# Discovery of a Cynomolgus Monkey Family With Retinitis Pigmentosa

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**Purpose.** To accelerate the development of new therapies, an inherited retinal degeneration model in a nonhuman primate would be useful to confirm the efficacy in preclinical studies. In this study, we describe the discovery of retinitis pigmentosa in a cynomolgus monkey (*Macaca fascicularis*) pedigree.

**Methods.** First, screening with fundus photography was performed on 1443 monkeys at the Tsukuba Primate Research Center. Ophthalmic examinations, such as indirect ophthalmoscopy, ERGs using RETeval, and optic coherent tomography (OCT) measurement, were then performed to confirm diagnosis.

**Results.** Retinal degeneration with cystoid macular edema was observed in both eyes of one 14-year-old female monkey. In her examinations, the full-field ERGs were nonrecordable and the outer layer of the retina in the parafoveal area was not visible on OCT imaging. Moreover, less frequent pigmentary retinal anomalies also were observed in her 3-year-old nephew. His full-field ERGs were almost nonrecordable and the outer layer was not visible in the peripheral retina. His father was her cousin (the son of her mother's older brother) and his mother was her younger half-sibling sister with a different father.

**Conclusions.** The hereditary nature is highly probable (autosomal recessive inheritance suspected). However, whole-exome analysis performed identified no pathogenic mutations in these monkeys.

Keywords: retinitis pigmentosa, monkey, whole-exome analysis

Recently, the clinical efficacy of gene therapy[1–4] and regenerative medicine[5,6] for intractable ocular diseases, including inherited retinal degeneration (IRD) has been reported. Studies evaluating the mechanisms of disease pathogenesis, as well as the efficacy of these new therapeutic treatments, have typically used rodent disease models.

There are many advantages to rodent models. Generally, they are relatively cheap and easy to breed and handle. A number of rodent disease models based on spontaneous mutations of genes that are also involved in human inherited retinal disorders have been described.[7] Moreover, it is easy to create a new hereditary disease model in rodents by using the genetic modification technique.[8] However, rodent models of retinal diseases have a major limitation due to the absence of the macula and the fovea found in the human retina.

In larger animals, body and organ sizes are closer to those of humans. There are special advantages to using nonhuman primates, which have phototransduction mechanisms and immune systems comparable to those of humans. Moreover, nonhuman primate eyes have the advantage of being similar in structure to the human eye, including a macula and fovea. Anatomically, retina of New World primates is almost the same as that of Old World monkeys, although some differences are present in the function, including color vision, compared with Old World monkeys and humans.[9,10] Discovery of an IRD model in nonhuman primates, especially in Old World monkeys, would be especially useful to confirm the efficacy of new therapies in preclinical studies and thereby accelerate their development.

In this study, the population of 1443 cynomolgus monkeys (*Macaca fascicularis*), one of the Old World monkeys, at Tsukuba Primate Research Center (TPRC) was screened ophthalmologically, and we fortunately found two genetically related individuals with retinitis pigmentosa (RP).

## Materials and Methods

### Animals

This study used cynomolgus monkeys bred at the TPRC, National Institutes of Biomedical Innovation, Health and Nutrition (NI-

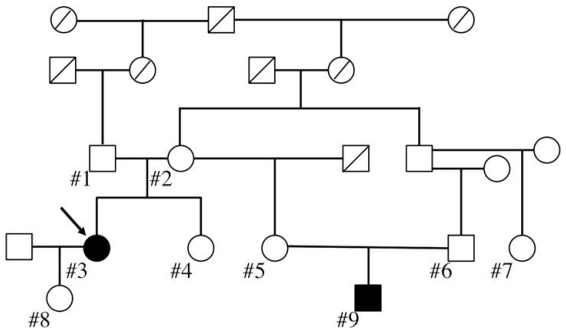


FIGURE 1. The pedigree chart. We performed the ophthalmic and genetic analysis for nine monkeys and assigned them identification numbers. *Arrow* indicates the index case (#3).

