneity of the genes responsible for CD and CRD in Japanese populations, as well as the efficacy of targeted exome sequencing-based screening of patients with inherited retinal degeneration.

Cone dystrophy (CD) and cone-rod dystrophy (CRD) are members of a heterogeneous group of inherited progressive retinal disorders featuring predominant cone cell dysfunction [1]. The prevalence of CD and CRD is approximately 1:40,000 worldwide, and the two diseases are considerably similar clinically and genetically: Both diseases are characterized by a loss of visual acuity, disturbance in color vision, and photophobia and result in a central scotoma. Furthermore, cone photoreceptor responses observed using electroretinography (ERG) are impaired or extinguished in CD and CRD cases. Patients with CD exhibit normal rod responses initially, but many of these patients develop rod dysfunction later in life; thus, definitively distinguishing between CD and CRD is not straightforward [2,3].

In the case of CD and CRD, all modes of gene inheritance have been detected, and to date, 30 genes have been reported as causative genes of these retinal diseases in the RetNet database (accessed on March 20, 2015). In previous studies, mutational analysis was performed mainly on single genes or on a set of genes by using Sanger sequencing or microarray techniques [1,4,5]. However, with the recent development of next-generation sequencing, all of the exons of multiple genes can be sequenced in parallel. Thus far, only a few studies have used systematic exome-sequencing analysis for patients with CD or CRD, but the studies suggest that the distribution of the causative genes might differ according to ethnicity [6-9]. Moreover, certain studies have shown that genes previously reported to be associated with other retinal dystrophies might cause CD or CRD [10].

In this study, we conducted a comprehensive molecular analysis of 43 Japanese patients with CD or CRD by using a targeted exome-sequencing approach. We analyzed all retinal and optic-nerve disease genes reported in the RetNet database at the time this study was designed (193 genes), including all causative genes of CD or CRD. We expect our results to contribute to the catalog of genetic variations in Japanese patients with CD and CRD.

METHODS

All procedures used in this study adhered to the tenets of the Declaration of Helsinki. The institutional review board and the ethics committee of each institution approved the

Correspondence to: Akio Oishi, 54 Kawahara, Shogoin, Sakyo, Kyoto 606-8507, Japan; Phone: +81-75-751-3248; FAX: +81-75-752-0933; email: aquio@kuhp.kyoto-u.ac.jp

study protocols. All patients and their relatives were fully informed of the purpose and procedures of this study, and written consent was obtained from each participant.

Study participants: We recruited 43 Japanese patients with CD or CRD who visited the Department of Ophthalmology and Visual Sciences at the Kyoto University Graduate School of Medicine, Kyoto, Japan, between January 2011 and December 2012 and agreed to participate in the study. An additional 26 patients with other inherited retinal diseases, carrying 33 identified variants, were investigated to evaluate the integrity of the current approach [11].

CD and CRD were diagnosed by two retina specialists (authors A.O. and M.O.) based on comprehensive ophthalmologic examinations. When the two diagnoses disagreed, another retina specialist (author K.O.) arbitrated. The criteria for inclusion in the CD diagnostic group were a progressive decline in visual acuity, the presence of a central scotoma, and reduced cone responses from full-field ERG but normal rod responses. Full-field ERG was performed according to the protocol of the International Society for Clinical Electrophysiology of Vision (ISCEV) [12]. Inclusion in the CRD diagnostic group was based on the following criteria: a progressive decline in visual acuity, the presence of a central scotoma, and reduced responses of cones and rods in full-field ERG, with cone functions equally or more severely impaired than rod function. Atrophic changes in the macula were confirmed in most of the patients by using ophthalmoscopy and optical coherence tomography (OCT) imaging. We excluded patients with presumed Stargardt disease who exhibited subretinal yellow-white flecks or the typical dark choroid sign in fluorescein angiograms. Patients with central areolar choroidal dystrophy, pattern dystrophy, vitelliform macular dystrophy, age-related macular degeneration, and syndromic disorders were also excluded from this study. A detailed family history was obtained through patient interviews, and the presumed inheritance traits were determined using this information. Genomic DNA was extracted and purified from the peripheral blood of the patients and their available family members by using a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan).

Target capture and next-generation sequencing: A capture panel of inherited retinal-disease genes was previously designed and assessed by our group [11]. The capture panel comprised 2,433,298 bp that covered all exons together with the flanking exon and intron boundaries (±25 bp) of 365 genes, including all 193 genes related to retinal and optic diseases that had been reported in RetNet at the time this study was designed (December 26, 2012). Precapture Illumina libraries were generated as previously described [11]. A custom-designed HaloPlex Target Enrichment Kit 2.5 Mb (Agilent Technologies, Santa Clara, CA) was used for target enrichment, according to the manufacturer's protocol. Subsequently, DNA libraries were quantified and sequenced as 100 bp paired-end reads by using the Illumina HiSeq 2500 system (Illumina, San Diego, CA) according to the manufacturer's protocols.

