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ARTICLE

LRRTM4-C538Y novel gene mutation is associated with hereditary macular degeneration with novel dysfunction of ON-type bipolar cells

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Abstract

The macula is a unique structure in higher primates, where cone and rod photoreceptors show highest density in the fovea and the surrounding area, respectively. The hereditary macular dystrophies represent a heterozygous group of rare disorders characterized by central visual loss and atrophy of the macula and surrounding retina. Here we report an atypical absence of ON-type bipolar cell response in a Japanese patient with autosomal dominant macular dystrophy (adMD). To identify a causal genetic mutation for the adMD, we performed whole-exome sequencing (WES) on four affected and four-non affected members of the family for three generations, and identified a novel p.C538Y mutation in a post-synaptic gene, *LRRTM4*. WES analysis revealed seven rare genetic variations in patients. We further referred to our in-house WES data from 1360 families with inherited retinal diseases, and found that only p.C538Y mutation in *LRRTM4* was associated with adMD-affected patients. Combinatorial filtration using public database of single-nucleotide polymorphism frequency and genotype–phenotype annotated database identified novel mutation in atypical adMD.

Introduction

The macula is the specialized central region of the eye responsible for visual acuity in primates. In the center of the macula, called the fovea, cone photoreceptors are packed in a region 0.2 mm in diameter, resulting in the highest density of cone cells in the retina. Outside the fovea, rod photoreceptors form a dense ring around the fovea, ~4.5 mm from the foveal pit [1]. To improve the efficiency of light perception, the macula is free of retinal vessels, and thus entirely dependent on the choriocapillaris for its blood supply. These anatomical specificities, in turn, render the macula susceptible to various types of stress, such as nutrient or oxygen deficiency or metabolic dysfunction, which can lead to severe visual impairment via photoreceptor dysfunction or degeneration.

Hereditary macular dystrophy is a general term for phenotypically and genetically highly heterogenous visual impairments, including Stargardt disease (STGD) [2], cone-rod dystrophy [3], vitelliform macular dystrophy (VMD) [4], X-linked juvenile retinoschisis [5], occult macular dystrophy [6], and North Carolina macular dystrophy (NCMD) [7]. Number of causal genes have been identified for each diseases, then, population of patients affected by the same disease caused by mutations in the same gene are very rare. The most prevalent case is the STGD, which has an estimated prevalence of 1 in 10,000 [8], and accounts for 7% of all inherited macular dystrophies. As most cases of the autosomal recessive STGD are caused by mutations in *ABCA4* gene, much lower prevalence is expected for other macular dystrophies caused by different genetic mutations, for example, autosomal dominant STGD caused by mutations in *ELOVL4* [9] and *PROM1* [10].

The rare appearance of disease-affected patient makes it difficult to find a second family with the same clinical

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phenotype, and with the same causal gene mutation. Whole-exome sequencing (WES) has remarkably accelerated the identification of causal genes for inherited retinal diseases [11], though, the analysis of a single pedigree often leaves number of candidate causal genes associating with disease-affected subjects even after eliminating the common variation using public single-nucleotide polymorphism databases. The Japan Eye Genetics Consortium (JEGC) was established in 2011 by 30 ophthalmology departments in Japanese universities to collect genotype–phenotype information of 36 inherited retinal diseases collecting 2240 DNA samples in 1360 pedigrees. WES have finished for 1536 individuals, and the sequence data are annotated with clinical characteristics.

Here, we report genetic analyses of a Japanese family with dominantly inherited macular degeneration for three generations. Patients showed a novel atypical loss of ON-type bipolar cell response in the whole-field electroretinogram (ERG). WES analysis and filtration of candidate mutations of this pedigree resulted with seven variations co-segregated with affected individuals. Further filtration using the JEGC genotype–phenotype database eliminated all candidate mutations detected in normal individual in other pedigrees leaving one mutation p.C538Y in *LRRTM4* (Leucine-Rich Repeat Transmembrane Neuronal 4) gene as disease-causing.

