A Novel Mutation in the *ELOVL4* Gene Causes Autosomal Dominant Stargardt-like Macular Dystrophy

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PURPOSE. To conduct clinical and genetic studies in a European family with autosomal dominant Stargardt-like macular dystrophy (adSTGD-like MD) and to investigate the functional consequences of a novel *ELOVL4* mutation.

METHODS. Ophthalmic examination and mutation screening by direct sequencing of the *ELOVL4* gene was performed in two affected individuals. Wild-type and mutant *ELOVL4* genes were expressed as enhanced green fluorescent protein (EGFP) fusion proteins in transient transfection in NIH-3T3 and HEK293 cells. To determine the subcellular localization of ELOVL4, an endoplasmic-reticulum (ER)–specific marker for pDsRed2-ER was cotransfected with ELOVL4 constructs. Transfected cells were viewed by confocal microscopy. Western blot analysis was performed to assess protein expression using an anti-GFP antibody.

RESULTS. Affected patients exhibited macular atrophy with surrounding flecks characteristic of adSTGD-like MD. A novel *ELOVL4* p.Tyr270X mutation was detected in affected individuals. In cell-transfection studies, wild-type ELOVL4 localized preferentially to the ER. In contrast, the mutant protein appeared to be mislocalized within transfected cells.

CONCLUSIONS. In a European family with adSTGD-like MD, a novel *ELOVL4* mutation was found to underlie the disorder. Transfection studies indicated that, unlike wild-type ELOVL4, the mutant protein does not localize to the ER but rather appears to be sequestered elsewhere in an aggregated pattern in the cytoplasm. Further analysis of the function of normal and mutant ELOVL4 will provide insight into the mechanism of macular degeneration. (*Invest Ophthalmol Vis Sci.* 2004;45: 4263–4267) DOI:10.1167/iovs.04-0078

Stargardt disease (STGD) is an early-onset hereditary macular dystrophy, characterized by decreased central vision, atrophy of the macula, and frequent appearance of orange-yellow flecks in the posterior pole of the retina.[1] STGD is most commonly inherited as an autosomal recessive trait, but numerous affected families have been described in which features of the disease showed autosomal dominant (ad) inheritance.[2–8] Mutations in the photoreceptor-specific *ABCA4* gene seem to account for all recessive forms of STGD (STGD1; MIM248200). Conversely, adSTGD is a genetically heterogeneous disorder, as two loci already have been identified. One locus for adSTGD was mapped to 4p (STGD4; MIM603786).[4] Another locus (STGD3, MIM600110) was localized to 6q14 in a large North American family.[2] Subsequently, several additional adSTGD-like families and ad macular dystrophy (adMD) families were mapped to the STGD3 locus.[5,6,9,10] Genealogy and haplotype analyses indicated that they were all linked through an ancestral founder.[5,7,10] Positional cloning revealed that a photoreceptor-specific gene, *ELOVL4* (elongation of very long chain fatty acids-like 4) is responsible for STGD3.[7] The ELOVL4 protein was shown to be homologous to a group of yeast proteins involved in the biosynthesis of very-long-chain fatty acids and is likely to play a central role in the biosynthesis of lipid components of the photoreceptor outer segment membrane. A 5-bp deletion in the *ELOVL4* gene was found to segregate with the disease in all five families.[7] Subsequently, the identification of a second mutation in the *ELOVL4* gene in a large unrelated pedigree confirmed *ELOVL4* as a disease-causing gene in adMD and adSTGD.[8] The second mutation consisted of two 1-bp deletions separated by four nucleotides, which occurred at the same location as the previously described 5-bp deletion and had an almost identical predicted truncating effect on the ELOVL4 protein.

Herein, we describe the identification of a third *ELOVL4* mutation in a European family with adSTGD-like macular dystrophy. To assess the functional consequence of this *ELOVL4* mutation, we investigated the subcellular location of normal and mutant ELOVL4. We demonstrated that the wild-type enhanced green fluorescent protein (EGFP)-ELOVL4 fusion protein localizes to the ER compartment in transfected cells. In contrast, the mutant EGFP-ELOVL4 fusion protein does not localize to the ER but rather appears to be sequestered elsewhere in an aggregate pattern in the cytoplasm.

