

Retinopathy in Danon Disease

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Objective: To evaluate visual function in 2 boys and their maternal aunt affected with Danon disease due to a mutation in the X-linked lysosome-associated membrane protein-2 (*LAMP2*) gene.

Methods: Linkage analysis using microsatellite markers from the X chromosome was done in family members from the paternal side. Visual acuity testing, fundus analysis, fluorescence angiography, and full-field electroretinography were performed in all 3 patients.

Results: Eye examinations confirmed the presence of retinopathy in the 2 boys and their maternal aunt, obligate carrier for the S157X mutation in *LAMP2*. The expression of the disease was milder in the female car-

rier than in the hemizygous boys, possibly due to lyonization.

Conclusions: Our report further expands the phenotype of Danon disease by describing retinopathy in 3 cases. A thorough clinical examination, including ophthalmic investigation, is needed in all cases of Danon disease.

Clinical Relevance: *LAMP2* belongs to a growing number of retinopathy genes. Genes involved in systemic diseases associated with poor survival may see their effect in other organs, not only in the eyes, becoming a major source of concern once a good and reliable therapy is available. This also represents a major issue for genetic counseling for patients undergoing gene therapy in the future.

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DANON DISEASE (DD) (Online Mendelian Inheritance in Man [OMIM] 300257), a lysosomal glycogen storage disease without acid maltase deficiency, is a rare X-linked dominant disease characterized by cardiomyopathy, myopathy, and various degrees of mental retardation. Danon disease results from mutations in the lysosome-associated membrane protein-2 (*LAMP2*) gene.¹ The first report of its occurrence seems to be from Danon et al² in 1981, although Antopol et al³ described in 1940 two brothers who died from heart failure due to a glycogen storage disease restricted to the heart and skeletal muscles. Byrne et al⁴ reported the involvement of 3 affected females and confirmed X-linked dominant inheritance of this disease. In the absence of treatment, patients usually experience various episodes of supraventricular arrhythmias and often die of sudden death. In a study of 20 male patients with proven DD, all patients but 1 died before the age of 30 years.⁵ The availability of implanted defibrillators and heart transplantation has dramatically changed life expectancy.

At the molecular level, nonsense and splice junction mutations leading to trun-

cated *LAMP2* proteins represent the main mutations observed and immunohistochemistry studies usually confirm complete absence of the protein in the affected tissues.¹ Autophagic vacuoles, a central aspect of DD, are primarily seen in the heart and muscles but can also be observed in the liver and spleen. *LAMP2* knockout mice also exhibit autophagic vacuoles in kidney, pancreas, small intestine, thymus, and spleen in addition to the classically affected organs.⁶

Recently, *LAMP2* mutations were observed in patients with unexplained left ventricular hypertrophy as the only clinical finding, thus concentrating the picture on cardiac involvement.⁷ We report on 3 patients with DD who had diffuse retinal dysfunction. Our report may serve as an example of evolving phenotype and stresses the importance of identifying all aspects of a disease, especially in the light of new and improved treatments, ie, gene therapy, that will prolong the life of patients who might otherwise die at an early age.

METHODS

FAMILY AND LINKAGE ANALYSIS

Two brothers and their maternal aunt originating from Switzerland were diagnosed with DD and referred to our ophthalmic clinics for

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Table. Linkage Analysis

Markers	Position on Chromosome	Recombination Fraction				
		0	0.1	0.2	0.3	0.4
DXS1211	138'030'857	$-\infty$	1.20	1.024	0.71	0.28
DXS8043	143'734'068	2.61	2.19	1.71	1.14	0.51
DXS1073	153'392'612	2.01	1.65	1.25	0.81	0.32

investigation of decreased visual performance. Furthermore, a familial history of retinitis pigmentosa was present in the father and several male individuals on his side of the family. Complete eye examinations were performed, including computerized static perimetry and fluorescein and indocyanine green angiography. Full-field electroretinography (ERG) (Utas 2000; LKC Technologies Inc, Gaithersburg, Md) was done under scotopic and photopic conditions according to standards from the International Society for Clinical Electrophysiology of Vision.⁸ Normal range consisted of mean values and 2 SD adjusted for age. Multifocal ERG (Veris; Electro-Diagnostic Imaging, Inc, Redwood City, Calif) was obtained in the female carrier. Previous analysis in the 3 patients identified an S157X mutation in the *LAMP2* gene.⁹ To confirm X-linked transmission of the paternal retinitis pigmentosa, linkage analysis was performed with markers from the X chromosome of the ABI Prism Linkage Mapping sets, version 2.5 (PE Biosystems, Foster City, Calif) as described by the manufacturer.