Methods

Subjects and clinical examination

The proband (Case III–2) was referred to our clinic because of strabismus at the ages of 4 year (yr). Her father (Case II-2), her aunt (Case II–1), and her grandfather (Case I–2) were examined in our hospital for the first time at the ages of 26yr, 39yr and 56yr, respectively (Table 1). These patients had consulted local clinics during childhood complaining of low visual acuity. We performed spectral-domain optical coherence tomography (OCT) (Cirrus HD OCT, version 6.5; Carl Zeiss Meditec, USA) on all family members. A molecular genetic study was performed at the Department of Ophthalmology of Juntendo University Hospital to investigate the clinical features and genotype of family members. Parents provided informed consent for the genetic analysis of their daughter and themselves. Salivary DNA samples were obtained from each patient. Full-field ERG of the proband (Case III–2) and two other affected patients (Case II–1 and II–2) were obtained as dictated by the standard protocol of the International Society for Clinical Electrophysiology of Vision (LE4000, Tomey Corporation, Aichi, Japan) [12]. ERG examination included: (i) dark-adapted response with dim flash (dark-adapted (DA) 0.01), (ii) dark-adapted response with bright flash (DA 10.0), (iii) light-adapted response with standard flash at 2 Hz (light-adapted (LA 3.0), (iv) light-

Table 1 Clinical information of each patient

Case	Sex	Age at first assesment (y)	visual acuity_1 (y)	visual acuity_2 (y)	Visual field (first assesment)	Funduscopy finding	Full-field ERG	additional comments
I–2	M	56	0.03, OS 0.01, OD (56)	N.E.	N.E.	Pigmented degeneration in the whole retina (56)	N.E.	Poor vision from childhood
II–1	F	39	0.03, OS 0.02, OD (39)	0.05, OS 0.05, OD (42)	N.E.	Pigmented degeneration in the whole retina with macular atrophy	Severely reduced rod and cone responses.	Poor vision from childhood
II–2	M	26	0.1, OS 0.1, OD (26)	0.06, OS 0.08, OD (41)	Central scotoma within 20° OU; preserved peripheral visual field OU.	Circular atrophy in macula	Reduced b-wave in DA 0.01, electronegative form in DA 10.0, square-shaped a-wave in LA 3.0, absence of b-wave and preserved d-wave in the long-flash ERG.	Poor vision from childhood
III–2	F	5	0.09, OS 0.03, OD (5)	0.03, OS 0.06, OD (17)	Central scotoma within 20° OD and 10° OS; Slight concentric contraction of peripheral visual field (40–60°) OU.	Pigmented degeneration in whole retina with macular atrophy	Severely reduced rod and cone responses, electronegative form in DA 10.0, relatively preserved d-wave in long-flash ERG.	

N.E. not examined, *OS*left eye, *OD*right eye, *OU*both eyes

Visual acuity_1and visual field are from the first clinical assesment. Other visual data (including OCT) were obtained at the ages same to the visual acuity_2 assesment, except in Case I-2

Table 2 Candidate gene mutations from the adMD family

Gene	Accession number	dbSNP	AA-change	Frequency			Allele conts in normal (JEGC)
				ExAC_EAS	1000G_EAS	HGVD	
LRRTM4	NM_001134745		exon4, c.G1613A, p.C538Y	0	0	0	0
NMS	NM_001011717	rs201430802	exon9, c.A438C, p.E146D	0.0003	0.001	0.0041	3
C6orf163	NM_001010868	rs200580279	exon4, c.T392C, p.M131T	0	0	0.0064	4
ASCC3	NM_006828	rs746115950	exon14, c.A2168C, p.H723P	0.0007	0	0.0057	1
HECTD1	NM_015382	rs143401795	exon30, c.C5413A, p.L1805I	0.0005	0.001	0.0030	2
FANCM	NM_020937	rs202171930	exon10, c.1741T, p.R581C	0.0023	0	0.0021	1
ARID4A	NM_002892	rs779291787	exon13, c.A1052T, p.Y351F	0.0003	0	0	2

ExAC_EAS East Asian data in Exome Aggregation Consortium database (ExAC); *000G_EAS* East Asian data in 1000 Genomes database, *HGVD* Human Genetic Variation Database *JEGC* Japan Eye Genetic Consortium

adapted response with standard flash at 30 Hz (LA 3.0 flicker), and (v) light-adapted long flash for 100 ms (long-flash ERG). This study was performed according to the tenets of the Declaration of Helsinki. All protocols were approved by the local ethics committee of Juntendo University School of Medicine (2014041).