BIOHN), and was conducted according to the rules for animal care and management of the TPRC,[11] the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan,[12] and the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals.[13] The research protocol was approved by the Animal Welfare and Animal Care Committee of NIBIOHN. All animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animals were housed under the following conditions: temperature, 23°C to 27°C; humidity, 50% to 70%; 12 air changes per hour; 12/12-hour light/dark cycle; and fed 70 g of commercial food (CMK-2; CLEA Japan, Inc., Tokyo, Japan) and 100 g of apples daily. Tap water was supplied ad libitum.

## Fundus Photography for the Screening

Approximately 20 minutes before examining the ocular fundi, a mixture of tropicamide and phenylephrine hydrochloride was instilled into both eyes of each animal to dilate the pupils. Then, the monkeys were anesthetized with an intramuscular injection of ketamine (10.0 mg/kg). Fundus photographs were taken with an ophthalmoscope camera (Kowa RC-2; Kowa Co. Ltd., Tokyo, Japan).

## Ophthalmic Data Collection for a Diagnosis of RP

The ophthalmic data collection for a diagnosis of RP was performed in the 14-year-old female monkey and her relatives. According to her pedigree chart (Fig. 1), her grandmothers were half-sisters of different mothers. The monkeys were anesthetized with an intramuscular injection of ketamine (6.0 mg/mL) and xylazine (1.2 mg/mL) and a mixture of tropicamide and phenylephrine hydrochloride was instilled into both eyes of each animal to dilate the pupils. Each monkey was kept in a dark room for at least 30 minutes under a dim red light. ERGs were recorded with the RETeval system (LKC Technologies, Gaithersburg, MD, USA). After the recording of dark-adapted ERGs, the monkeys were exposed to a white adapting field for at least 10 minutes, and their light-adapted ERGs were recorded. Then, slit-lamp biomicroscopy of the anterior segment and fundoscopic examination by both direct and indirect ophthalmoscopy were carried out and details of the lens, vitreous, and fundus were observed. Fundus photographs were taken with an ophthalmoscope camera (AFC-330; Nideck Co. Ltd., Gamagori, Aichi, Japan). OCT measurements were obtained for all monkeys by spectral domain OCT (RS-3000 Advance; Nideck Co. Ltd.).

## Target Exome Analysis

Whole-exome sequencing was carried out using the DNA from nine monkeys, including two affected individuals (labeled 1–9 in

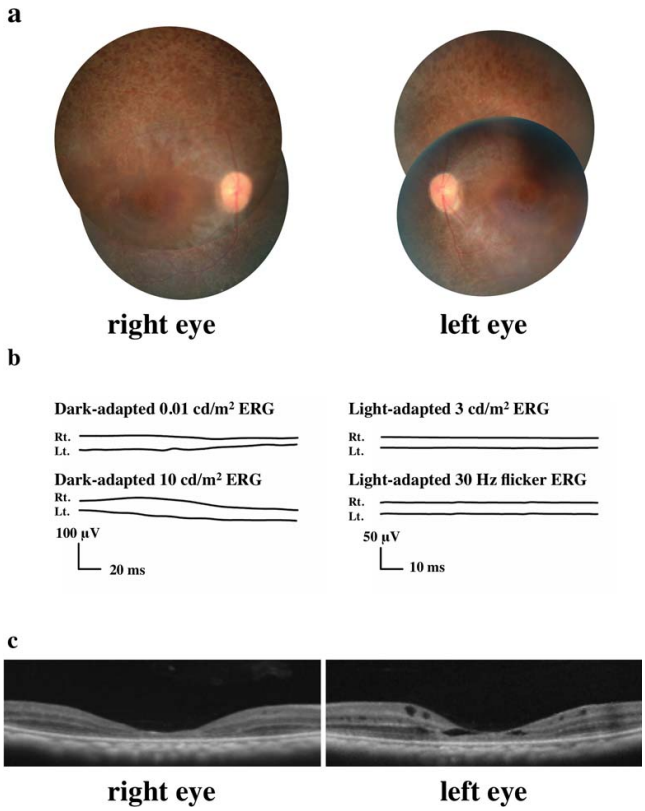


FIGURE 2. Ophthalmic data from a 14-year-old female monkey (#3). (**a**) Fundus photographs. Retinal degeneration with cystoid macular edema was observed. (**b**) Full-field ERGs. Both dark-adapted and light-adapted ERGs were nonrecordable. Rt., right eye; Lt., left eye. (**c**) Images of horizontal OCT macular scan. The outer layer of the retina in the parafoveal area was not visible and cystoid spaces were observed.