LAMP2 EXPRESSION

The human *LAMP2a* and *LAMP2b* variants were amplified by polymerase chain reaction after reverse transcription from messenger RNA extracted from ARPE-19, a human retinal pigment epithelial (RPE) cell line.

ARPE-19 cells were obtained from the American Type Culture Collection (ATCC; LGC Promochem, Teddington, England) and maintained in 1:1 (volume-to-volume ratio) mixture of Dulbecco modified Eagle and Ham F12 medium (No. 31330 DMEM/F12; Invitrogen, Carlsbad, Calif) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and grown with 1% penicillin/streptomycin (Invitrogen) in a humidified incubator at 37°C in 5% carbon dioxide.

Expression of *LAMP2a* and *LAMP2b* was examined using reverse-transcriptase polymerase chain reaction. Total RNA from ARPE-19 cells was extracted using TRIZOL reagent (Invitrogen) according to the supplier's procedure and further purified and treated with DNase-I on column using RNeasy Mini kit (Qiagen, Valencia, Calif). Five micrograms of total RNA in a 50- μ L reaction was used for complementary DNA synthesis using oligo (dT)18 according to the manufacturer's procedure (StrataScript Reverse Transcriptase; Stratagene, La Jolla, Calif). The equivalent of 50 ng of original total RNA was used for polymerase chain reaction amplification using Taq DNA polymerase (Taq PCR Mastermix kit; Qiagen) and 10 pmol of sense and antisense primers. The following primers were used for amplifications: hLAMP2-F1 (5'-CAA TGA TAC TTG TCT GCT GG-3'), hLAMP2a-R1 (5'-GCT CAT ATC CAG CAT GAT GG-3'), and hLAMP2b-R1 (5'-TCA TCC AGC GAA CAC TCT TGG-3'). Primer pairs hLAMP-F1/hLAMP2a-R1 and hLAMP2-F1/hLAMP2b-R1 generated products of 541 and 431 base pairs, respectively. Polymerase chain reaction cycle parameters were conducted with a pre-amplification denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. Samples of the amplification reactions were resolved on 1% Tris-borate-ethylenediaminetetraacetic acid agarose gel.

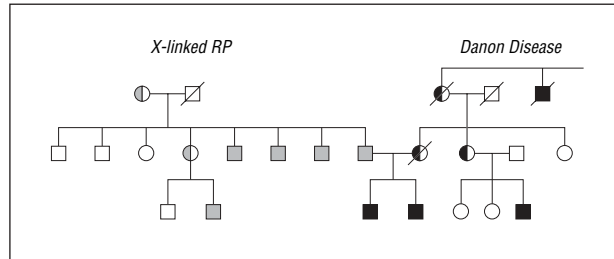


Figure 1. Pedigree of the 2 branches of the family showing 2 X-linked transmissions: on the paternal side, a retinitis pigmentosa (RP) disease (shaded symbols) and on the maternal side, Danon disease (black symbols). Circles denote female; squares, male; half-filled symbols, heterozygous carriers; filled symbols, affected members; symbols with slashes, deceased individuals.

IMMUNOHISTOCHEMISTRY

Six-millimeter-thick retina sections were obtained from an enucleated eye. Sections were heated at 60°C before being processed. For immunohistochemistry, the H4B4 antiserum was used after acetone fixation at a dilution of 1:50 together with a peroxidase second antibody.

RESULTS

MAPPING OF PATERNAL RETINITIS PIGMENTOSA

Maximum logarithm of odds scores of 2.61 and 2.01 at $\theta=0$ were obtained for markers DXS8043 and DXS1073, respectively (**Table**), thus confirming linkage to the X chromosome of the retinitis pigmentosa (RP) on the paternal side (**Figure 1**) and therefore eliminating any contribution of this disease to the ophthalmic status of the 2 children.

