Retina

Specific Alleles of CLN7/MFSD8, a Protein That Localizes to Photoreceptor Synaptic Terminals, Cause a Spectrum of Nonsyndromic Retinal Dystrophy

Kamron N. Khan,1–4 Mohammed E. El-Asrag,3,5 Cristy A. Ku,6 Graham E. Holder,1,2,7 Martin McKibbin,3,4 Gavin Arno,1 James A. Poulter,3 Keren Carss,8,9 Tejaswi Bomimreddy,4 Saghar Bagheri,10 Benjamin Bakall,11,12 Hendrik P. Scholl,10 F. Lucy Raymond,8,13 Carmel Toomes,3 Chris F. Inglehearn,3 Mark E. Pennesi,6 Anthony T. Moore,1,2,14 Michel Michaelides,1,2 Andrew R. Webster,1,2 and Manir Ali3; for NIHR BioResource-Rare Diseases and UK Inherited Retinal Disease Consortium

1University College London Institute of Ophthalmology, London, United Kingdom
2Inherited Eye Disease Service, Moorfields Eye Hospital, London, United Kingdom
3Section of Ophthalmology and Neuroscience, Leeds Institute of Biomedical and Clinical Sciences, University of Leeds, St. James’s University Hospital, Leeds, United Kingdom
4Department of Ophthalmology, St. James’s University Hospital, Leeds, United Kingdom
5Department of Zoology, Faculty of Science, Benha University, Benha, Egypt
6Casey Eye Institute, Oregon Health & Science University, Portland, Oregon, United States
7Department of Electrophysiology, Moorfields Eye Hospital, London, United Kingdom
8National Institute for Health Research BioResource-Rare Diseases, Cambridge University Hospitals, Cambridge Biomedical Campus, Cambridge, United Kingdom
9Department of Haematology, University of Cambridge, Cambridge, United Kingdom
10Wilmer Eye Institute, Johns Hopkins University, Baltimore, Maryland, United States
11Associated Retina Consultants, Phoenix, Arizona, United States
12University of Arizona, College of Medicine, Phoenix, Arizona, United States
13Department of Medical Genetics, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, United Kingdom
14Department of Ophthalmology, University of California–San Francisco School of Medicine, San Francisco, California, United States

Correspondence: Manir Ali, Section of Ophthalmology and Neuroscience, Leeds Institute of Biomedical and Clinical Sciences, University of Leeds, Leeds LS9 7TF UK. medna@leeds.ac.uk.

Kamron N. Khan, Medical Retina Service, Moorfields Eye Hospital, 162 City Road, London EC1V 2PD, UK; kamron.khan@moorfields.nhs.uk.

The work presented here and should therefore be regarded as equivalent first authors.

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PURPOSE. Recessive mutations in CLN7/MFSD8 usually cause variant late-infantile onset neuronal ceroid lipofuscinosis (vLINCL), a poorly understood neurodegenerative condition, though mutations may also cause nonsyndromic maculopathy. A series of 12 patients with nonsyndromic retinopathy due to novel CLN7/MFSD8 mutation combinations were investigated in this study.

METHODS. Affected patients and their family members were recruited in ophthalmic clinics at each center where they were examined by retinal imaging and detailed electrophysiology. Whole exome or genome next generation sequencing was performed on genomic DNA from at least one affected family member. Immunofluorescence confocal microscopy of murine retina cross-sections were used to localize the protein.

RESULTS. Compound heterozygous alleles were identified in six cases, one of which was always p.Glu336Gln. Such combinations resulted in isolated macular disease. Six further cases were homozygous for the variant p.Met454Thr, identified as a founder mutation of South Asian origin. Those patients had widespread generalized retinal disease, characterized by electoretinography as a rod-cone dystrophy with severe macular involvement. In addition, the photopic single flash electoretinograms demonstrated a reduced b- to a-wave amplitude ratio, suggesting dysfunction occurring after phototransduction. Immunohistology identified MFSD8 in the outer plexiform layer of the retina, a site rich in photoreceptor synapses.