Genomic DNA extraction and whole-exome analysis

Genomes of four affected and four unaffected family members were extracted with Oragene (OG-500, Genotek) and purified with Nucleospin Blood L (Takara Bio). Exon capture was performed with SureSelect XT Human Exon kit ver.4 + UTRs (Agilent Technologies). WES was performed with a HiSeq 2000 (Illumina). Reads were mapped to the reference human genome (1,000 Genomes, phase 2 reference, hs37d5) with Burrows-Wheeler Alinger software version 0.7.10 [13]. Duplicate reads were removed by Picard Mark Duplicates module version 1.129. Mapped reads surrounding insertion–deletion polymorphisms (INDELs) were realigned, and base-quality scores were recalibrated with Genome Analysis Toolkit (GATK) version 3.3–0 [14]. Calling of mutations was performed with the GATK HaplotypeCaller module. Called single-nucleotide variants and INDELs were annotated with snpEff version 4.1B [15] and Annovar version 2015-04-24 [16]. Mutations were annotated with snpEff score ("HIGH", "MODERATE", or "LOW") and with allele frequency in the 1000 Genomes database, Exome Aggregation Consortium (ExAC) database, and Human Genetic Variation Database (HGVD) (Kyoto University, version 1.42). Mutations were filtered to obtain mutations with a HIGH or MODERATE snpEff score (indicating that the amino-acid sequence would be functionally affected) and frequency <1% in the above databases. Mutations were filtered with the family's hereditary information (Table 2). We additionally selected variations that were not found in the in-house control (WES data of 14 individuals without ocular disease). Mutations were filtered with the family's hereditary information. Finally, we selected mutations that were not found in unaffected individuals in our in-house WES data (Japan Eye Genetics Consortium, JEGC, http://www.eye.go.jp/) [17].

Real time-polymerase chain reaction (RT-PCR)

All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Vision Research. Protocols were approved by the Tokyo Medical Center Experimental Animal Committee. Total RNA was isolated from mouse and monkey retina with Trizol (Thermo Fisher Scientific) and reverse-transcribed with Superscript III (Thermo Fisher Scientific). The long isoform of *LRRTM4* was amplified with specific primers: (cynomolgus monkey (449 bp): forward: 5′-TGGTGATCTATGTGTCTTGG-3′, reverse: 5′-ACTCGAGGTTTGCAATTCTCTCTAGGTAG-3; mouse (1698 bp): forward: 5′-CCTGCTTGCTTCAACAGCTG-3′, reverse: 5′-CTGGCAGTACCCAATCACTG-3′).

Results

Clinical information of the autosomal dominant Macular dystrophy in the Japanese family

The pedigree for a Japanese family showed dominant-pattern macular dystrophy for three generations (Fig. 1a). Cases I–2, II–1, and II–2 consulted local clinics because of poor vision during their childhoods. The proband (Case