METHODS

Patients

Two individuals with adSTGD-like MD, a woman in a Belgian family and her affected daughter, were available for clinical and molecular investigation (patients III:2 and IV:1, Fig. 1). Both patients gave their
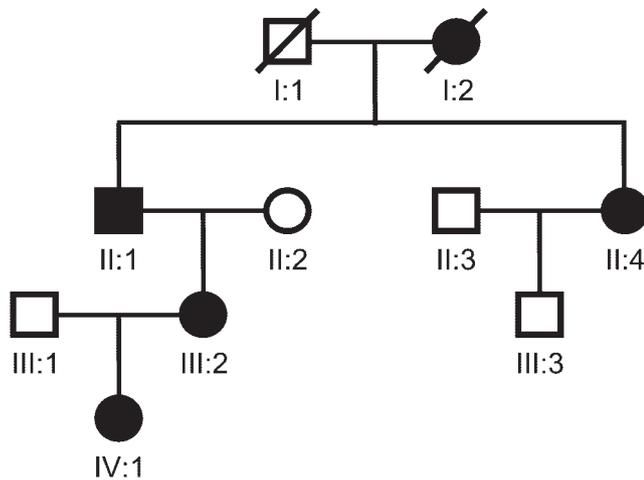
FIGURE 1. Pedigree of the autosomal dominant STGD family described in this study.

informed consent before inclusion in the study as part of a protocol that adhered to the tenets of the Declaration of Helsinki. Clinical investigation included visual acuity measurement, dilated fundus examinations, and fluorescein angiography.

Mutation Analysis

DNA of both patients was screened by direct sequence analysis of all six exons and flanking splice sites of the *ELOVL4* gene using primers and conditions as reported in Table 1. The identified novel *ELOVL4* mutation was tested on a panel of 96 ethnically matched control individuals, using an amplification-refractory mutation specific (ARMS) technique.[11] Wild-type-allele–specific and mutant-allele–specific forward primers used for ARMS were, respectively, 5′-AACTTCTACAT-TCGGACATAC-3′ and 5′-AACTTCTACATTCGGACATAG-3′. The reverse primer was 5′-TCAACAACAGTTAAGGCCCA-3′.

Transfection Studies

Media and reagents for cell culture and transfection were purchased from Invitrogen-Gibco (Grand Island, NY). Anti-EGFP monoclonal antibody and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis,

MO). Chamber slides were purchased from Nalge Nunc (Rochester, NY).

Generation of Expression Constructs. Wild-type and mutant *ELOVL4* cDNAs were cloned separately into a pEGFPC1 vector (BD-Clontech, Palo Alto, CA). This vector utilizes a cytomegalovirus (CMV) promoter and expresses EGFP after transfection into mammalian cells. PCR was performed with one forward primer containing a *Kpn*I site (5′-CGGGGTACCGCGATGGGGCTCCTGGACTC-3′), and two reverse primers containing a *Bam*H1 site (5′-CGGGATCCCGTTAATC-TCCTTTTGCTTTTC-3′ for the wild-type *ELOVL4* and 5′-CGGGATC-CCGCTATGTCCGAATGTAGAAG-3′ for the mutant 270X *ELOVL4*), with wild-type cDNAs used as a template. The resultant PCR products were digested with *Kpn*I and *Bam*HI, and cloned into the *Kpn*I and *Bam*HI sites of a pEGFPC1 vector in frame at the C-terminal end of EGFP. The recombinant plasmids containing EGFP-ELOVL4 fusion constructs were verified by direct DNA sequencing, amplified and purified with a plasmid isolation kit (Qiagen, Inc., Valencia, CA).

Cell Transfection and Imaging. NIH3T3 and HEK293 were used for all transfection studies. The recombinant plasmids were transfected into the cell lines using Lipofectamine Reagent 2000 (Invitrogen-Gibco) according to the manufacturer's protocol. Cells were monitored for fluorescence between 7 and 36 hours after transfection, by epifluorescence microscopy.