Data analysis and interpretation of genetic variants: Data were processed using our in-house pipeline, and several filtering steps were applied to determine the pathogenicity of the variants as previously described [11]. The analysis was focused specifically on the variants of the 193 genes reported in the RetNet database, which included all identified causative genes of CD or CRD. Because CD and CRD are rare Mendelian genetic disorders, we excluded variants that exhibited an allele frequency >0.5% (for recessive variants) or >0.1% (for dominant variants) in any of the following databases: 1000 Genomes [13], NHLBI GO Exome Sequencing Project (ESP6500), and Human Genetic Variation Database, which contains the genetic variations of 1,208 Japanese patients. To predict the effect of missense variants, we used dbNSFP [14], which contains seven well-established in silico prediction programs (Scale-Invariant Feature Transform [SIFT], Polyphen2, LRT, MutationTaster, MutationAssessor, PhyloP, and GERP++); only variants that were predicted to be pathogenic by at least five of the seven algorithms are reported here. For splice-site variants, we used the prediction program MaxEntScan [15], and variants whose scores differed by >5 between the wild-type and mutant sequences were considered pathogenic. The Human Gene Mutation Database (HGMD) was used to screen mutations reported in published studies.

All mutations and potential pathogenic variants were validated using conventional Sanger sequencing methods. Segregation analysis was performed if DNA from family members was available. As for novel mutations, we checked the Exome Aggregation Consortium (ExAC) database (Cambridge, MA; accessed November 2015) and confirmed that none of them were registered at the time of analysis.

RESULTS

Before the sequence analysis, CD and CRD were diagnosed in 17 and 26 of the 43 participants, respectively. The inheritance patterns of the causative genes were determined to be autosomal dominant (ad) in nine patients, autosomal recessive (ar) in eight patients, and simplex in 26 patients. These cases included one consanguineous pair of patients with adCRD (a parent–child relationship).

Targeted sequencing and data processing: The targeted gene-capture and sequencing analysis described in the preceding

Table 1. Known mutations and novel potentially causative changes detected in patients.

ID	Type	Phenotype	Gene	Mutation		Reference	rs ID
Patients harboring known mutations							
K1741	simplex	CRD	ABCA4	c.6445C>T	p.R2149* (homo)	[19,21]	rs61750654
K2022	ad	CD	PRPH2	c.589A>G	p.K197E (hetero)	[38]	rs62645931
K2039	simplex	CRD	ABCA4	c.1760+2T>G (homo)		[20,30]	rs61751385
K3341	ad	CD	GUCY2D	c.2512C>T	p.R838C (hetero)	[39]	rs61750172
K6073	ad	CRD	PROM1	c.1117C>T	p.R373C (hetero)	[40]	rs137853006
K6120	ar	CRD	ABCA4	c.1957C>T	p.R653C (homo)	[21,31]	rs61749420
K6205	ad	CRD	PROM1	c.1117C>T	p.R373C (hetero)	[40]	rs137853006
K6343	simplex	CD	CRX	c.121C>T	p.R41W (hetero)	[41]	rs104894672
Patients harboring at least one novel mutation							
K2044	ar	CRD	ABCA4	c.3050+1G>A (homo)		Novel	NA
K6140	simplex	CD	CDHR1	c.386A>G	p.N129S (homo)	Novel	NA
K6247	ar	CRD	CRB1	c.652+1_652+4del (homo)		Novel	NA
K6496	simplex	CD	KCNV2	c.529T>C	p.C177R (hetero)	[42]	NA
				c.454G>A	p.D152N (hetero)	Novel	NA
K6345	simplex	CRD	CRX	c.284delG (hetero)		Novel	NA
K3479	ar	Bradyopsia	RGS9BP	c.211G>T	p.E71* (homo)	Novel	NA

CD: cone dystrophy; CRD: cone-rod dystrophy; ad: autosomal dominant; ar: autosomal recessive; hetero: heterozygous; homo: homozygous; NA: not available. K6345 and K3479 were classified as unresolved cases based on the criteria used in this study.

section was applied to the 43 Japanese patients with CD or CRD, and the data were processed using our bioinformatics pipeline [11]. In a few cases (7/43, 16.3%), the coverage obtained was lower than that in others, but the mean and median coverages of the 193 RetNet genes in all samples were 269X and 260X, respectively. Within the targeted region, 93.2% of the bases showed >10X coverage and 90.1% showed >20X coverage, which suggested that sufficient coverage was achieved [11]. Among the 3,619 targeted exons, only 1.46% were covered at <5X (Appendix 1).