CLINICAL CHARACTERISTICS OF FAMILY MEMBERS WITH *LAMP2* MUTATIONS

The family history on the mother's side revealed that many members were affected with DD, another X-linked disease, and a hemizygous S157X mutation was previously identified in the 2 boys (**Figure 1**).⁹ The aunt was heterozygous for the same mutation. The mother of the 2 affected brothers as well as a son of the carrier aunt died suddenly from a myocardial infarction many years ago and no material was available for molecular analysis. As expected, the father did not carry the S157X mutation.⁹

Patient 1 was 14 years old at the time of examination and was complaining of poor vision. Best-corrected visual acuity was 20/30 in both eyes, and a slight but diffuse loss of sensitivity was present in both eyes on computerized visual field examination (**Figure 2**). Fundus examination was remarkable for small bilateral macular RPE hypopigmentation and a peripheral salt-and-pepper retinopathy (**Figure 3**). On fluorescein angiography, the macular changes appeared as window defects, and a peripheral slightly mottled appearance of the RPE (**Figure 3**) was present in both eyes. Indocyanine green angiography was unrevealing. Scotopic full-field ERG was slightly abnormal in both eyes. The ampli-

tudes of the a-wave and b-wave were within the normal range for both scotopic stimuli (-24 dB and 0 dB), but the culmination time of the b-wave after stimulation with the weakest flash (0 dB) was delayed in both eyes (111.0 milliseconds OD and 102.5 milliseconds OS; normal upper limit, 99.7 milliseconds). Photopic full-field ERG was moderately abnormal in both eyes. The b-wave amplitudes were decreased in both eyes with either the single photopic 0 dB flash (69.5 μ V OD, 56.3 μ V OS; normal lower limit, 94.5 μ V) or the 30 Hz flicker (41.7 μ V OD, 31.5 μ V OS; lower limit, 62.9 μ V) with only a 0.5-millisecond delay in the culmination time of the b-wave with either flash.

Patient 2, the older brother of patient 1, was 17 years old at the time of examination and was asymptomatic. On examination, best-corrected visual acuity was 20/25 in both eyes and results of a visual field examination showed anomalies very similar to those of his brother with a slight but diffuse loss of sensitivity in both eyes (Figure 2). Fundus examination results were remarkable for a peripheral annular salt-and-pepper retinopathy. On fluorescein angiography, only a few temporal paramacular moderately hyperfluorescent spots were present (window defects) and peripheral mottled appearance of the RPE was present. Indocyanine green angiography was unrevealing. Scotopic full-field ERG was slightly abnormal with a significant interocular asymmetry. The b-wave amplitudes were moderately reduced after maximal stimulation (scotopic 0 dB) in the right eye whereas they were normal in the left eye (301.2 μ V OD, 586.6 μ V OS; normal lower limit, 383.6 μ V). However, a slight delay of the b-wave culmination time was present in both eyes (3-millisecond delay for the -24 dB stimulus, 0.5-millisecond delay for the 0 dB stimulus). No interocular asymmetry was present on the photopic full-field ERG. The b-wave amplitudes were normal after the single photopic 0 dB flash but were moderately decreased in both eyes after the 30 Hz flicker stimulation (41.7 μ V OD, 39.0 μ V OS; normal lower limit, 61.5 μ V). The culmination time of the photopic b-wave was normal after the 0 dB stimulation but was slightly delayed after the 30 Hz flicker (33.0 milliseconds OD, 33.1 milliseconds OS; normal upper limit, 31.3 milliseconds).