NIH3T3 cells were seeded onto poly-L-lysine–coated, four-well chamber glass slides at a confluence of 20% to 30% and transfected with recombinant plasmids containing wild-type or mutant *ELOVL4*. Cotransfection with an ER-organelle–specific marker pDsRed2ER was performed with wild-type and mutant ELOVL4 to determine the subcellular localization of ELOVL4. Transfected cells were incubated at 37°C for 24 hours, washed with PBS, fixed in methanol-acetone (50:50, vol/vol), and mounted on glass slides with antifade medium (Vectashield; Vector Laboratories, Inc., Burlingame, CA) for microscopy. To assess subcellular localization of wild-type and mutant ELOVL4 protein, transfected cells were observed at different time intervals. However, all data were collected at approximately the same time point after transfection.

Images of green fluorescence were collected with a confocal laser scanning microscope (IX70; Olympus, Tokyo, Japan) using a 488-nm excitation source and a 505- to 550-nm band-pass barrier filter. A red fluorescence (DsRed2) marker for ER was examined using 568-nm excitation light from the Kn laser, a 575-nm dichromic mirror, and a 580- to 625-nm filter. The cells were illuminated sequentially to avoid

TABLE 1. Oligonucleotide Primer Sequences and Conditions Used for *ELOVL4* Mutation Analysis

Exon	Forward Primer	Forward Primer Sequence (5′ → 3′)	Reverse Primer	Reverse Primer Sequence (5′ → 3′)	T (°C)	Mg (mM)
1	8310	ttgaggagcaggagaagacg	8311	tgatccgcagcatccgaaag	66	1.5
2	8312	ttgggactcaaaggacagtg	8313	atgccagaacagctaataagg	56	1.5
3	8314	cacagtaacttctagcaatcg	8315	cataccactgcacttcagtc	56	1.5
4	8316	ccatgccttgtacattttgtg	8317	tgacagagcgagactccatc	58	2.0
5	8318	acaactgtgaaagtcctttgc	8319	ataactgcgatatagctggag	56	1.5
6	8320	ttgagttgtgaatgaggagc	8321	acactttactcagtctaagag	56	1.5

TABLE 2. Clinical Features of the Patients with Autosomal Dominant Stargardt Disease