After automated variant detection, a mean of 507 raw variants and small insertions or deletions per sample were identified in the exons and corresponding splice junctions. When we used our systematic data-filtering method, we detected an average of 6.88 rare variants that were likely to cause a deleterious protein-coding change. All variants present in the 26 positive-control samples were successfully detected after the stringent filtering steps, which excluded putative false-positive variants and reconfirmed the integrity of our approach [11].

Mutation analysis of patients: Pathogenic mutations were identified in 12 of the 43 patients (27.9%). A total of 14 distinct mutations were identified, including ten reported mutations and four novel mutations. Furthermore, one novel heterozygous putative pathogenic mutation was identified in *CRX* (OMIM: 602225; c.284delG), and one novel homozygous nonsense mutation was identified in *RGS9BP* (OMIM 607814; c.211G>T (p.E71*)), which is a causative gene for bradyopsia (Table 1). Table 2 lists the novel missense mutations predicted to be pathogenic by using a combination of in silico prediction tools. Gene reference numbers are shown in Appendix 2. Figure 1 shows the pedigrees of the 12 families carrying the pathogenic mutations, and the phenotype data of these patients are shown in Appendix 3 and Figure 2, Figure 3, and Figure 4.

Patients carrying mutations in known CD or CRD genes: Among the 12 resolved patients, eight known mutations were identified in the genes *ABCA4* (OMIM: 601691), *CRX*, *GUCY2D* (OMIM 600179), *KCNV2* (OMIM 607604), *PROM1* (OMIM 604365), and *PRPH2* (OMIM 179605), whereas four novel mutations were identified in *ABCA4*, *CDHR1* (OMIM 609502), *CRB1* (OMIM 604210), and *KCNV2* (Table 1). The pair of familial-related cases (K6073 and K6205) was included in this group. We included proband K6247, who carried the novel homozygous splice-site mutation c.652+1_652+4del in *CRB1*, in this group because patients harboring the *CRB1* mutation occasionally exhibit CRD or macular dystrophy phenotypes [10,16-18]. Three probands (K1741, K2039, and K6120) were discovered to carry

homozygous mutations in *ABCA4* (c.6445C>T (p.R2149*), c.1760+2T>G, and c.1957C>T (p.R653C), respectively) that are known to cause Stargardt disease. The mutations are associated with early onset macular degeneration [6,19,20], vessel attenuation [6], patchy parafoveal atrophy surrounded by numerous yellow-white flecks [21], or retinitis pigmentosa [20] in certain cases. The three patients in this study (K1741, K2039, and K6120) had experienced early onset visual-acuity decline and presented pan-retinal degeneration but did not exhibit yellowish-white flecks; this agrees with previous reports suggesting the phenotypic variation. Additional information is provided in Appendix 4, which lists all the other potentially pathogenic rare variants identified in patients who carried pathogenic mutations in known CD or CRD genes.

In one simplex case (K6345), we identified a heterozygous putative pathogenic mutation, c.284delG, in *CRX*, which has been reported to cause CD and CRD in families who exhibit autosomal dominant inheritance patterns. However, this variant was a novel mutation, and segregation analysis was not performed because DNA samples from the patient's family members were unavailable. Therefore, we classified this case as unresolved, according to the criteria used in this study (see "Data analysis and interpretation of genetic variants" in Materials and Methods and our previous report [11]). Furthermore, probands K3445 and K1909 were discovered to carry mutations in *ABCA4* and *RDH5*, respectively, which are recognized to cause CD and CRD in families who exhibit recessive inheritance patterns. Although these two variants were reported to be pathogenic mutations, a second copy of the mutations was not detected using the current approach, and the cases were ultimately classified as unresolved.

Patients carrying mutations in other retinal disease-related genes: Among the remaining cases, one patient was identified to carry mutations in genes that are known to cause other retinal diseases. Proband K3479 carried a novel homozygous nonsense mutation, c.211G>T (p.E71*), in *RGS9BP*; mutation of this gene is recognized to cause bradyopsia, which is characterized by difficulty in tracking moving objects or adapting to sudden changes in illuminance. Careful reassessment of the clinical symptoms revealed that the patient showed difficulty in adjusting to illuminant changes, photophobia, a moderately reduced visual acuity (RV=0.6 and LV=0.3), and visual field defects with a loss of central sensitivity. Observation of the fundus with ophthalmoscopy and OCT imaging indicated no apparent retinal degeneration, except drusen and epiretinal membrane. All of these symptoms were compatible with bradyopsia, but because the patient developed a malignant lymphoma after inclusion in the study, we could not complete follow-up confirmation of the characteristic ERG findings, such as a reduced response to consecutive stimuli. However, these clinical symptoms were consistent with those of bradyopsia, and this disorder was molecularly diagnosed in the proband.

DISCUSSION

In this study, we performed a comprehensive molecular screening of 43 Japanese patients with CD or CRD by using next-generation sequencing techniques, and we successfully obtained molecular diagnoses for 12 cases (27.9%).