CONCLUSIONS. This study highlights a hierarchy of MFSD8 variant severity, predicting three consequences of mutation: (1) nonsyndromic localized maculopathy, (2) nonsyndromic widespread retinopathy, or (3) syndromic neurological disease. The data also shed light on the underlying pathogenesis by implicating the photoreceptor synaptic terminals as the major site of retinal disease.

Keywords: macular degeneration, retinal dystrophy, DNA sequencing, electoretinography, immunohistology, photoreceptor synapse
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herited retinal dystrophies (IRD) are a complex group of genetically heterogeneous eye disorders that are caused by Mendelian mutations in over 200 genes (see https://sph.uth.edu/retnet/; provided in the public domain by The University of Texas Health Science Center, Houston, TX, USA). They are initially diagnosed according to the patient’s clinical symptoms, their age of onset, and the cell type that is primarily affected by the condition. Knowledge of the underlying genetic defect allows for a more comprehensive description, though the assumption that each genetic disorder may have a unique clinical signature is oversimplified since molecular screening has revealed profound heterogeneity, both clinical and genetic, in inherited retinal diseases. Additionally, we are now starting to appreciate that isolated retinal disease can be associated with variants in genes previously identified as only causing syndromic disease.1

The neuronal ceroid lipofuscinoses (NCLs) are a group of lysosomal storage disorders resulting from single gene defects in lysosomal hydrolases or lysosomal/endoplasmic reticulum membrane proteins.2 They are characterized by early onset neurodegeneration associated with accumulation of autofluorescent intracellular inclusions in neurons and extraneuronal tissues.3 Collectively, they are the most common cause of neurodegeneration associated with accumulation of autofluorescent intracellular inclusions in neurons and extraneuronal tissues.4–8 Symptoms of vLINCL usually start between the ages of 3 and 6 years, with seizures and developmental regression followed by speech failure, ataxia, visual loss, myoclonus, and ultimately premature death. Recently, recessive mutations in MFSD8 have also been associated with nonsyndromic macular dystrophy in two families.3 Retinal dysfunction in the absence of syndromic association had not previously been reported. Despite many advances in NCL research, the biological basis for neurodegeneration remains eluding. The present study provides novel mechanistic insights gleaned from detailed phenotypic investigations in 12 patients with MFSD8-associated retinopathy, together with immunolocalization studies in the murine retina. Possible genotype-phenotype correlations underlying nonsyndromic and syndromic disease are also discussed.

Materials and Methods
Patient Ascertainment and Clinical Evaluation

The study was approved by the appropriate local research ethics committees and the protocol adhered to the tenets of the Declaration of Helsinki. Patients were ascertained from the inherited eye disease clinics at Moorfields Eye Hospital, London (Patients GC17967, GC17967, GC13428, GC18458, GC19832, GC3716, and GC4694), Bradford Royal Infirmary, Bradford (Patient LE1), St. James’s University Hospital, Leeds (Patients LE2 and LE3), Casey Eye Institute, Portland (CEI1), and Wilmer Eye Institute, Baltimore (CEI2). A diagnosis of inherited retinal disease was made by an experienced ophthalmologist based on signs and symptoms, including progressive and symmetrical retinal disease.

Detailed clinical investigations included best-corrected visual acuity (VA; Snellen chart), confrontation visual fields, and pattern and full-field electroretinography (PERG, ERG) using techniques that incorporated the International Society of Clinical Electrophysiology of Vision (ISCEV) recommendations.9,10 Retinal imaging was performed using color fundus photography (Topcon TRC 50IA retinal camera, Topcon Corp., Tokyo, Japan), spectral-domain optical coherence tomography (Spectralis HRA-I OCT, Heidelberg Engineering, Heidelberg, Germany), and 488-nm wavelength fundus autofluorescence (AF) with either a 30° or 55° field (Spectralis HRA-I OCT, Heidelberg Engineering).

