Neurobiology

Generalized Lysosome-Associated Membrane Protein-2 Defect Explains Multisystem Clinical Involvement and Allows Leukocyte Diagnostic Screening in Danon Disease

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Danon disease, an X-linked dominant disorder, results from mutations in the lysosome-associated membrane protein-2 (LAMP2) gene and presents with hypertrophic cardiomyopathy, skeletal myopathy, and mental retardation. To investigate the effects of LAMP2 gene mutations on protein expression in different tissues, we screened LAMP2 gene mutations and LAMP-2 protein deficiency in the skeletal muscle of nine unrelated patients with hypertrophic cardiomyopathy and vacuolar myopathy. We identified three novel families (including one affected mother) with unreported LAMP2 gene null mutations and LAMP-2 protein deficiency in skeletal and myocardial muscle, leukocytes, and fibroblasts. LAMP-2 protein deficiency was detectable in various tissues, including leukocytes, explaining the multisystem clinical involvement. Skeletal muscle immunopathology showed that mutant protein was not localized in the Golgi complex, vacuolar membranes expressed sarcolemmal-specific proteins, and the degree of muscle fiber vacuolization correlated with clinical muscle involvement. In our female patient, muscle histopathology and LAMP-2 protein analysis was inconclusive, indicating that diagnosis in females requires mutation identification. The random X-chromosome inactivation found in muscle and leukocytes excluded the possibility that selective involvement of some tissues in females is due to skewed X-chromosome inactivation. Therefore, biochemical analysis of leukocytes might be used for screening in male patients, but genetic screening is required in females.

Danon disease is a rare X-linked dominant disorder predominantly affecting striated muscle. The disorder was originally described as a "lysosomal glycogen storage disease with normal acid maltase" because the pathological hallmarks are cytoplasmic vacuoles containing autophagic material and glycogen in skeletal and cardiac muscle cells. At the clinical level, the triad of hypertrophic cardiomyopathy, skeletal myopathy, and mental retardation typically characterizes the disease in male patients. Cardiac symptoms usually begin during adolescence, and patients die of heart failure in their third decade. In contrast, skeletal myopathy is usually mild, weakness and atrophy predominantly affect shoulder girdle and neck muscles, but distal muscles may also be involved. Mental retardation is present in 70% of male patients (rare in females) and it is usually mild. In female patients, the disease predominantly involves the cardiac muscle and has a later onset than in males.

Danon disease is caused by primary deficiency of lysosome-associated membrane protein-2 (LAMP-2), whose gene (LAMP2) maps to chromosome region Xq24. In the human LAMP2 gene, exon 9 exists in two forms, 9a and 9b, which are alternatively spliced and produce two protein isoforms called LAMP-2a and LAMP-2b, respectively. LAMP-2a is distributed rather ubiquitously, whereas LAMP-2b is expressed predominantly in striated muscle and brain. By analogy with LAMP2 knockout mice, tissues other than striated muscle are likely to be involved in the human form of the disease.

LAMP-2 and LAMP-1 are highly glycosylated homologous proteins that share 37% sequence identity and...
similar molecular weight\(^{5-7}\) and constitute a significant fraction of the total lysosomal membrane proteins. Although LAMP-2 and LAMP-1 have many structural and biochemical similarities, they differ in the expression level in skeletal muscle and might have different roles in lysosomes.\(^{3,8,9}\) The lysosomal membrane plays a crucial role in the function of this organelle by sequestering many of the acid hydrolases that are responsible both for the degradation of foreign materials and for specialized autolytic functions. LAMP-2 coats the inner surface of the lysosomal membrane and consists of a large intraluminal head, a transmembrane domain, and a small cytoplasmic tail containing a lysosomal membrane-targeting signal.\(^{10}\) LAMP-2 is supposed to be involved both in the fusion of lysosomes with other membranes and in the maturation of autophagic vacuoles.\(^{3,8,9}\) LAMP-2 also acts as a receptor for proteins to be imported and degraded within lysosomes in chaperone-mediated autophagy.\(^{8,11}\)

The molecular diagnosis of Danon disease has so far been assessed by the demonstration of LAMP-2 protein deficiency in skeletal or cardiac muscle and/or the identification for LAMP2 gene mutations. In this study, we systematically investigated, at both biochemical and molecular levels, a group of patients who presented vacuolar myopathy and hypertrophic cardiomyopathy to identify families affected by Danon disease. We performed an immunopathological study on skeletal muscle from LAMP2 mutant patients to correlate the extent of pathological changes in different cellular compartments with the severity of skeletal myopathy and cardiomyopathy. Furthermore, we analyzed the expression of LAMP-2 protein in different tissue other than striated muscle because the defect of LAMP-2 in various cell types could explain the multisystem involvement observed at the clinical level and could provide an easier and less invasive diagnostic tool in Danon disease.