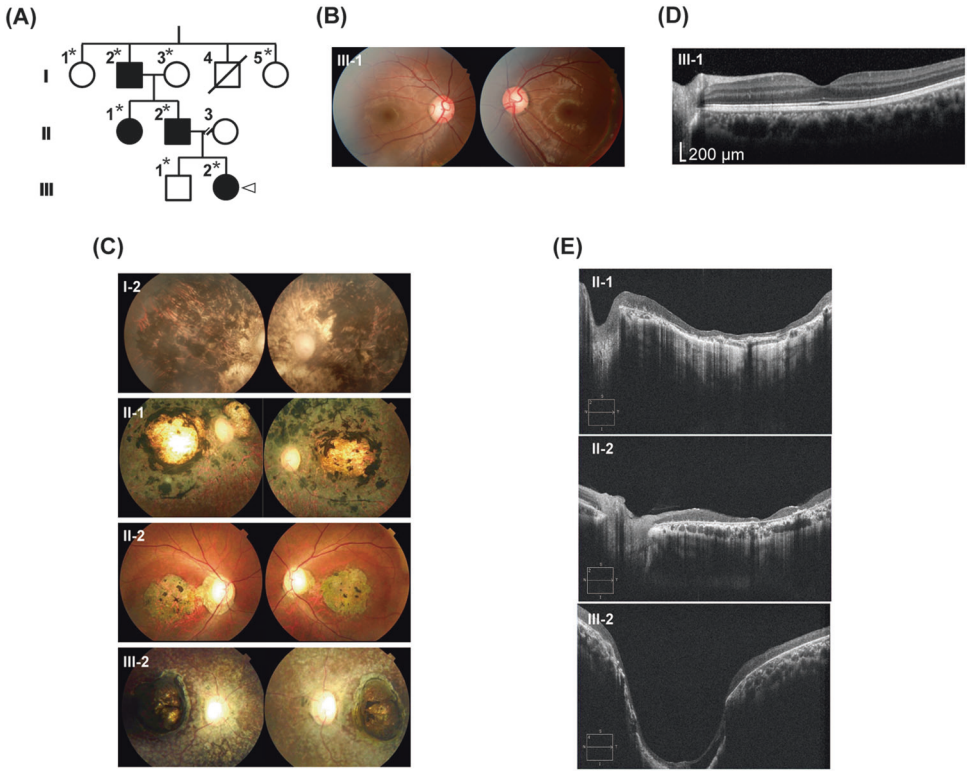

Fig. 1 Pedigree and clinical examinations for patients with adMD. **a** Family pedigree showing an autosomal dominant heredity pattern for macular dystrophy. Affected individuals are indicated in black. Arrowhead indicates proband (Case III–2). Genomes were collected from the individuals marked with asterisks. **b**, **c** Funduscopy images from one unaffected individual (Case III–1) **b** and four affected patients (Case I–2, II–1, II–2, and III–2) **c**. **d**, **e** OCT images from one unaffected individual (Case III–1) **d** and three affected patients (Case II–1, II–2, and III–2) **e**. Retinal degeneration in Cases II–1 and III–2 extended to the entire retina, whereas degeneration was observed only in the macula in Case II–2

III–2) was referred to our clinic because of strabismus at the age of 4 years. The other cases (I–2, II–1, and II–2) were first examined in our clinic at the ages of 56 yr, 39 yr, and 26 yr, respectively. Detailed clinical information is provided in the supplemental text and summarized in Table 1. Funduscopic examination of affected individuals revealed circular degeneration in the vascular arcade and thinning of all retinal layers (Fig. 1c, e). Affected individuals (except Case II–2) showed severe retinal degeneration throughout the retina, circular choroidal atrophy in the macula (Fig. 1c), attenuation of retinal vessels, and optical atrophy. A circular atrophic region was observed in the macula of Case II–2, but the peripheral retina was relatively preserved. OCT results showed severe thinning throughout the retina, affecting the outer nuclear, photoreceptor and retinal pigment epithelium layers, in all three cases. A large choroidal excavation of the macula was observed in Case III–2 (Fig. 1e).

Patient ERGs suggest impairment of ON-type bipolar cells

ERG responses were severely reduced under both DA and LA conditions in all patients (Fig. 2). In Case II–2 (Fig. 2c), where retinal degeneration was observed only in the macula, ERG results suggested particular abnormalities in ON-type bipolar cells. ERG testing of this patient revealed a severely reduced b-wave in the DA response with dim flash (DA 0.01), an electronegative form in the DA response with bright flash (DA 10.0), a square-shaped a-wave in the LA response with standard flash (LA 3.0), and absence of the b-wave and preserved d-wave with long flash [18]. In Case III–2 (Fig. 2d), where retinal degeneration extended throughout the retina, the DA response with dim flash (DA 0.01) was extinguished, but the DA response with bright flash (DA 10.0) showed the electronegative form. The a- and b-waves in the LA response with standard flash (LA 3.0) were severely reduced, but the d-wave in the long-flash ERG was relatively preserved.