Molecular Genetic Analysis

Genomic DNA from affected patients was extracted and analyzed either by whole exome (SureSelect All Exon XT Sequencing kit, Agilent Technologies, Santa Clara, CA, USA) or by whole genome sequencing (Illumina TruSeq DNA PCR-free sample preparation kit, Illumina, San Diego, CA, USA, followed by Illumina HiSeq 2500 sequencing as part of the NIH BioResource–Rare Diseases project) according to the manufacturer’s instructions. FastQ files were aligned to the GRCh37 (hg19) reference genome. Potentially pathogenic variants were identified, filtered based on functionality, minor allele frequency (MAF) in public and in-house databases, and on calculated pathogenicity scores. Putative mutations were validated by Sanger sequencing and checked for segregation.

Immunofluorescence and Confocal Microscopy

Sagittal sections from the eyes of 30-day-old mice were incubated with goat polyclonal anti-MFSD8 (S-14; Santa Cruz Biotechnology, Heidelberg, Germany), followed by an Alexa Fluor 568-conjugated donkey anti-goat immunoglobulin (Molecular Probes, Inc., Eugene, OR, USA). Nuclei were counterstained with DAPI (Vector Laboratories, Orton Southgate, UK). As controls, a section was stained with secondary and another was treated with primary antibody that had been preincubated with peptide antigen followed by secondary. For the co-localization experiments, sections were incubated with anti-MFSD8 and either rabbit polyclonal anti-SNAP-25 (ab5666; Abcam, Cambridge, UK) or rabbit polyclonal anti-PD-95 (ab18258; Abcam) followed by Alexa Fluor 568-conjugated donkey anti-goat immunoglobulin and Alexa Fluor 488-conjugated chicken anti-rabbit immunoglobulin (Molecular Probes, Inc.), and counterstained with DAPI. Confocal images were obtained using an Eclipse TE2000-E system microscope (Nikon Corp., Tokyo, Japan) and processed using Nikon EZ-C1 3.50 software.

Results

Molecular Analysis

Nine families were identified by next generation sequencing of genomic DNA from patients with nonsyndromic retinal dystrophy, which included 12 individuals carrying biallelic variants in MFSD8 (Fig. 1). Six of the cases (GC19741, LE1–LE3, CEI1, and CEI2) were compound heterozygotes that carried the variant, c.1006G>A, p.Glu336Gln, paired with one of three other disease-causing alleles. This allele was identified in trans with a null variant (c.103C>T, p.Arg35Ter for GC19741 or c.235G>A, p.Trp78Ter for CEI1 and CEI2) or a missense change (c.1394G>A, p.Arg465Gln) for LE1–LE3). The remaining six cases (GC17967, GC14328, GC18458, GC19832, GC3716, and GC4694) were homozygous for the missense variant, c.1361T>C, p.Met454Thr. Three of these individuals (GC17967, GC14328, and GC3716) were the offspring of three separate consanguineous marriages. A common shared founder haplotype of South Asian origin, spanning approximately 1.47 Mb was identified by examining single nucleotide variants in
and below each individual, with M representing the mutant allele as shown (LE3, CEI1, and CEI2). The genotypes for all subjects tested are shown (GC17967, GC14328, GC18458, GC19832, GC3716, GC4694, LE1–LE3, CEI1, and CEI2). The genotypes for all subjects tested are shown below each individual, with M representing the mutant allele as shown and + representing the wild-type allele.

the WGS data at the MFSD8 locus from four cases with the homozygous variant c.1361T>C (Supplementary Table S1). The variants were found to co-segregate with the disease in all family members from whom DNA was available (Fig. 1).

**Clinical Investigations**

The clinical features of the 12 cases are summarized in the Table. Central visual loss was the primary complaint in all patients. Patients with isolated maculopathy (GC19741, LE1-LE3, CEI1, and CEI2) developed symptoms at a later age than those with generalized retinopathy (GC17967, GC14328, GC18458, GC19832, GC3716, and GC4694). Patients with a compound heterozygous genotype that included c.1006G>C, p.Glu336Gln had isolated macular disease. In contrast, cases with a homozygous c.1361T>C, p.Met454Thr genotype had more widespread retinopathy, with the majority also reporting night-blindness. No signs or symptoms of neurological disease were apparent. Fundus examination findings ranged from minimal changes (GC19741) to those typical of advanced retinitis pigmentosa (GC3716; Fig. 2A). Corresponding structural changes were present in all cases when imaged using AF and optical coherence tomography (OCT; Figs. 2B, 2C).