| No.	Age at Last Examination (y)	Age at Onset (y)	Visual Acuity OD	Visual Acuity OS	Fundus Characteristics	Fluorescein Angiogram	ERG Scotopic	ERG Photopic	ERG 30 Hz
III:2	39	11	20/400	20/400	Macular pigmentary changes; perimacular yellow flecks	Macular RPE window defects	Normal	Subnormal (71%)	Reduced (50%)
IV:1	16	9	20/400	20/400	Macular pigmentary changes; progressive increase of yellow flecks in the posterior pole	Macular RPE window defects	Normal	Subnormal	Normal
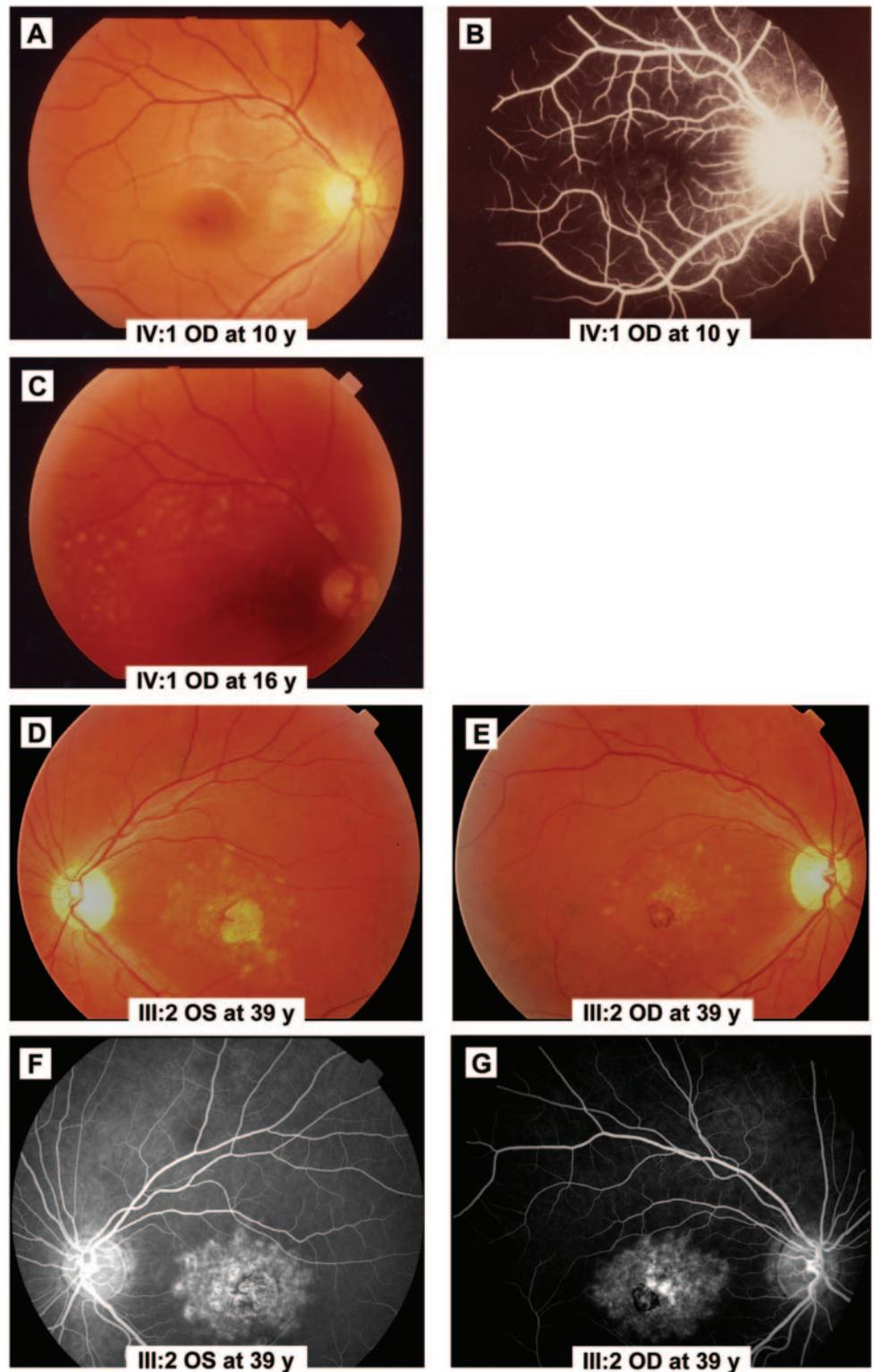
FIGURE 2. Fundus photographs and fluorescein angiograms of patients IV:1 (**A–C**) and III:2 (**D–G**), carrying the p.Tyr270X ELOVL4 mutation. (**A, B**) Patient IV:1 at 10 years of age. (**A**) Fundus photograph showing discrete macular alterations but no flecks. (**B**) Fluorescein angiography demonstrating window defects in the macula. (**C**) Fundus photograph of patient IV:1 at age 16. Yellow flecks extend beyond the arcade vessels. (**D–G**) Patient III:2 at the age 39. (**D, E**) Characteristic yellow flecks in the perimacular area, RPE atrophy, and pigmentary changes are shown. (**F, G**) Fluorescein angiography photographs of the left eye (**F**) in a middle phase and of the right eye (**G**) in the late venous phase, showing early fluorescence in the macular area due to RPE atrophy.

bleed through (3.7 seconds per frame for EGFP and DsRed2), and images were collected in a single optical section of 0.35 μm, where they were compared for colocalization analyses.