Our results identified causative *ABCA4* mutations in four patients. The *ABCA4* mutation is a major cause of arCD and CRD in Europe: >31–65% of patients with arCD or CRD carry mutations of *ABCA4* [22-24]. In contrast, a study conducted in China reported that the prevalence of *ABCA4* mutations in Chinese patients with CRD was only 2.1% [6]. This discrepancy indicates that ethnic differences exist in the prevalence of causative gene alleles for CD and CRD, as in the case of retinitis pigmentosa [11]. Here, the prevalence of *ABCA4* mutations was 9.3% (4/43), which lies between the prevalence rates measured for European and Chinese populations and confirms the ethnic difference in the prevalence of *ABCA4* mutations.

The results of this study hold implications for the role of *CRB1* in CRD. *CRB1* is widely recognized as a causative gene for retinitis pigmentosa and Leber congenital amaurosis; moreover, mutations in this gene were reported to be associated with the various phenotypes of retinal dystrophies, including CRD [10,16-18]. Here, proband K6247 carried the homozygous splice-site mutation c.652+1_652+4del in *CRB1*, and the patient exhibited the CRD phenotype. The patient's

Table 2. Results of seven in silico programs of novel missense mutations.

ID: mutation	SIFT	Polyphen2 Hvar	LRT	Mutation taster	Mutation assessor	GERP++	PhyloP
K6140: *CDHR1* c.386A>G (p.N129S)	1 (D)	0.885(P)	1 (D)	1 (D)	4.55(H)	5.09	1.917
K6062: *CRX* c.142C>T (p.R48W)	1 (D)	0.996(D)	1 (D)	1 (D)	2.385(M)	1.47	0.246
K6496: *KCNV2* c.454G>A (p.D152N)	1 (D)	1 (D)	1 (D)	1 (D)	3.95(H)	5.07	2.352

D: damaging; P: possibly damaging; H: high; M: medium.