Patients with clinically isolated macular disease (CEI1 and GC19741) initially showed only pattern ERG abnormalities, though as the disease progressed the full field ERG could become abnormal. With the photopic single flash ERG (CEI1) showing a disproportionate loss of b- to a-wave leading to a significantly reduced b- to a-wave ratio (Fig. 3). Patients with peripheral retinal signs (GC17967 and GC14328) also had highly abnormal full-field ERGs, in keeping with a severe rod-cone dystrophy with marked macular involvement (Fig. 3). The photopic single flash ERGs (GC17967 and GC14328) again showed reduction in the b to a amplitude ratio. Although there was a lower amplitude b-wave than a-wave in the bright flash dark-adapted ERG, that is likely to be a reflection of a cone isolated retina, with all signals arising from dark-adapted cones, rather than inner retinal dysfunction with the rod system.

**CLN7/MFSD8 Localization in the Retina**

Confocal immunofluorescence microscopy in the murine retina showed that MFSD8 predominantly localized to the outer plexiform layer (OPL), a region rich in synaptic connections between photoreceptors and second order neurons—bipolar and horizontal cells (Fig. 4). Co-localization studies highlighted considerable overlap in the staining pattern for MFSD8 localization and PSD-95, but not SNAP-25, suggesting that MFSD8 is predominantly located at the photoreceptor synaptic terminals rather than bipolar or horizontal cells.

**DISCUSSION**

Mutations in MFSD8 usually cause vLINCL, a severe, early-onset neurodegeneration characterized by seizures, progressive mental and motor deterioration, myoclonus, visual failure, and ultimately premature death. Atypical presentations also exist, either commencing at a later age associated with mild disease, or a recently described, nonsyndromic form causing only macular disease. The present study describes a fourth consequence of MFSD8 dysfunction: widespread rod-cone dystrophy with severe macular involvement. Longitudinal study is needed to determine whether patients with isolated retinal disease eventually develop neurological involvement.
<table>
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<th>GC3716</th>
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<th>LE2</th>
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<td>+</td>
<td>+</td>
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<td>Choroidal atrophy, INL/ONL cysts</td>
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+ present; – absent; arMD, autosomal recessive macular dystrophy; arRCD, autosomal recessive rod-cone dystrophy; BCCA, best-corrected visual acuity; CFP, color fundus photography; D, dioptr; ELM, external limiting membrane; INL, inner nuclear layer; MSE, mean spherical equivalent; nd, not done; ONL, outer nuclear layer; POS, photoreceptor outer segments.
Figure 3. ERG and PERG of four patients (CEI1, GC19741, GC17967, and GC14328) and a normal subject. The ages of the subjects at examination are shown. The ISCEV standard ERGs, with conventional nomenclature, are shown. DA, dark-adapted; LA, light-adapted. The subsequent number indicates flash strength in cd.s.m\(^{-2}\). All are single flash responses other than the LA 50-Hz response, which uses computerized signal averaging. Blink artefact in CEI1 (at 49 and 54 years), GC17967 (at 43 years), and GC14328 has been replaced by broken lines. CEI1 and GC19741, who have a macular dystrophy phenotype, show normal full-field ERGs (for initial CEI1 and GC19741 recordings) but a subnormal PERG (when available). The later recording of CEI1 shows a normal dark-adapted response but a reduced b- to a-wave ratio in the LA 3.0 response in keeping with a progressive loss of inner retinal cone function. The initial recordings of GC17967 show marked loss of rod system function with generalized cone system dysfunction. The DA 0.01 response is undetectable, the DA 10.0 shows a markedly abnormal a-wave in keeping with photoreceptor dysfunction, LA 3.0 30 Hz and LA 3.0 are subnormal and markedly delayed, and the PERG is detectable but subnormal. These findings indicate a rod-cone dystrophy with relatively mild macular involvement. Later recordings show an undetectable PERG in keeping with worsening macular function, and all detectable ERGs show profound deterioration. GC14328 and the later recordings of GC17967 show a lower b- than a-wave in the DA 10.0 response suggesting a dark-adapted cone origin with all rod function having been lost. The photopic ERGs are subnormal and delayed, but over time there is the development of a broadened a-wave in the LA 3.0 response with marked reduction in the b to a ratio in keeping with additional inner retinal cone system dysfunction. The recordings from a normal control subject are included for comparison.
though it is reassuring that the three oldest patients reported herein developed no neurological abnormalities despite being in their seventh or eighth decade of life.