Fig. 2). Exons and flanking sequences were captured on SureSelect V5 (Agilent Technologies, Santa Clara, CA, USA) designed for human genetic study, which resulted in an average of 80,425,534 reads. An average of 80,333,774 readouts (99.89%) were mapped to the macaque reference genome (Macaca_fascicularis_5.0) using BWA-mem (ver.0.7.15) (in the public domain, http://arxiv.org/abs/1303.3997).[14] We then excluded PCR-duplicated reads, and extracted uniquely mapped and properly paired reads with an insert size within ± 2 SD of the mean. Variant calling was performed in exonic regions (69.06 Mb), and regions within 150 bp upstream/downstream of the exonic regions (total 135.14 Mb). Variant calling was performed using GATK (in the public domain, https://software.broadinstitute.org/gatk/), samtools (in the public domain, http://samtools.sourceforge.net), and in-house programs, as previously reported.[15] This resulted in 47,481,230 (average) mapped reads with average read depth of 61.7 (median: 15.6) in the targeted regions. At this point, the variants were annotated with regard to amino acid sequences using ANNOVAR (in the public domain, http://annovar.openbioinformatics.org/).[16] Protein coding annotations were obtained from the National Center for Biotechnology Information (in the public domain, ftp://ftp.ncbi.nih.gov/genomes/MapView/Macaca_fascicularis/sequence/ANNOTATION_RELEASE.100/initial_release/seq_gene.md.gz) and Ensembl (in the public domain, ftp://ftp.ensembl.org/pub/pre/gtf/macaca_fascicularis/Macaca_fascicularis.MacFas_5.0.pre.gtf). On average, 289,613 single nucleotide variants (SNVs) and 20,227 indels were identified. Of these, 899 SNVs and 32 indels were nonsynonymous. Only those nonsynonymous variants that were found in homozygous states in the affected monkeys (#3 and #9), but not in the unaffected