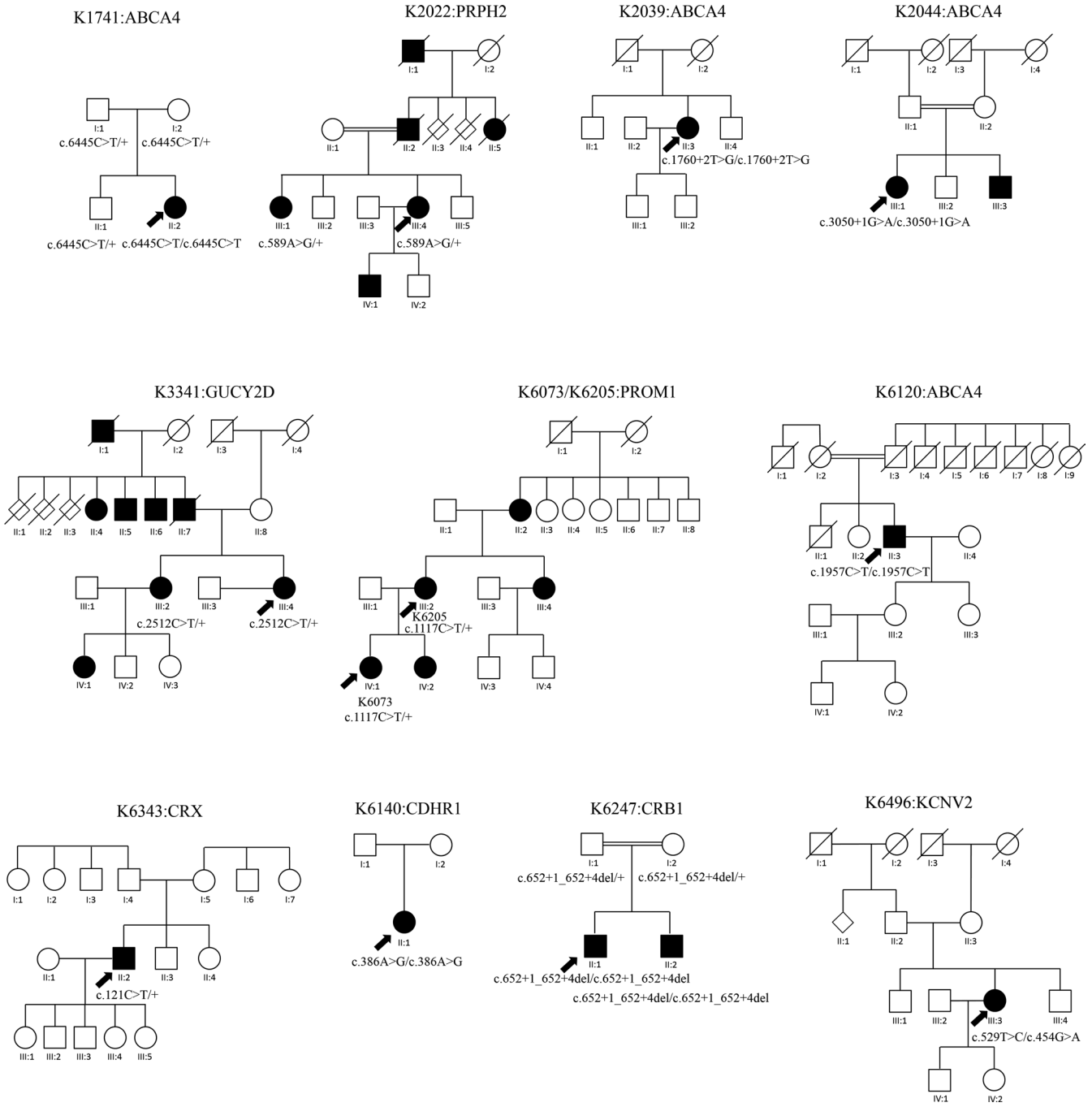

Figure 1. Pedigrees of the 12 patients with cone or cone-rod dystrophy carrying pathogenic mutations. The patients' IDs and the corresponding genes are shown above the pedigrees; +: wild-type allele.

parents were a consanguineous couple, and the brother (not included in this study) harbored the same mutation and developed the same phenotype. The early onset phenotype and autosomal recessive heritability agreed with the results of a previous study [18], and the presence of cystoid macular edema and predominantly nasal involvement agreed with the results of another report [17]. The current study further supports the notion that mutations in *CRB1* cause CRD.

This study revealed that one patient carried a putative disease-causing homozygous mutation in *RGS9BP*. RGS9BP plays a crucial role together with RGS9 in the recovery phase of visual transduction [25-27]. Mutations in *RGS9*