ERG localizes the consequences of MFSD8 dysfunction more precisely. The scotopic bright flash ERG is a mixed rod-cone response, dominated by the rod-system. Most of the negative going a-wave reflects photoreceptor hyperpolarization, with the later positive going b-wave generated in the bipolar cells. The photopic single flash ERG a-wave is generated by cone photoreceptors and Off-(hyperpolarizing) bipolar cells, with the positive b-wave being an effectively synchronized response from On- and Off-bipolar cells. Those patients homozygous for the p.Met454Thr allele demonstrated a reduction in the photopic b to a ratio, a feature not typically associated with primary photoreceptor degeneration. Although a reduced b- to a-wave ratio can occur in the bright flash dark-adapted ERG in cone isolated retinas due to vitamin A deficiency, or RDH5 mutation, when rod loss is so severe that all remaining signals arise in dark-adapted cones, the photopic ERGs do not exhibit such a phenomenon. The reduced photopic b to a ratio in the present series thus indicates dysfunction after phototransduction beyond the photoreceptor outer segment. This contention is further supported in a patient carrying the p.Glu356Gln variant allele, who has isolated macular disease but developed a reduced photopic ERG b- to a-wave ratio over a 6-year period, indicating progressive “inner retinal” cone dysfunction. Immunofluorescence of the murine retinal sections demonstrated that the strongest MFSD8 signal arises in the OPL, in the photoreceptor synaptic terminals, in keeping with the electrophysiological data.

Thus, two independent lines of evidence implicate the OPL in MFSD8-associated retinal disease. The localization of MFSD8 to the photoreceptor presynaptic terminals, together with the presence of a post-phototransduction ERG abnormality in patients with MFSD8 mutations, strongly suggests a synaptic origin for the defect. Synaptic alterations have previously been suggested as initiating events causing NCL in the CLN5-knockout sheep and in a mouse model of lysosomal disease due to a defect in cathepsin D function (CLN10).

Overall, the data suggest that macular photoreceptors appear to be most sensitive to mutations in MFSD8. Extramacular photoreceptors are the next most vulnerable, with cortical neurons being the most resistant of the affected cell types. There are anatomical and physiological differences between photoreceptor and conventional synapses that could account for this differential vulnerability. Photoreceptor terminals release neurotransmitter continuously, with light turning off vesicle release, whereas cortical neurons are usually switched off and are only triggered by action potentials. To permit these unique properties of photoreceptors, the terminals contain many more synaptic vesicles of which ~85% are freely mobile, actively participating in glutamate release, compared to ~20% in conventional synapses.

Furthermore, photoreceptor terminals possess a synaptic ribbon for vesicle docking, necessary for maintaining the higher rates of neurotransmitter release over a sustained period of time. It is also relevant that peripheral cones contain twice as many ribbons as central cones, suggesting that this, or a similar synaptic modification, may underlie the different photoreceptor sensitivities.

To date, MFSD8 has been shown to be a lysosomal membrane protein with an MFS transporter domain that is characteristic of proteins that transport small solutes through chemiosmotic ion gradients. The substrates for MFSD8 are impaired autophagy, and cell death, as well as accompanying inflammation that occurs both in the brains of MFSD8-deficient patients and in the MFSD8-knockout dog and mouse models.