Patient 3 was 53 years old and was asymptomatic at the time of examination. Visual acuity was 20/20 OU and color vision was slightly abnormal in both eyes (10/13 OD and 11/13 OS on Ishihara pseudoisochromatic plates). The same pigmentary disturbances were found on fluorescein angiography in the macular area (Figure 4). Peripheral sensitivity loss was present in both eyes on visual field examination (Figure 2), affecting the left eye slightly more than the right eye (mean sensitivity of 22.7 dB vs 23.2 dB). Peripheral salt-and-pepper retinopathy was present in both eyes with marked chorioretinal atrophy of the nasal retina in both eyes. These retinal changes were more obvious on fluorescein angiography. Indocyanine green angiography was not performed. A significant asymmetry of the scotopic ERG results was present, the amplitudes of both the a-wave and b-wave being diminished, albeit within the normal limits, in the right eye with normal culmination times in both eyes. The amplitude of the photopic b-wave was normal

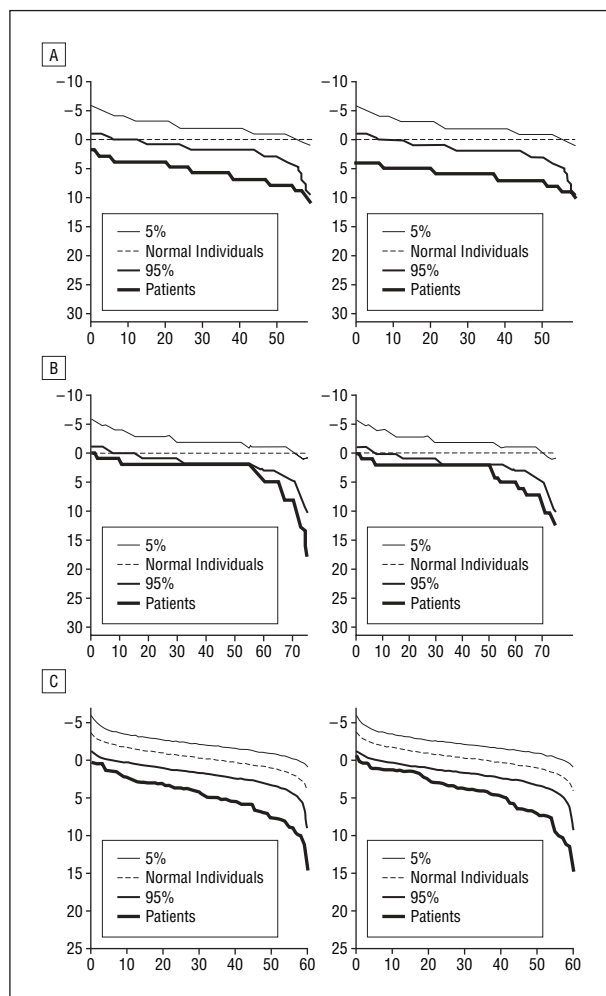


Figure 2. Result of static automated perimetry. The Bebie curves of the right eye and left eye of patient 1 (A), patient 2 (B), and patient 3 (C) showed diffuse decreased sensitivity in both eyes.

in both eyes, but the culmination time was slightly delayed in both eyes after either the single photopic flash or the 30 Hz flicker (delay of 1-1.5 milliseconds). Multifocal ERG was also performed, revealing a significant interocular asymmetry with lower amplitudes in the left eye and a slight decrease in ERG amplitudes in the nasal superior retina of both eyes (Figure 4).

The two variants, *LAMP2a* and *LAMP2b*, were observed by reverse-transcriptase polymerase chain reaction in the human ARPE-19 RPE cell line (Figure 5). Immunohistochemistry analysis of a human retina showed strong expression of *LAMP2* in the RPE cells but did not show expression, or not at detectable levels, in other retinal layers (Figure 6).

COMMENT

The family history of our patients is remarkable in the sense that the 2 boys were at risk for 2 diseases, RP and DD. Cases of RP were diagnosed on the paternal side. Linkage analysis confirmed that the RP was linked to the X chromosome. Sixty percent to 90% of X-linked RP cases are RP3 cases and are caused by mutations in the *RPGR*