Materials and Methods

Selection Criteria of Patients

Our skeletal muscle biopsy tissue bank, which contains more than 6000 specimens, was surveyed for patients affected with an unidentified form of vacuolar myopathy and hypertrophic cardiomyopathy. Nine skeletal muscle biopsies matched our selection criteria and were selected for the screening of both LAMP-2 protein deficiency (by immunohistochemical and Western blot analysis) and LAMP2 gene mutations. Three patients in whom LAMP2 gene mutations were identified were the objects of the present study; the remaining six patients are currently undergoing the search for an alternative molecular diagnosis.

Case Reports

Patient 1 (Figure 1, Family 1, II-2)

This 20-year-old male of Balkan origin complained of easy fatigability, anorexia, and abdominal pain. He progressively developed diffuse muscle hypotrophy and showed Wolff-Parkinson-White (WPW) syndrome and heart failure. He had several syncopal episodes. Elevated creatine kinase (CK) level was found. At 22 years, he was hospitalized to assess his eligibility to cardiac transplantation. Chest X-ray showed cardiomegaly and pleural effusion. Echocardiography showed end-stage hypertrophic cardiomyopathy, ie, with severe reduction of left ventricular ejection fraction (20%), and cardiogenic ascites. A pacemaker was implanted to control the severe bradi-arrhythmias in presence of atrial fibrillation. On neurological examination, he had cachectic appearance, marked atrophy of trunk and paraspinal muscles, marked weakness in upper girdle muscles, and diminished deep tendon reflexes. Electromyography was myogenic. CK level was 283 U/L (normal values 0–190). A quadriceps femoris muscle biopsy showed severe vacuolar myopathy with storage of glycogen and filamentous material. The physical examination showed tender and enlarged liver as well as ascites. Ultrasounds revealed hepatosplenomegaly and small kidneys. Laboratory tests (bilirubin, transaminases, prothrombin time; partial thromboplastin time; international normalized ratio, albumin, creatinine clearance, and microalbuminuria) were abnormal. Viral hepatitis was excluded by serological tests. He had mild mental retardation and attended special schools in childhood. Spirometry showed severe restrictive pulmonary insufficiency. He died at 22 years of age (1 month after hospitalization) of heart failure.

Patient 2 (Figure 1, Family 2, III-1)

At 9 years of age this male showed scleral jaundice, abnormal hepatic laboratory tests, and chronic hepatitis at liver biopsy with normal serology. At age 18, an electrocardiography revealed WPW syndrome. Since age 22, he noticed exertion dyspnea and difficulty in climbing stairs and complained of several syncopal episodes;
hypertrophic cardiomyopathy was diagnosed. At age 24, an implantable-cardioverter defibrillator (ICD) was implanted, and 1 year later, cardiac transplant was considered. Echocardiography showed end-stage hypertrophic cardiomyopathy. Cardiac single photon emission computed tomography and positron emission tomography showed reduced caption. Endomyocardial biopsy, which was obtained during heart catheterization, suggested a form of hypertrophic cardiomyopathy with glycogen storage disease. On neurological examination, he had mild waddling gait with hyperlordosis, winging scapulae, diffuse muscle hypotrophy, diminished tendon reflexes, Gower’s sign, arachnodactyly, and weak facial muscles. CK was 558 U/L. A quadriiceps femoris muscle biopsy showed vacular myopathy with storage of periodic acid-Schiff (PAS)-positive material. On physical examination, he had cachectic appearance and hepatomegaly, and on abdominal ultrasound, he had also a mild reduction of kidney size. Laboratory tests (bilirubin, transaminases, creatinine clearance, and proteinuria) were abnormal. No mental retardation was present. Spirometry showed severe restrictive pulmonary insufficiency. The patient died at age 29 years of heart failure while waiting for cardiac transplant.

Patient 3 (Figure 1, Family 3, III-10)