Electrophoresis and Immunoblot Analysis. To analyze the expression of wild-type and mutant ELOVL4, we grew transfected cells for 24 hours, harvested them from the plates, and briefly washed them with PBS. Cells were lysed on ice for 20 minutes with a buffer containing 1% Triton X-100, 0.01% SDS, 0.05 M Tris-HCl, and 1 mM

EDTA (pH 7.5). The cell lysates were centrifuged at 4000 rpm for 5 minutes and the supernatants used for electrophoresis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli.[12] Ten microliters of sample ($\sim$7 μg) was loaded onto a 9% polyacrylamide gel and electrophoresed at 110 V for 1 hour. The resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) and blocked for 2 hours at room temperature with 5% skim milk in Tris-
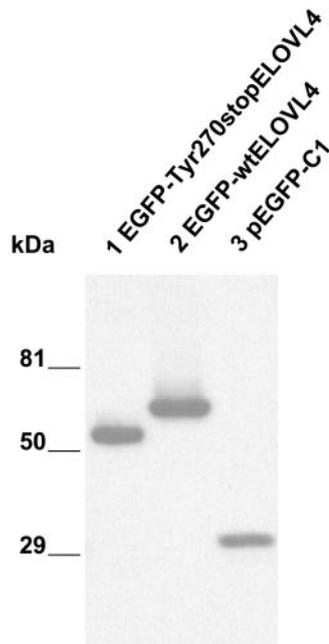
FIGURE 3. Western blot analysis of ELOVL4 expression in NIH3T3 cells. Transfected cells were allowed to express EGFP-ELOVL4 fusion proteins (*lane 1*: Tyr270stopELOVL4; *lane 2*: wtELOVL4) and EGFP alone (*lane 3*) for 24 hours, followed by immunoblot analysis with anti-GFP monoclonal antibody.

buffered saline containing 0.05% Tween 20 (TTBS). The membrane was incubated for 2 hours with monoclonal anti-GFP antibody diluted 1:2000 in 5% milk containing TTBS, and then probed with peroxidase-conjugated anti-mouse antibody (1:4000 dilution in TTBS; Amersham Biosciences, Piscataway, NJ) for 1 hour and developed with a chemiluminescence detection kit according to the manufacturers' protocol (ECL; Amersham Bioscience).

RESULTS

Clinical Investigation

Clinical features of both patients included in the study (patients III:2 and IV:1) are summarized in Table 2.

Patients experienced vision loss at young age (11 and 9 years, respectively). At 26 years of age, the mother (III:2) had a visual acuity of 20/400 in both eyes. At 16 years of age, the visual acuity of her daughter (IV:1) also had decreased to 20/400 in both eyes. In both patients, the fundus examinations showed macular pigmentary changes and the appearance of

yellow flecks characteristic of adSTGD-like MD (Figs. 2C–E). Fluorescein angiography revealed macular RPE defects in both individuals (Figs. 2B, 2F, 2G).

ELOVL4 Mutation Analysis

Because mutations in the *ELOVL4* gene had been associated with adSTGD, we screened both patients for mutations in *ELOVL4*. In both patients, we identified a C-to-G change at nucleotide c.810 in exon 6 of the *ELOVL4* gene. This change resulted in a novel substitution of a stop codon for tyrosine 270, leading to a truncated protein lacking the last 45 amino acids. The mutation was not found in 96 healthy control individuals.

Characterization of EGFP-ELOVL4 Fusion Proteins

We expressed wild-type and mutant ELOVL4 as EGFP fusion proteins to facilitate direct visualization of subcellular localization. Western blot analysis confirmed the synthesis of EGFP-ELOVL4 fusion proteins, and single bands were visualized for each construct at ~61 kDa for wild-type ELOVL4 and ~56 kDa for the EGFP-ELOVL4 truncated mutant (Fig. 3).