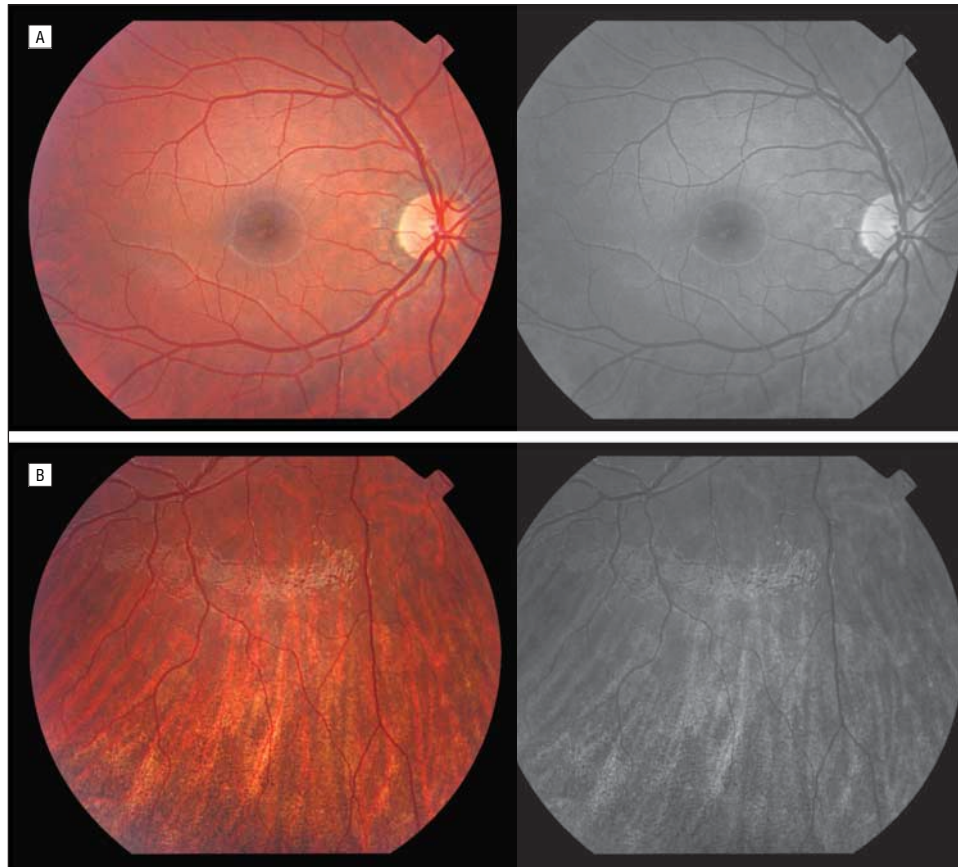


Figure 3. Fundus photographs (left column) and arteriovenous phase fluorescein angiography (right column). A, Posterior pole of the right eye of patient 1 revealed hypopigmentary alterations of the retinal pigment epithelium showing as window defects on fluorescein angiography. B, Inferior retina of the right eye of patient 2 showed a peripheral salt-and-pepper retinopathy with multiple corresponding zones of hyperfluorescence/hypofluorescence on fluorescein angiography.