At 12 years of age, this male patient had subclinical jaundice, but viral hepatitis was excluded by serology. At 18 years, he noticed easy fatigueability after mild effort. At 19 years, ECG revealed bradycardia and WPW syndrome. At age 22, he had very low aerobic resistance by Tread-Mill test. At age 27, chest X-ray showed mild cardiomegaly. An ophthalmologic examination revealed severe myopia with pigmented epithelial retinal dystrophy. Abdominal echography showed hepatomegaly and chronic pathology, but hepatic biopsy was normal. The patient was hospitalized at age 28 to investigate muscle weakness and high CK (1094 U/L). Skeletal muscle biopsy showed vacular myopathy with accumulation of PAS-positive material. Muscle CT scan revealed moderate proximal and distal atrophy of lower limbs. Echocardiography showed mild hypertrophy of intraventricular septum. Mild restrictive ventilator dysfunction was present on spirometry. At age 30, he complained of mild difficulty in climbing stairs and lifting weights. On physical examination, he had mild waddling gait with hyperlordosis, Gower’s sign, distal leg muscle hypotrophy, and mild weakness of proximal and distal girdles, neck flexor, and facial muscles. Non-obstructive hypertrophic cardiomyopathy with moderate septum hypertrophy (21 mm) was diagnosed by further echocardiography. On neuropsychological evaluation, he had delayed psychomotor development, poor school achievements, and an IQ (WAIS-R) of 77. Electroencephalography revealed diffuse slow dys-rhythmia.

Patient 4 (Figure 1, Family 3, II-5)

This female is the mother of patient 3. She has suffered since age 26 of palpitations and easy fatigability; at age 29, an ECG showed WPW syndrome with paroxysmal atrial flutter, which was later cardioverted, first electrically and then pharmacologically. A few years later, echocardiography revealed hypertrophic cardiomyopathy. At 38 years, aspecific hepatitis was diagnosed on the basis of echographic hepatosplenomegaly and hyp-transaminasemia with negative serology. At age 47, she developed a moderate bilateral neurosensory hypoacusia and progressive heart failure with effort dyspnea, orthopnea, worsening asthenia, and several syncopal episodes. Aerobic capacity was markedly reduced. Echocardiography showed end-stage hypertrophic cardiomyopathy with severe reduction of left ventricular ejection fraction (33%) and diffuse hypokinesia. Three years later after colecistectomy, she had an episode of acute heart failure complicated by ventricular fibrillation, and an ICD was implanted. At age 52, she underwent cardiac transplantation. Since then, she did not have cardiac complaints but still reported muscular weakness and myalgia. At age 54, on neurological examination, she had mild proximal and flexor muscle weakness, facial hypomimia, and distal upper and lower limb atrophy. Severe myopia and weakly reactive pupils were recorded. Liver was tender to palpation. CK level was normal. On neuropsychological examination, her cognitive records were normal (IQ = 82 by WAIS-R scale). No electroencephalography abnormalities were present. A mild reduction of cortical kidney thickness and some cystic lesions were demonstrated by ultrasounds. Quadriiceps femoris muscle biopsy showed mild myopathic changes without fiber vacuolization.

Cardiological Evaluation

A cardiac evaluation was obtained from clinical history and noninvasive methods (ECG, Holter monitoring, M-mode, and two-dimensional and Doppler echocardiography). The presence of ventricular pre-excitation was diagnosed on short PR (between P and R waves) interval, δ waves, or both and left ventricular hypertrophy on voltage.12 Hypertrophic cardiomyopathy was diagnosed on echocardiography showing unexplained left ventricular hypertrophy (maximal wall thickness ≥15 mm). Two patients underwent invasive cardiac techniques (heart catheterization and endomyocardial biopsy), and one patient underwent heart transplantation.

Fibroblast Cell Culture

Fragments of skin biopsy were sterilely collected at the time of biopsy, placed in 10% dimethylsulfoxide in rich medium (70% M199 medium [Seromed, Berlin, Germany] and 30% fetal calf serum [Euroclone, Torquay Devon, UK]), gradually frozen, and then stored in liquid nitrogen. To start the cell culture, specimens were thawed quickly and incubated in a freshly prepared mixture of 80% rich medium and 20% human plasma that was filtered directly on the disk. When fibroblast proliferation was visible, the specimens were trimmed and placed on gelatinized dishes. Fibroblasts were cultured in Dulbecco’s modified Eagle’s medium nutrient medium (Seromed) supple-
ment with 10% fetal calf serum, l-glutamine, and penicillin-streptomycin in 75 mm² flasks at confluence, collected, and directly dissolved in electrophoresis loading buffer.