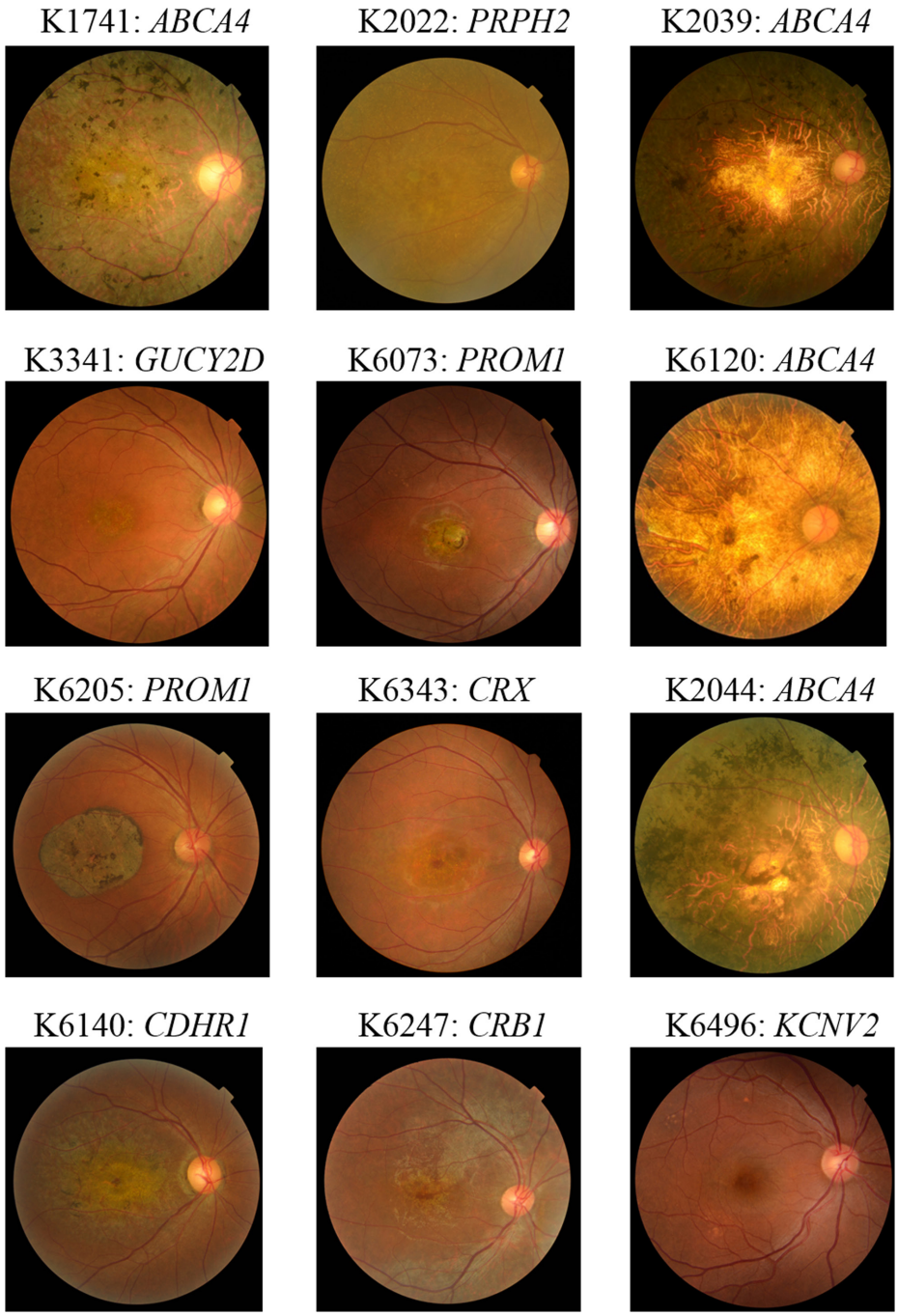

Figure 2. Color fundus photographs of the patients with cone or cone-rod dystrophy carrying pathogenic mutations.

and *RGS9BP* are known to cause bradyopsia (slow vision), which is characterized by delays in adaptation to changes in light and darkness, photophobia, moderate loss of visual acuity, difficulty in seeing moving objects, normal color vision, and a normal fundus [25,28,29]. Proband K3479 carried the novel homozygous nonsense mutation c.211G>T (p.E71*) in *RGS9BP*. The symptoms, fundus appearance, and standard ERG findings of the proband were compatible with bradyopsia. Rod responses were within the normal range, whereas cone responses were non-recordable by using ISCEV ERG standards. The patient's parents were healthy but consanguineous, and three of the six siblings of the patient showed similar disease phenotypes, which suggested an autosomal recessive inheritance pattern. We could not confirm the characteristic ERG findings (i.e., a reduction in cone and rod responses after the first stimulation with prolonged