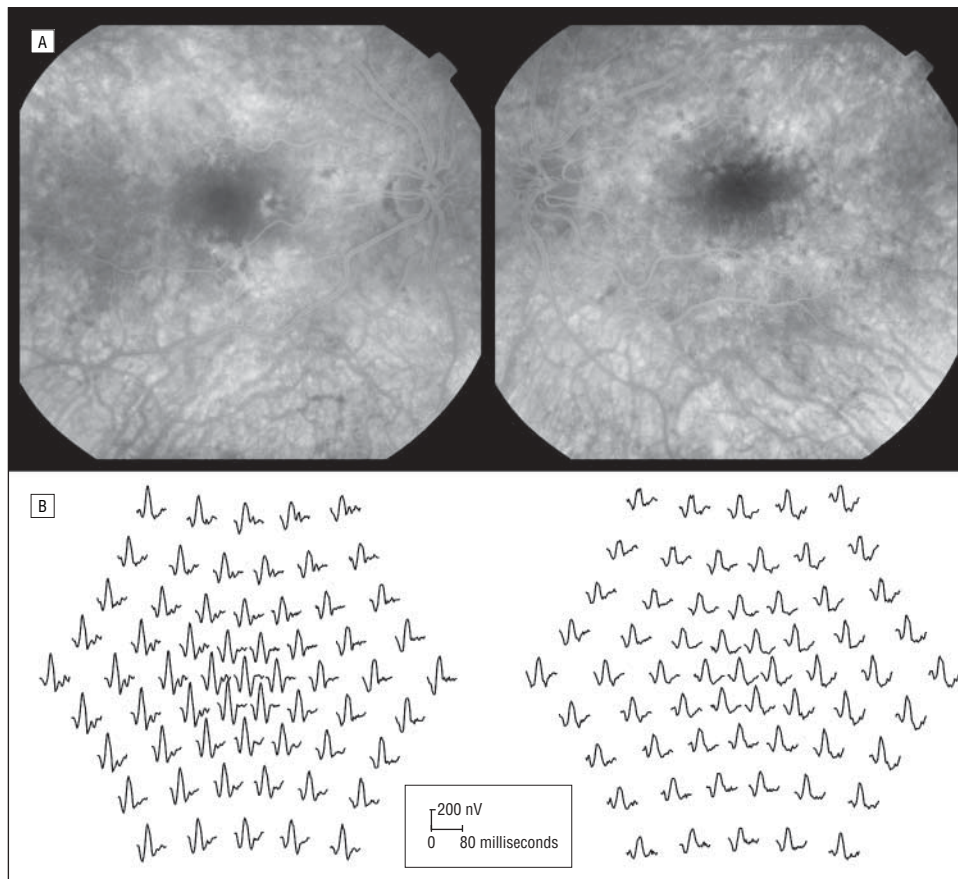


Figure 4. Patient 3 imaging data. A, Fluorescein angiography (midphase) showed bilateral maculopathy with pigmentary alterations. B, Multifocal electroretinography displayed that retinal potentials revealed a significant interocular asymmetry with lower amplitudes in the left eye.

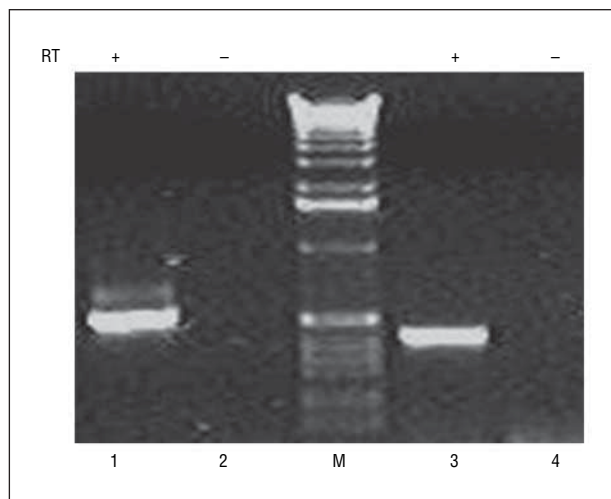


Figure 5. Analysis by reverse-transcriptase polymerase chain reaction of human *LAMP2a* (lanes 1 and 2) and *LAMP2b* (lanes 4 and 5) messenger RNA expression in ARPE-19, a human retinal pigment epithelial cell line. Lane 3 is commercially supplied size marker (M). Reverse transcription was performed with (+) or without (-) reverse transcriptase (RT).

gene on Xp21.1.¹⁰ Haplotype analysis clearly excluded this region and showed linkage to RP24, an RP locus on Xq26-q27. This locus was identified in a single family, but the gene has yet to be identified.¹¹ Thus, this family could represent the second occurrence of RP24.¹² Genetic counseling was given to the father and his 2 boys. Based on this linkage analysis, X-linked transmission was stated and a zero risk of transmission of the affected haplotype from their father to the 2 boys was given. Several years later, cardiomyopathy and arrhythmia were observed in the 2 boys and the diagnosis of DD was established.

Because of the ubiquitous expression of *LAMP2* and systemic nature of the disease, complete ophthalmic evaluation was performed in the 2 brothers and their maternal aunt, all carriers of the S157X *LAMP2* mutation. All 3 patients showed a diffuse but mild retinopathy and maculopathy. The retinopathy was characterized by a salt-and-pepper appearance of the midperipheral retina over 360°. Visual dysfunction was moderate in the youngest boy and minimal in the oldest brother. In their aunt, sensitivity loss was noted mostly in the peripheral visual field. All 3 patients showed photopic ERG abnormalities that were more pronounced in the hemizygous boys than in the heterozygous carrier. The 2 brothers further showed subtle scotopic ERG abnormalities. In the female carrier, multifocal ERG (mostly reflecting cone function) revealed a diffuse albeit asymmetrical loss, possibly related to lyonization. A significant interocular asymmetry on full-field ERG was also present in patient 2. Altogether these ERG abnormalities suggested a diffuse retinopathy, most likely inherited, affecting the cones more than the rods. Recently, a case of DD associated with Charcot-Marie-Tooth disease and maculopathy was described, although no details on ophthalmic examination were provided.¹³ Earlier, Lacoste-Collin et al¹⁴ reported a progressive decrease of bilateral visual acuity related to diffuse choriocapillary atrophy. Here again, no ophthalmic description was provided. Based on these ob-