Subcellular Localization of ELOVL4

Confocal microscopy was used to determine the subcellular localization of wild-type and mutant EGFP-ELOVL4 in transfected NIH3T3 cells. Wild-type EGFP-ELOVL4 localized preferentially to the endoplasmic reticulum (ER) compartment (Fig. 4). In contrast, mutant EGFP-ELOVL4 showed a markedly different subcellular localization pattern in the transfected cells. It did not localize to the ER. Instead, the pattern of its subcellular distribution is consistent with an aggregated form in the cytoplasm (Fig. 4). As a control, EGFP when expressed alone, localized to the cytoplasm (data not shown).[13]

DISCUSSION

Autosomal dominant STGD-like MD is a juvenile macular dystrophy. Most affected families in North America have been linked to the STGD3 locus on 6q14.[2,5,6,9,10] Subsequently, haplotype and *ELOVL4* mutation analyses have shown that all affected individuals share the same disease haplotype[5,7,10] and harbor a 5-bp deletion in the *ELOVL4* gene.[7] The only unrelated family described up to now was also of North American origin and carried a 2-bp deletion mutation that, oddly enough, occurred at the same location as the previously described 5-bp deletion.[8]

Herein, we describe the identification of a novel *ELOVL4* mutation in a family of European origin with adSTGD-like MD. This c.810C>G mutation predicts a p.Tyr270X change.
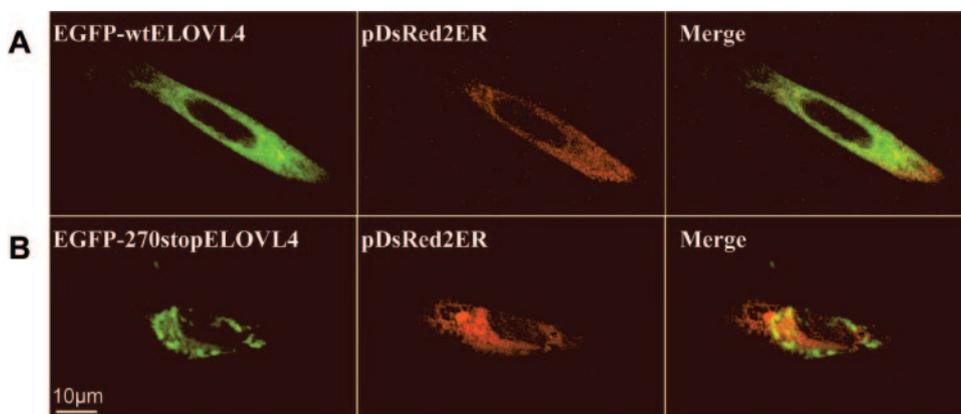

FIGURE 4. Localization of ELOVL4 with ER-organelle–specific markers in NIH3T3 cells. Twenty-four hours after cotransfection, the cells were imaged by confocal microscopy. (**A**) Cotransfected cell with EGFP-wtELOVL4 and pDsRed2-ER, specific for endoplasmic reticulum. (**B**) Cotransfected cell with EGFP-mtELOVL4 and pDsRed2-ER. *Green*: expression of ELOVL4; *red*: ER organelle. Merged image: superimposed image of expression of ELOVL4 and the ER organelle.

Both previously identified *ELOVL4* mutations were small deletions that generate a frameshift leading to a truncated protein lacking the last 51 amino acids.[7,8] The p.Tyr270X mutation results in the absence of the last 45 amino acids. In all three cases, a KXKXX dilysine-targeting signal at the carboxyl terminus of the ELOVL4 protein is deleted. This signal is known to be responsible for the retention of transmembrane proteins in the ER,[14] the site of very-long-chain fatty acid biosynthesis.[15] As expected, subcellular localization studies showed that, unlike wild-type ELOVL4, the mutant does not localize to the ER. The mislocalized protein seems to be sequestered in another subcellular compartment exhibiting dense fluorescence-positive aggregates. Similar results were also observed with 5-bp ELOVL4 mutants.[16,17] Future studies with additional cell markers and/or electron microscopy will elucidate the subcompartmental localization and underlying mechanism of the ELOVL4 mutant proteins.