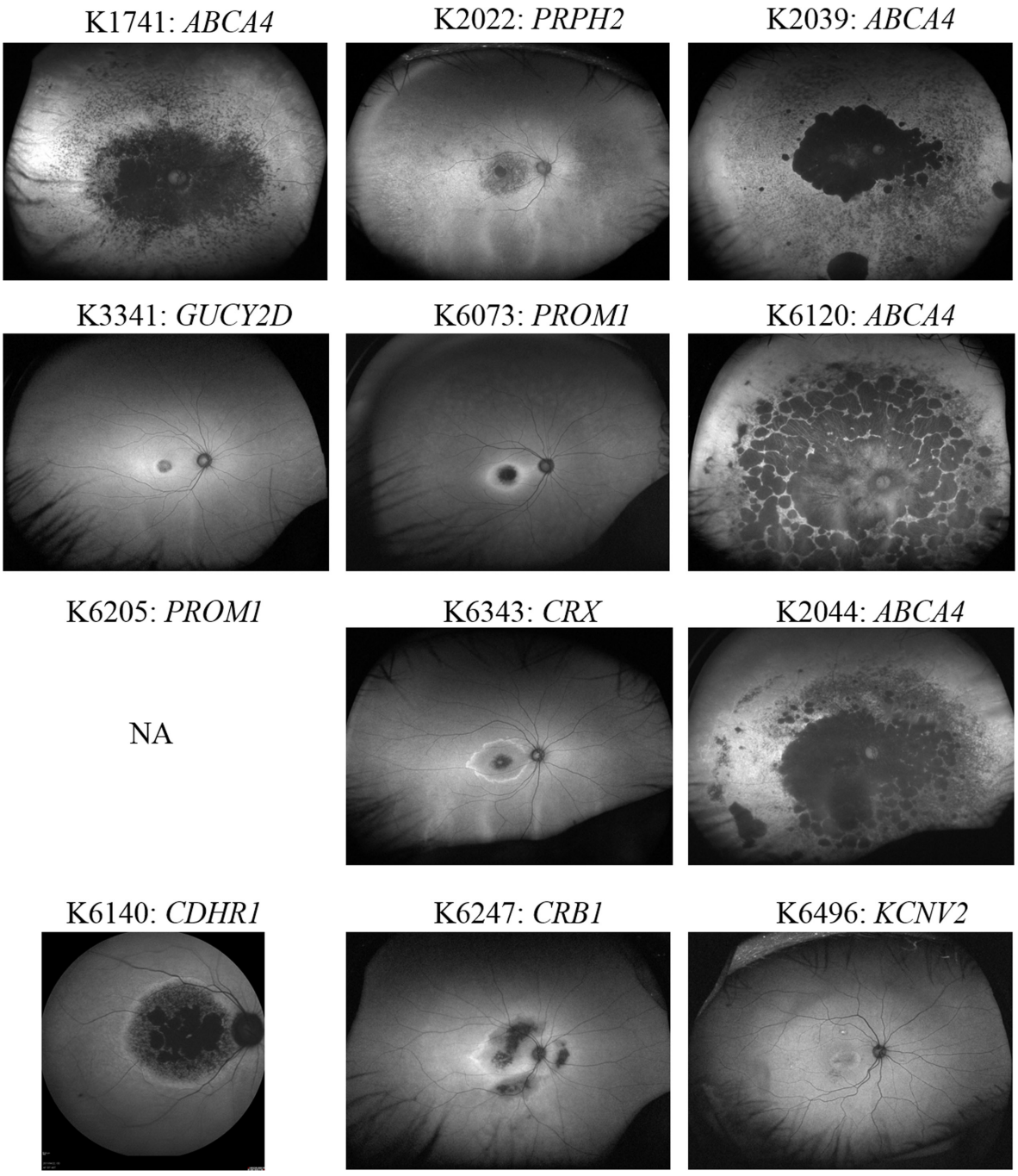

Figure 3. Wide-field fundus autofluorescence images of the patients with cone or cone-rod dystrophy carrying pathogenic mutations. Fundus autofluorescence was imaged with Optos200Tx (Optos, Dunfermline, UK) except K6140 (HRA2; Heidelberg Engineering, Heidelberg, Germany). Most of the examinations for K6205, the mother of K6073, were performed at another institution and were not available.

recovery) because the proband had died by the time we identified the mutation. However, the clinical symptoms and mode of inheritance were consistent with those of bradyopsia, and thus, in this patient, we diagnosed bradyopsia instead of CRD.

Probands K1741, K2039, and K6120 were identified to carry the homozygous mutations c.6445C>T, c.1760+2T>G, and c.1957C>T in *ABCA4*, respectively, which have been reported to cause Stargardt disease [19-21,30,31]. *ABCA4* is a causative gene for autosomal recessive CD, CRD, retinitis pigmentosa, and Stargardt disease. Before the era of genetic diagnostics, these diseases were differentiated based on clinical findings; however, Stargardt disease, CD,

and CRD are currently recognized to show considerable overlap. For instance, the same mutation is associated with Stargardt disease and CRD [24,32]. Furthermore, the mutation c.1760+2T>G identified in proband K2039 was reported to cause retinitis pigmentosa [20]. These three cases in this study highlight the phenotypic variations, and these patients should be treated as patients with *ABCA4*-associated retinopathy and not designated arbitrarily as patients with Stargardt disease or CRD [33-35].

In this study, molecular diagnoses were made in 27.9% of the patients, and 62.1% of the cases remained unresolved. The detection rate was slightly higher than that for the Chinese population (21.28%) but lower than that for the European population (62.1%), which indicates the ethnic differences in the prevalence of causative gene alleles for CD and CRD [6,9]. The methodological limitations of our approach and the technical limitations of next-generation sequencing technology might be one of the reasons for not determining the molecular cause of disease in these patients [11]. Here, we did not assess intronic mutations, synonymous mutations, and small insertions or deletions that do not cause frameshift. For instance, some intronic mutations in *ABCA4* reported in European populations were not screened in this study [36]. Furthermore, next-generation sequencing technology occasionally does not allow easy reading of certain regions such as GC-rich regions, repeated sequences, copy-number variations, and large deletions or insertions. Moreover, the sequencing of seven samples (16.9%) yielded lower coverage