Filtration of causal gene mutation from family genomes by WES analysis

Because of the early onset and severity of macular degeneration in the proband, Case III–2, we first screened for mutations common to Leber congenital amaurosis (*CRX*, *LCA5*, *AIPL1*, and *IMPDH1*) by Sanger direct sequencing. No mutations were detected. We therefore performed WES on the genomes of the four patients (Cases I–2, II–1, II–2, and III–2) and four unaffected individuals (Cases I–1, I–3, I–5, and III–1) who were members of the family (Fig. 1a). Averaged sequence depth was at least 47 for each individual, and > 89% regions were covered with more than five reads. Use of the filtration protocol described below identified the single disease-associated gene mutation, p.C538Y in leucine-rich repeat transmembrane neuronal 4

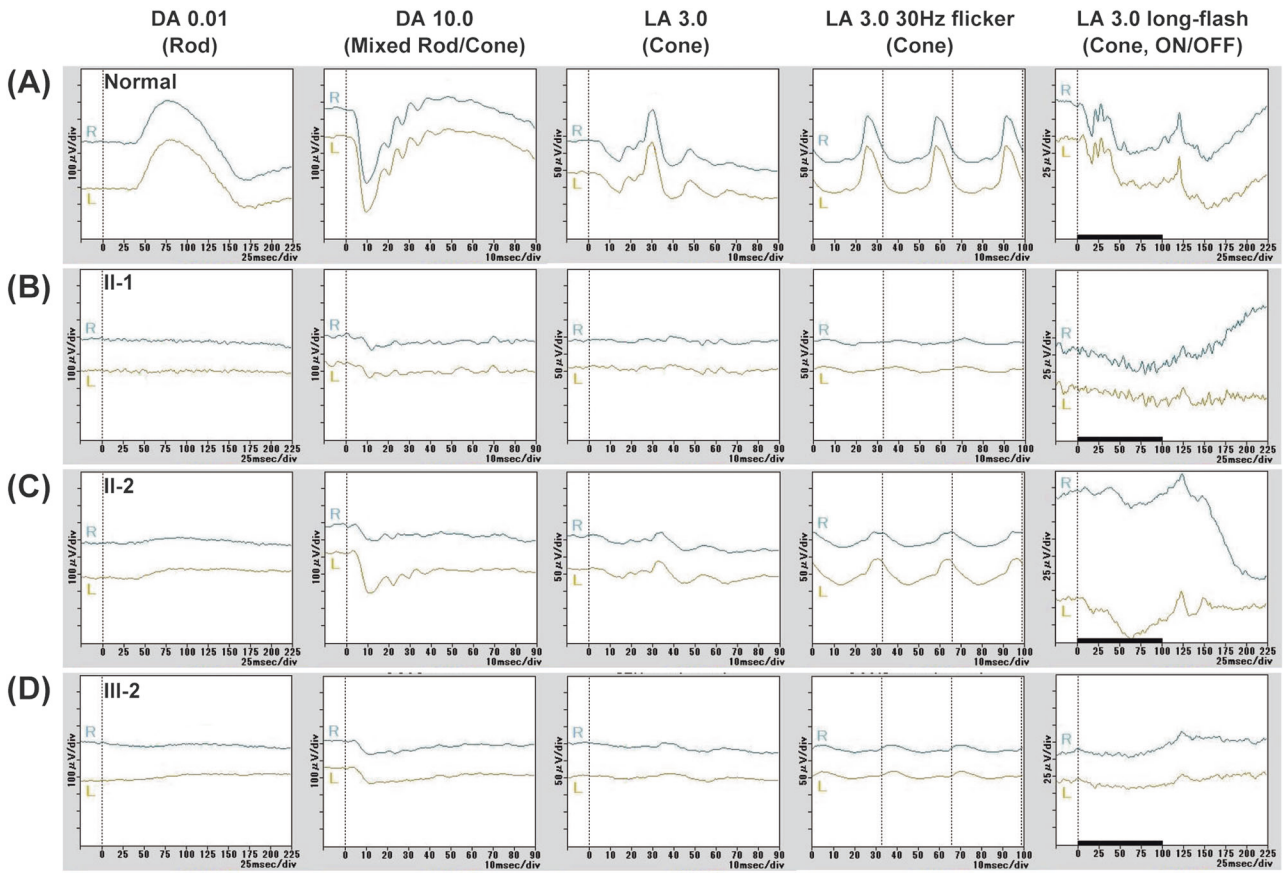

Fig. 2 Full-field ERGs from three patients affected with macular dystrophy and one unaffected control. **a** Unaffected individual, **b** Case II–1, **c** Case II–2, and **d** Case III–2. In case II–2, the ON-type bipolar cell response (DA 0.01) was severely reduced, but cone responses (LA 3.0, LA 3.0 30 Hz flicker, and LA 3.0 long flash) remained. Cases II–1 and III–2 did not show either photoreceptor (rod or cone) or bipolar cell responses

(*LRRTM4*). Comparing patient sequences with the reference human genome (hs37d5) revealed 93,315 variations in the patients (Fig. 3a). We filtered the results for variations that could change the amino-acid sequence and excluded common variations in the 1000 Genomes database, ExAC database, Human Genetic Variation Database and our in-house control (see Methods). We filtered the resulting 571 candidate mutations using the family's pattern of inheritance, and