It is interesting that all three *ELOVL4* mutations were clustered in the C terminus; so far, no mutations in the N terminus have been reported. This may suggest a dominant negative nature of the mutant ELOVL4 protein, rather than a mechanism of haploinsufficiency. Consistent with this notion, the 5-bp deletion mutant causes cell death when transfected into cultured cells.[17]

The identification of the third mutation in the *ELOVL4* gene further supports its important role in macular degeneration, and provides another entry point from which ELOVL4 function in retinal physiology and disease can be investigated.

Acknowledgments

References

1. Stargardt K. Uber familiäre, progressive degeneration in der Maculagegend des Auges. *Graefes Arch Ophthalmol.* 1909;71:534–550.

2. Stone EM, Nichols BE, Kimura AE, Weingeist TA, Drack A, Sheffield VC. Clinical features of a Stargardt-like dominant progressive macular dystrophy with genetic linkage to chromosome 6q. *Arch Ophthalmol.* 1994;112:765–772.

3. Zhang K, Bither PP, Park R, Donoso LA, Seidman JG, Seidman CE. A dominant Stargardt's macular dystrophy locus maps to chromosome 13q34. *Arch Ophthalmol.* 1994;112:759–764.

4. Kniazeva M, Chiang MF, Morgan B et al. A new locus for autosomal dominant Stargardt-like disease maps to chromosome 4. *Am J Hum Genet.* 1999;64:1394–1399.

5. Edwards AO, Miedziak A, Vrabec T et al. Autosomal dominant Stargardt-like macular dystrophy: I. clinical characterization, longitudinal follow-up, and evidence for a common ancestry in families linked to chromosome 6q14. *Am J Ophthalmol.* 1999;127:426–435.

6. Lagali PS, MacDonald IM, Griesinger IB, Chambers ML, Ayyagari R, Wong PW. Autosomal dominant Stargardt-like macular dystrophy segregating in a large Canadian family. *Can J Ophthalmol.* 2000;35:315–324.

7. Zhang K, Kniazeva M, Han M, et al. A 5-bp deletion in *ELOVL4* is associated with two related forms of autosomal dominant macular dystrophy. *Nat Genet.* 2001;27:89–93.

8. Bernstein PS, Tammur J, Singh N, et al. Diverse macular dystrophy phenotype caused by a novel complex mutation in the ELOVL4 gene. *Invest Ophthalmol Vis Sci.* 2001;42:3331–3336.

9. Griesinger IB, Sieving PA, Ayyagari R. Autosomal dominant macular at 6q14 excludes CORD7 and MCDR1/PBCRA loci. *Invest Ophthalmol Vis Sci.* 2000;41:248–255.

10. Donoso LA, Frost AT, Stone EM, et al. Autosomal dominant Stargardt-like macular dystrophy: founder effect and reassessment of genetic heterogeneity. *Arch Ophthalmol.* 2001;119:564–570.

11. Newton CR, Graham A, Heptinstall LE, et al. Analysis of any point mutation in DNA: the amplification refractory mutation system (ARMS). *Nucleic Acids Res.* 1989;17:2503–2516.

12. Laemmli UK. Cleavage of structural proteins during assembly of head of bacteriophage-T4. *Nature.* 1970;227:680–685.

13. Niswender KD, Blackman SM, Rohde L, Magnuson MA, Piston DW. Quantitative imaging of green fluorescent protein in cultured-cells: comparison of microscopic techniques, use in fusion proteins and detection limits. *J Microsc.* 1995;180:109–116.

14. Jackson MR, Nilsson T, Peterson PA. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic-reticulum. *EMBO J.* 1990;9:3153–3162.

15. Cinti DL, Cook L, Nagi MN, Suneja SK. The fatty-acid chain elongation system of mammalian endoplasmic-reticulum. *Prog Lipid Res.* 1992;31:1–51.

16. Ambasudhan R, Wang XF, Jablonski MM, et al. Atrophic macular degeneration mutations in ELOVL4 result in the intracellular misrouting of the protein. *Genomics.* 2004;83:615–625.

17. Karan G, Yang ZL, Zhang K. Expression of wild type and mutant ELOVL4 in cell culture: subcellular localization and cell viability. *Mol Vis.* 2004;10:248–253.