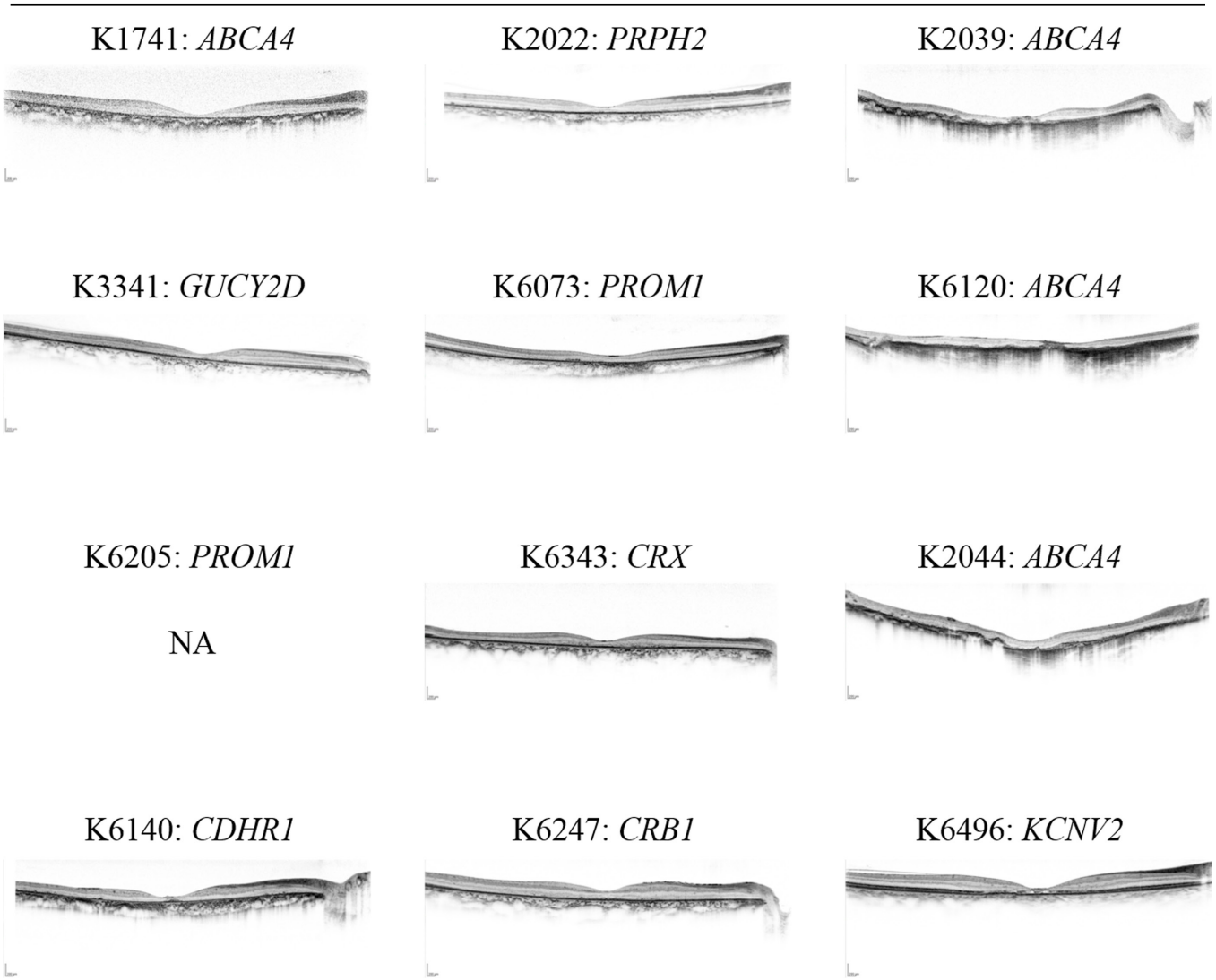

Figure 4. Optical coherence tomography of the patients carrying pathogenic mutations. Images were obtained by using Spectralis (Heidelberg Engineering, Heidelberg, Germany). Most of the examinations for K6205, the mother of K6073, were performed at another institution and were not available.

than that in the case of other samples due to a technical reason: generation of the precapture libraries. Although the mean coverage of these samples was sufficient for the detection of genetic variants (233X), the percentage of bases showing >10X coverage was 81%, and low-coverage variants were excluded. To compensate for the deficiencies of our approach, additional target screening of low-depth regions could be conducted using conventional direct sequencing, and this might serve as an effective tool for improving genetic diagnosis [37]. Another reason for the limited detection rate could be our stringent criteria for determining the pathogenicity of variants; we included only those variants that were predicted to be pathogenic by five out of the seven in silico programs used. When less stringent criteria were employed, such as prediction by one out of five in silico programs, two additional patients were assessed as carrying disease-causing mutations.

In conclusion, this study screened the largest sample of Japanese patients with CD and CRD to date and indexed the genetic constitution of this cohort. Our results confirmed the efficacy of next-generation sequencing-based molecular diagnosis of patients with CD or CRD.

APPENDIX 1. LOW-COVERAGE REGIONS IN THE 193 CAUSATIVE GENES OF HERITABLE EYE DISORDERS.

CD: cone dystrophy; CRD: cone-rod dystrophy; Chr: chromosome. To access the data, click or select the words "Appendix 1."

APPENDIX 2. LIST OF GENE REFERENCE NUMBERS.

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

APPENDIX 3. PHENOTYPE DATA OF THE PATIENTS WHO CARRIED MUTATIONS IN KNOWN CD OR CRD GENES.

VA: visual acuity; NR: non-recordable; NA: not available. *Visual acuity at the current age. †Data of the right eyes. To access the data, click or select the words "Appendix 3." K6205 is a mother of K6073 and most examinations were performed in another institution. K6140 underwent electroretinogram more than 15 years before and the current data is not available.

APPENDIX 4. LIST OF ADDITIONAL POTENTIALLY PATHOGENIC RARE VARIANTS IDENTIFIED IN PATIENTS WHO CARRIED MUTATIONS IN KNOWN CD OR CRD GENES.

To access the data, click or select the words "Appendix 4." CD: cone dystrophy; CRD: cone-rod dystrophy; ad: autosomal dominant; ar: autosomal recessive; hetero: heterozygous; NA: not available.

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