obtained seven candidate genetic mutations (Table 2). Then, we again referred to our in-house WES database (JEGC data) to see if the seven variations were shared among Japanese inherited retinal disease patients and/or unaffected individuals. All the seven variations were found in a heterozygous manner. *LRRTM4 (p.C538Y)* was detected only among the autosomal dominant macular dystrophy (adMD)-affected subjects in this family. *NMS* (p.E146D), *C6orf167* (p.M131T) and *HECTD1* (p.L1805I) were found in more than two unaffected individuals from different families. *ARID4A* (p.Y351F) was found in two unaffected individuals from a single family with retinitis pigmentosa. Heterozygous *ASCC3* (p.H723P) and *FANCM* (p.R581C) were found in one unaffected, independently, in the JEGC data. Following these steps, *LRRTM4* (p. C538Y) was identified as the only disease-associated mutation (Fig. 3a).

Genetic investigation revealed the candidate causal mutation to be the heterozygous mutation p.C538Y (c.G1613A) in the long isoform of *LRRTM4* (RefSeq accession numbers: NM_001134745 for mRNA, NP_001128217 for protein; Fig. 3b). The 538th cysteine residue (Fig. 3b, asterisk) resided in the long isoform specific region, which is conserved among vertebrates (Fig. 3b). The p.C538Y mutation was predicted as damaging/disease-causing by Polyphen-2 (score = 0.995), SHIFT (score = 0) and Mutation Taster (score = 0.974).

LRRTM4 long isoform was expressed in the primate retina

Human *LRRTM4* gene is composed of four exons. The short isoform uses the stop codon in the middle of the exon3, encoding a protein with 518 amino acids (aa). On the other hand, the long isoform splice out part of the exon3 including the stop codon, but uses exon4, which is