Table. Annotations of Cosegregating Candidate Variants

| Type | Gene Name | Mutation | Human Disease | Function | Conservation | Conclusion |
|---|---|---|---|---|---|---|
| SNV | *LEFTY1* | c.C1012T:p.P338S | | Embryonic left-right asymmetry | | Unlikely |
| SNV | *ARHGAP30* | c.A563T:p.E188V | Cancer | Actin dynamics and cell adhesion | | Unlikely |
| **SNV** | ***CCDC13*** | **c.G35A:p.G12D** | | **Interact with RD genes** | | **Possible** |
| SNV | *ANO10* | c.A49G:p.K17E | Cerebellar ataxia | | | Unlikely |
| SNV | *FAM184A* | c.C105G:p.D35E | | Poorly characterized | | Uncertain |
| SNV | *PSAPL1* | c.T325C:p.W109R | | Poorly characterized | | Uncertain |
| SNV | *EVC2* | c.C137T:p.P46L | Ellis-van Creveld syndrome, Weyers acrofacial dysostosis | | | Unlikely |
| SNV | *SLC2A9* | c.G919A:p.V307I | Hypouricemia, renal, 2 | | | Unlikely |
| SNV | *SDAD1* | c.A1855T:p.M619L | | Poorly characterized | | Uncertain |
| SNV | *MTHFD2L* | c.C31G:p.R11G | Megaloblastic anemia, hemolytic uremic syndrome, severe combined immune deficiency | | | Unlikely |
| SNV | *GABRA6* | c.A994G:p.T332A | | KO mice have no phenotype | | Uncertain |
| SNV | *CHRNA7* | c.C1081G:p.Q361E | Schizophrenia | | | Unlikely |
| SNV | *ISLR2* | c.C935A:p.P312Q | | KO mice die soon after birth | | Unlikely |
| SNV | *ISLR* | c.C890T:p.A297V | | KO mice die soon after birth | No | Unlikely |
| SNV | *IL16* | c.C1700T:p.P567L | | Migration of mouse Th1 cells | | Unlikely |
| SNV | *IL16* | c.C2771A:p.P924Q | | Migration of mouse Th1 cells | | Unlikely |
| SNV | *LOC102131556* | c.G131A:p.R44Q | | Poorly characterized | | Uncertain |
| SNV | *CEBPD* | c.C491A:p.P164Q | | Regulation of corneal stem cells | | Unlikely |
| SNV | *LOC101926285* | c.G10A:p.G4R | | Poorly characterized | | Uncertain |
| inframe ins | *ZNF503* | * | | Embryonic left-right asymmetry | | Unlikely |
| SNV | *TBATA* | c.G877A:p.V293M | | | No | Unlikely |
| SNV | *OBFC1* | c.C299T:p.A100V, | Cerebroretinal microangiopathy | | | Unlikely |
| inframe del | *NEFH* | c.1524_1547del:p.508_516del | Charcot-Marie-Tooth disease | | | Unlikely |
| SNV | *KIAA1551* | c.C1118T:p.T373M | | | No | Unlikely |
| SNV | *TRIM51* | c.G10A:p.G4R | | Poorly characterized | | Uncertain |
| **inframe del** | ***CEP164*** | **c.1595_1597del:p.532_533del** | **Nephronophthisis** | **Role in primary cilia** | | **Possible** |
| SNV | *CEP164* | c.A1703C:p.Q568P | Nephronophthisis | | No | Unlikely |
| SNV | *LOC102130732* | c.T14A:p.I5N | | Poorly characterized | | Uncertain |

Two candidate variants that remained after the filtering steps are in bold. KO, knockout.
* c.66_67insGGCGGCGGA:p.G22delinsGGGG.

monkeys were selected (Table). Variants in genes listed as causing retinal disease in RetNet (in the public domain, http://www.sph.uth.tmc.edu/RetNet) were highlighted. DNA collected from unaffected monkeys served as controls.

## Results

To identify cynomolgus monkeys associated with IRD among the pedigreed population at TPRC, we performed screening with fundus photography on 1443 monkeys. Fortunately, retinal degeneration (RD) with cystoid macular edema was observed in both eyes of one 14-year-old female monkey (Fig. 2a).

Next, ophthalmic examinations were performed to confirm the diagnosis in this monkey and her relatives. In her examinations, full-field ERGs were nonrecordable, and the outer layer of the retina in the parafoveal area was not visible on OCT imaging (Figs. 2b, 2c). She was diagnosed with RP. Moreover, less frequent pigmentary retinal anomalies were observed in the fundus of both eyes of her 3-year-old nephew (#9) (Fig. 3a). Dark-adapted ERGs were almost nonrecordable