All previously published MFSD8 mutation combinations associated with disease are listed at http://www.ucl.ac.uk/ncl/
Many of the missense alleles cause isolated macular dystrophy when paired either with a null variant on the other allele or with a missense variant (p.Arg465Gln) as shown here. In direct contrast, the p.Met454Thr variant, when paired with a null allele, causes vLINCL rather than isolated retinal disease. The other allele of interest, p.Met454Thr, may be considered a "moderate" allele since homozygous variants cause a more severe rod-cone dystrophy but not vLINCL. The severe neurological phenotype may only occur when the p.Met454Thr variant is paired with a more severe allele. In addition, an atypical patient with late-onset vLINCL was homozygous for the p.Ala157Pro missense variant, suggesting that allele may be hypomorphic, accounting for the less severe phenotype. All other reported cases with homozygous missense alleles cause vLINCL. A study that overexpressed two vLINCL-causing missense mutations, p.Thr294Iys and p.Pro412Leu, found enhanced proteolytic cleavage of mutant MFSD8 by cysteine proteases, inactivating the protein. It was suggested that some of the missense alleles may represent functional null mutations. Based on the pathogenicity prediction scores of the missense mutations (Supplementary Table S2) and the topology prediction diagram that maps the mutations on the protein sequence (Fig. 5) there appears to be no obvious correlation between the mutations and whether the patient develops vLINCL or nonsyndromic eye disease. The missense changes, p.Glu336Gln and p.Met454Thr, that are associated with nonsyndromic eye disease may disrupt normal protein function either by reducing the transporter function of MFSD8 or by abrogating a yet to be defined specific function that is unique to photoreceptor synapses, but absent from conventional synapses.

To conclude, this study describes patients with isolated retinal disease due to biallelic mutations in MFSD8, a gene more usually associated with vLINCL. The data suggest a genotype/phenotype relationship such that a mild reduction in function results in an isolated later-onset maculopathy; moderate reduction results in an earlier-onset generalized rod-cone dystrophy; and much wider central nervous system pathology if both alleles are functionally null. ERG demonstrates additional dysfunction occurring after phototransduction in the cone system, and immunofluorescence localizes the putative lysosomal transporter MFSD8 to the photoreceptor presynaptic terminals in the retinal OPL. Together these observations support a synaptic defect as the primary basis of pathology for this type of vLINCL and related diseases.

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References

MFSD8 Mutations Cause Synaptic Disease


APPENDIX

The NIHR BioResource-Rare Diseases Consortium includes Timothy Aitman, Hana Alachiak, Sonia Ali, Louise Allen, David Allsup, Gautum Ambegaonkar, Julie Anderson, Richard Antrobus, Ruth Armstrong, Gavin Arno, Gururaj Arumugakani, Sophie Ashford, William Astle, Antony Attwood, Steve Austin, Chiara Bacchelli, Tamam Bakchoul, Tadbir K. Bariana, Helen Baxendale, David Bennett, Claire Bethune, Shahnaz Bibi, Maria Bittner-Glindzik, Marta Bleda, Harm Boggard, Paula Bolton-Maggs, Claire Booth, John R. Bradley, Angie Brady, Michael Brown, Andrew DaCosta, Louise Daughery, Sophie Davies, John Davis, Minka De Vries, Patrick...
MFSD8 Mutations Cause Synaptic Disease


The UK Inherited Retinal Disease Consortium includes Graeme Black, Georgina Hall, Stuart Ingram, Rachel Taylor, Simon Ramsden, Forbes Manson, Panagiotis Sergouniotis, Andrew Webster, Alison Hardcastle, Michel Michaelides, Vincent Plagnol, Michael Cheetham, Gavin Arno, Alessia Fiorentino, Nikolas Pontikos, Shomi Bhattacharya, Anthony Moore, Kamron Khan, Chris Inglehearn, Barn Whaleer, Manir Ali, Martin McKibbin, James Poulter, Emma Lord, Andrea Nemeth, Susan Downes, Stephanie Halford, Jing Yu, Stefano Lise, and Veronica van Heyningen.