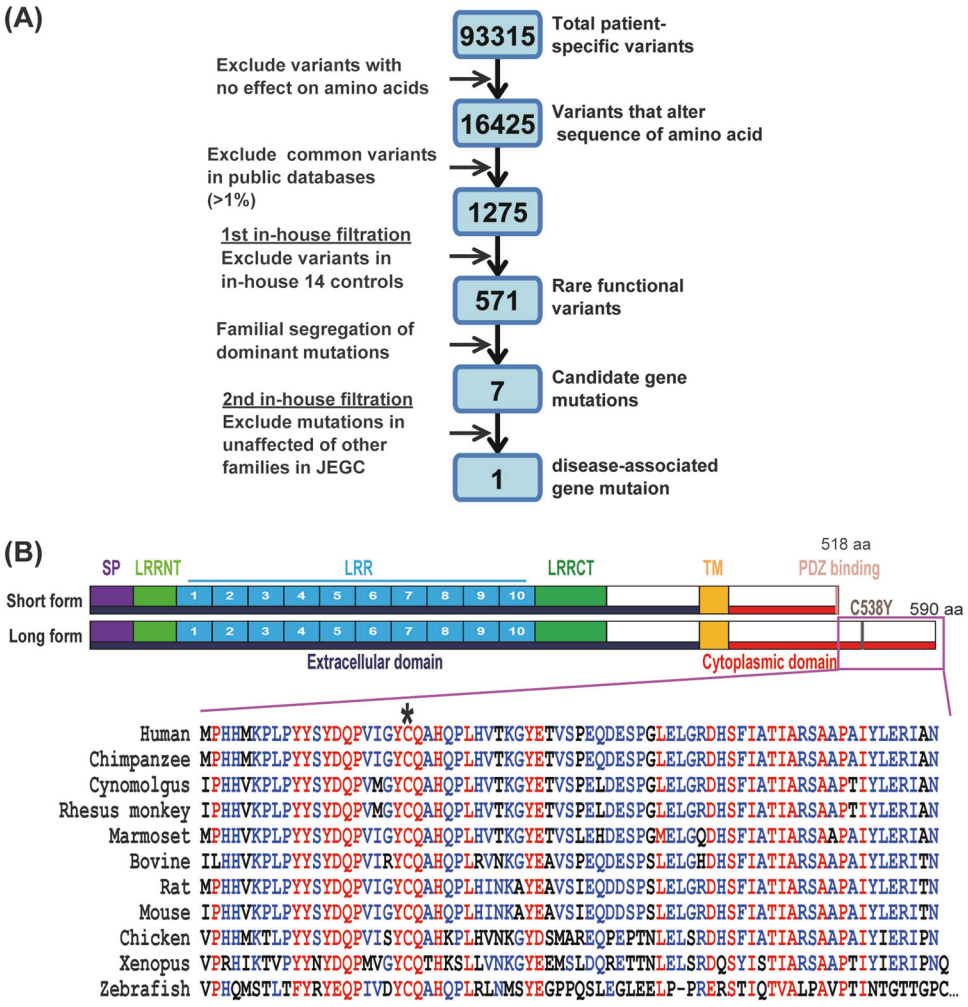

Fig. 3 Flow diagram for filtering WES results. **a** Among the patient-specific variants, those which did not cause amino-acid changes, or common variants (found in 1000 Genomes, ExAC, or HGVD) were excluded. LRRTM4 C538Y was the least common mutation among the dominantly inherited candidate mutations. **b** Protein domain structure of the short and long isoforms of LRRTM4, and alignment of long isoform-specific domain of C-terminal LRRTM4 (magenta box) among vertebrate species. Asterisk indicates the mutated site (538th Cysteine), which is conserved from primates to fish. SP, signal peptide; LRRNT, leucine-rich repeat N-terminal; LRR, leucine-rich repeat motif; LRRCT, leucine-rich repeat C-terminal; TM, transmembrane domain; PDZ binding, PSD95 binding site as PDZ binding motif

translated into a protein with 590 aa. Expression of long and short isoforms of *LRRTM4* was previously shown in mouse brain, but their expression in the retina was not reported. To determine whether the long isoform of *LRRTM4* was expressed in the retia, we analyzed mRNA expression in the mouse and monkey retinal tissues by RT-PCR. Forward primer was designed on the exon3′, and reverse primer was on the exon4 (see Methods). RT-PCR showed that the *LRRTM4* long isoform was expressed in the mouse and monkey retinal tissues (Fig. 4b).