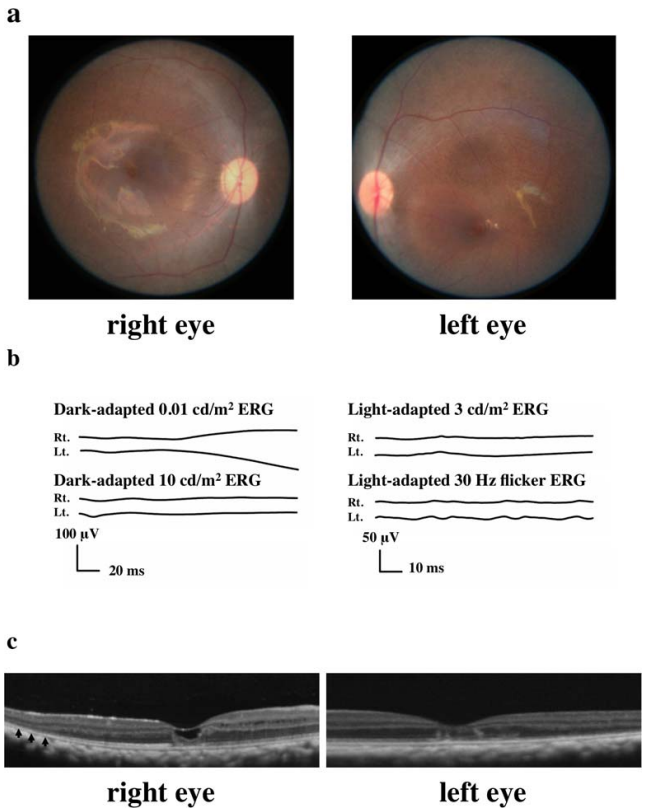


FIGURE 3. Ophthalmic data from a 3-year-old male monkey (#9). (**a**) Fundus photographs. Slight retinal degeneration was observed around the vascular arcade. (**b**) Full-field ERGs. Dark-adapted ERGs and were almost nonrecordable and light-adapted ERGs were extremely reduced. (**c**) Images of horizontal OCT macular scan. Disappearance of the outer layer in the peripheral retina (*arrows*) and the outer layer of the foveal area was partly disarranged. Retinal cystic change was observed in the right eye.

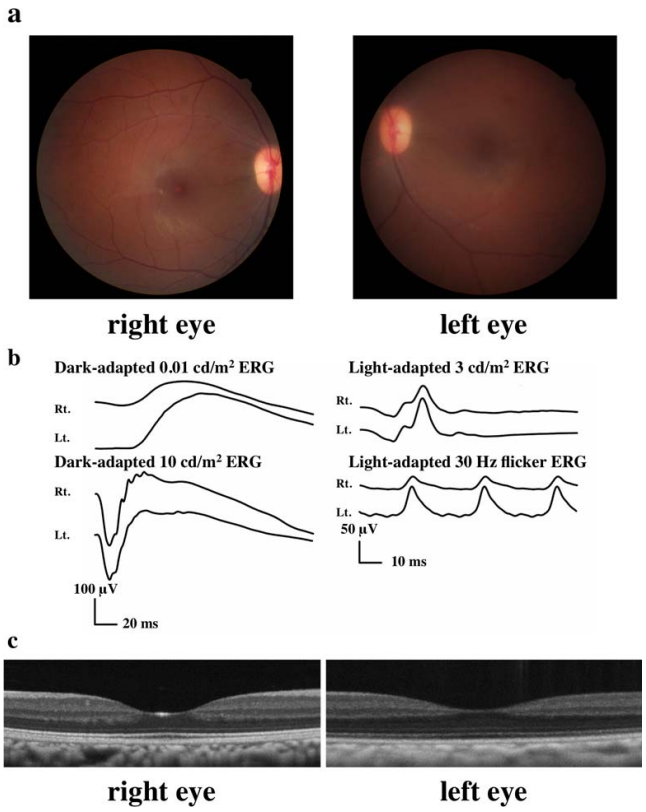


FIGURE 4. Ophthalmic data from a 9-year-old female monkey (#5). (**a**) Fundus photographs. Retina was normal. (**b**) Full-field ERGs. Both dark-adapted and light-adapted ERGs were recordable. (**c**) Images of horizontal OCT macular scan. Retinal structure was preserved.

and light-adapted ERGs were extremely reduced (Fig. 3b). In addition, OCT showed disappearance of the outer layer in the peripheral retina (Fig. 3c, arrows). He was also diagnosed with RP. His father (#6) was her cousin (the son of her mother's older brother) and his mother (#5) was her younger half-sibling sister with a different father. The hereditary nature of the RD seemed highly probable (autosomal recessive inheritance suspected). In the ophthalmic examinations of her other eight relatives, the fundus findings were normal and full-field ERGs were recordable (Fig. 4; #5).