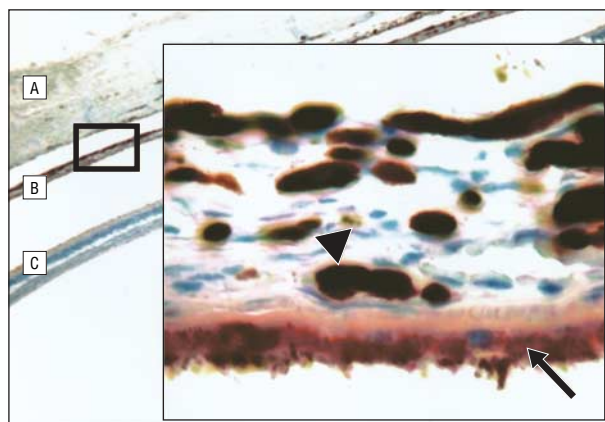


Figure 6. Immunohistochemistry analysis of a human retina. A indicates sclera; B, choroid; C, retina; arrowhead, melanocytes; arrow, *LAMP2*-positive reaction.

servations and ours, we propose that the phenotype of DD may include a diffuse cone-rod dystrophy and a maculopathy in addition to mental retardation, cardiomyopathy, and myopathy. A systematic ophthalmic examination of patients with DD will allow for a true estimation of the ocular involvement or whether our observation reflects a linkage disequilibrium with a new RP gene in the vicinity of *LAMP2*.

Lysosomes represent the final recycling location of numerous endocytic, autophagic, and secretory molecules marked for destruction. In addition, they also play a capital role in inactivating pathogenic organisms, repairing molecular membranes, and recycling photoreceptor outer segments by RPE.¹⁵ These acidic structures come in many different shapes and contents. Classically, lysosomes are defined as hydrolase-rich, acidic organelles lacking the cation-dependent and cation-independent mannose 6-phosphate receptors. Their membrane is composed of 2 abundant glycoproteins of high molecular mass called lysosomal-associated membrane protein 1 (*LAMP1*) and 2 (*LAMP2*). Being heavily glycosylated, these proteins are responsible for the very low pH levels found in lysosomes.¹⁶

LAMP2 produces at least 2 isoforms by an alternative usage of exon 9, its most 3' exon. *LAMP2a*, and more specifically 4 positively charged amino acids only present in the cytosolic tail of this variant, is required for *LAMP2* chaperone-mediated autophagy.^{17,18} We showed by reverse-transcriptase polymerase chain reaction that both *LAMP2a* and *LAMP2b* variants were present in the ARPE-19, thus suggesting that this function may also be conserved in RPE.

Tanaka et al⁶ generated a *LAMP2* knockout mouse that reproduced many signs found in DD: early death and massive accumulation of autophagic vacuoles in numerous tissues, including liver, muscle, and heart. Although the initial report did not mention any abnormal accumulation of material in the mouse RPE, the high expression of *LAMP2* in human RPE and the phenotype we described here would justify a thorough re-examination of the eyes of these models. In addition to the hearts, these mice could also be very valuable for understanding the mechanism underlying photoreceptor degeneration in DD.

Numerous genes are needed to build an eye and maintain vision throughout life. The ones that have been identified so far are those with direct ophthalmic implications. It is clear that many genes involved in systemic diseases associated with poor survival prognosis will see their effect in other organs, not only in the eyes, become a major source of concern once a good and reliable therapy is available. This will also represent a major issue for genetic counseling for patients undergoing gene therapy in the future.

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