Discussion

LRRTM4 is a neuronal and post-synaptic protein whose short isoform (NP_079269) has been well-studied for its role in the instruction of excitatory pre-synapses [19], but the role of the long isoform of LRRTM4 has not been extensively studied. Moreover, the expression and roles of LRRTM4 have not been examined in the retina. This is the

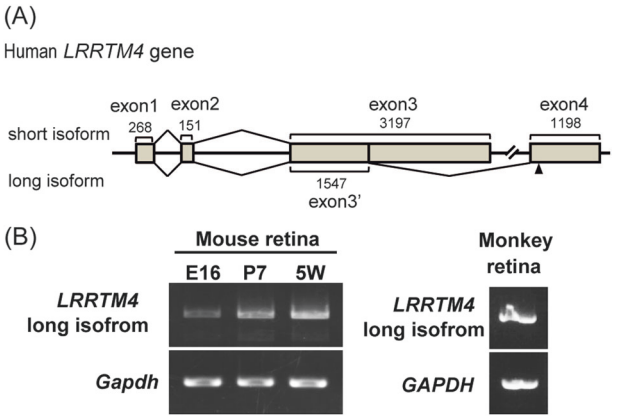

Fig. 4 LRRTM4 is expressed in the mouse and monkey retinal tissues. **a** Schematic of human *LRRTM4* gene structure. Boxes indicate exons. LRRTM4 long isoform consisted of four exons: exon 1, exon 2, partial exon3, and exon4. Variant 2 consisted of three exons: exon 1, exon 2, and full-length exon3. **b** Detection of *LRRTM4* variant 1 mRNA in mouse and monkey retina by RT-PCR. Arrowhead indicate the c. G1613A mutation

first report of *LRRTM4* as a causal gene for autosomal dominant macular dystrophy.

LRRTM4 is one of four members in the leucine-rich-repeat transmembrane (LRRTM) protein family. LRRTM4, as well as other LRRTM family members, is associated with proteins and instructs the formation of excitatory pre-synaptic structure [19]. A recent report showed that both the short and long isoforms of LRRTM4 had equivalent pre-synapse formation activity in vitro [20]. Furthermore, *LRRTM4* knock-out mice showed reductions in dendritic spine density and miniature excitatory post-synaptic current frequency in the dentate gyrus [21]. Given the importance of *LRRTM4* in excitatory synapse development in brain, LRRTM4 would work as a post-synaptic adhesion molecule also in the retina. Genetic studies indicate that intragenic deletion or copy number variations in *LRRTM4* are associated with autism spectrum disorders [22–25]. However, the patients included in this study, either the family members, did not noticed symptoms of neuropsychiatric or memory disorders, implying that the long isoform–specific C538Y mutation induced a disease phenotype through a different mechanism.

Inherited macular degeneration is a general name, including STGD, VMD, X-linked juvenile retinoschisis, occult macular dystrophy, and NCMD. Like in other reported pedigrees with inherited macular degeneration [26, 27], phenotypic variability was observed among our patients by clinical examinations. Retinal degeneration was limited to the central region in the case II–2, but was extended to the whole-retina in the cases II–1 and III–2. In addition, decrease of ON-type bipolar cell response was detected in the case II–2. One explanation for this phenotypic variability is that disease progress was different between the patients. The *LRRTM4* C538Y mutation might affect bipolar cell response at first, then, caused photoreceptor degeneration from macular to the surrounding area. Another possibility is that the case II–2 had additional genetic mutation that caused the decrease of ON-type bipolar cell response, which is classically characteristic in the congenital stationary night blindness (CSNB). For the latter possibility, we compared our whole-exome data of cases II–1, II–2, and III–2, and selected the variations found only in the case II–2. We found 34 heterozygous variations and 11 homozygous variations specific for the case II–2, but no known CSNB causal gene was included. Evidences suggest the effect of genetic modifiers to the variability of phenotypes in human eye diseases, however, the identification of the modifier molecule is still limited [28]. Further functional analyses of *LRRTM4* C538Y mutation will help us to understand the disease-causing mechanism of the variable phenotypes observed.

This finding provides evidence of severe macular degeneration caused by mutation in gene expressed in retinal synapse. Further investigation of long isoform of LRRTM4 in relation to degeneration of the entire macular opens new field of photoreceptors–bipolar cells interaction in addition to the regular neurotransmission and therapeutic for individuals suffering from autosomal dominant macular dystrophy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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