Moreover, we collected DNA from the two affected and seven unaffected monkeys in the family and carried out whole-exome sequencing and variant calling followed by candidate variant prioritization (Fig. 5 for summary). After mapping and annotation of the sequencing results of the whole exome using the macaque genome as the reference, a total of 289,613 SNVs and 20,227 insertions or deletions were detected. The search for nonsynonymous variants that segregated with the disease while assuming an autosomal recessive mode of inheritance and mutations being in the homozygous state only in the affected monkeys revealed 28 candidate variants (25 single nucleotide substitutions [SNVs] and three insertions and/or deletions [indels]) in 26 genes, including two variants in *CEP164*, which has been reported to cause RD in humans (Table). After excluding variants in disease genes known to cause other pathologies unrelated to RD in humans or mice, nonsynonymous variants at nonconserved sequences in mammals, and variants in poorly characterized genes, only two candidate variants in two genes (*CEP164* and *CCDC13*) remained. *CEP164* was previously associated with syndromic RD and *CCDC13* directly interacts with multiple genes known to cause RD.[17] However, after screening for these variants in the DNA of 48 unaffected monkeys, both were found in a homozygous state in multiple animals, making their association with RD unlikely (data not shown).

## DISCUSSION

IRD, including RP, is an intractable disease; no known treatment exists at the present time. If we could use an IRD

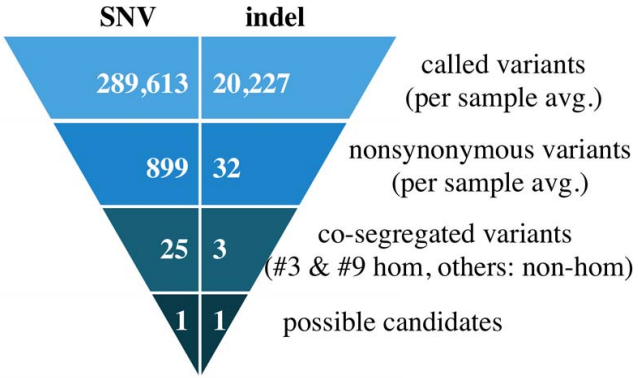


FIGURE 5. Summary of candidate variant prioritization. Analysis workflow and summary of each step. Note, the workflow assumes autosomal recessive mode of inheritance and mutations being homozygous state only in the affected monkeys (#3 and #9).

model in nonhuman primates, studies evaluating the mechanisms of disease pathogenesis, as well as the efficacy of these new therapeutic treatments, would be more reliable. To our knowledge, there are few reports demonstrating IRD in nonhuman primates.[18,19] In this study, we first discovered a cynomolgus monkey family with RP among the pedigreed population.

Although we could not determine their clinical features, such as night blindness in RP patients, the ophthalmic data (fundus photography, full-field ERGs, and OCT imaging) we collected suggest they have night blindness, and the 14-year-old female monkey is in an advanced stage. In both eyes of the 3-year-old monkey on OCT imaging, the outer layer of the peripheral retina was not visible (Fig. 3c, arrows). On the other hand, the structure of the foveal area was almost preserved. It suggests that the rod photoreceptor cells were predominantly affected, compared with the cone photoreceptor cells. No evidence of inflammatory disorder existed in their eyes. Based on the above findings, we diagnosed RP.

RP in monkeys is surprisingly rare, considering that such monkeys have never been reported to the best of our knowledge and that these monkeys are inbred. Therefore, it is likely that the disease in these two related families is caused by the same mutation. However, the possibility that different mutations account for the two monkeys cannot be completely ruled out. Unfortunately, the whole-exome sequencing analysis we performed could not identify the pathogenic mutations in these monkeys. This may have been due to the variable capturing efficiency, which could be overcome by the application of whole-genome sequencing. Nevertheless, in principle, the data we show indicate that a search for pathogenic mutations using a human whole-exome sequencing kit is feasible and may be indicated for the screening of autosomal recessive genetic diseases in macaques.

In conclusion, we discovered novel cynomolgus monkeys with RP in their pedigrees. In the future, we will breed this disease colony to contribute to the development of therapies for RP.

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