Histopathology and Immunohistochemistry

Sections of skeletal muscle biopsies from patients and controls were routinely stained to evaluate overall muscle morphology or were used for immunohistochemistry. The same reactions were also performed in myocardial biopsy from patient 2, whereas histoenzymatic and immunohistochemical reactions in the explanted heart from patient 4 were prevented by embedding it in paraffin. We used a panel of antibodies to study the following subcellular compartment or cell component: lysosomal membrane, anti-LAMP-2 antibody (H4B4 luminal domain; Developmental Studies Hybridoma Bank, Iowa City, IA); plasma membrane, anti-caveolin-3 (Transduction Lab., Lexington, KY) and anti-dystrophin (DYS2; Novocasta, Newcastle on Type, UK); nuclear membrane, anti-lamin A/C (LAM-A/C; Novocasta) and anti-emerin (Novocasta); basal lamina, anti-laminin A (1924; Chemicon, Temecula, CA); proliferation of Golgi apparatus, anti-Golgi (1271; Chemicon); early endosome membrane, anti-rab5 (Transduction); cytoskeleton, anti-desmin (1698; Chemicon); muscle regeneration, anti-fetal myosin (MHCn; Novocastra); and inflammatory features, anti-macrophages (1271; Chemicon). Sections were incubated for 1 hour with primary antibodies (diluted 1:100, except LAMP-2, which was diluted 1:20). After washes in phosphate-buffered saline, antibodies (diluted 1:100, except LAMP-2, which was diluted 1:20). After washes in phosphate-buffered saline, sections were incubated for 30 minutes with anti-mouse Cy-3-conjugated Ig (1:100; Caltag, Burlingame, UK) and examined by fluorescence microscopy.

Western Blot Analysis

Except for the endomyocardial biopsy sample, which was insufficient for this analysis, LAMP-2 Western blotting was conducted in a panel of tissues and cell types from patients and controls. Skeletal muscle biopsies, leukocytes, and skin fibroblasts were dissolved in the electrophoresis-loading buffer and processed as previously reported,13 using the same anti-LAMP-2 antibody used for immunohistochemistry (diluted 1:300). LAMP-1 antibody (H4A3; Developmental Studies Hybridoma Bank) was used to evaluate a potential cross-reaction with LAMP-2 protein (for both proteins, the immunogen was the native protein). LAMP-2 and LAMP-1 antibodies (diluted 1:200) were used in duplicate in adjacent lanes of the same gel. The protein quantity of each sample was normalized to the amount of tissue loaded, as determined by the skeletal myosin or the actin bands in the post-transfer Coomassie blue-stained gels. The amount of protein in patients was determined by densitometry (ImageJ software v.1.34) and expressed as percentage of control.

DNA and RNA Analysis

Polymerase Chain Reaction (PCR) Amplification of Genomic DNA Sequence

Genomic DNA was extracted from blood leukocytes or muscle biopsy, using the GenElute Mammalian Genomic DNA kit (Sigma, St. Louis, MO). The entire coding sequence of the LAMP2 gene was amplified in 10 amplicons using primer sequences (available on request) designed using the human LAMP2 sequence as reference (GenBank accession no. AC002476.1). PCR reactions were performed under standard conditions.13,14

DNA Sequencing

PCR products were purified by enzyme reaction (Exo-Sap-IT; Amersham, Buckinghamshire, UK), quantified on agarose gel, and directly sequenced using the Big Dye dideoxy-terminator cycle sequencing kit and the 377 ABI-PRISM automated sequencer at the Centro Interdipartimentale di Ricerca e Servizi per le Biotecnologie Innovative Biotechnology Centre, University of Padova.

PCR Amplification with Allele-Specific Primers (ARMS-PCR)

To confirm two of the three novel mutations found by direct DNA sequencing (W98X and 796–797insC), we used appropriate ARMS-PCR tests by allele-specific primers designed ad hoc. PCR amplifications were performed under standard conditions using a touchdown PCR protocol.13,14 An ARMS-PCR test was not required to confirm the third mutation (22-bp deletion), because the reduced size of the amplicon was easily detectable by 2% agarose gel electrophoresis.

RNA Isolation and Semiquantitative RT-PCR

Total RNA was isolated from muscle biopsies using the SV total RNA isolation system (Promega Corp., Madison WI) according to the manufacturer’s protocol, including DNase treatment. To estimate the amount of LAMP2 RNA in skeletal muscle, total RNA isolated from control and patients’ muscle biopsies was reverse transcribed using Superscript III reverse transcriptase (Invitrogen, San Diego, CA) and oligo-dT primer in a 20-μl reaction volume, according to the manufacturer’s recommendations. The full-length LAMP2 gene cDNA was amplified using specific primers (forward, 5’-TGGTGTTGCAGCTGTTG-3’; reverse, 5’-CGTAAGCAATCACTATAACGATA-ATCAA-3’) designed on the human LAMP2 mRNA sequence (GenBank accession no. NM_013995) by Primer Express 2.0 software (Applied Biosystems, Foster City, CA). The amplification product was expected to be 1418 bp in size. A fragment of 838 bp of the β-actin cDNA was used as an internal control for normalization. A primary solution of 150 μl was prepared and distributed in 10 aliquots of 15 μl, each corresponding to different points of the amplification curve measured during the exponen-
Table 1. Clinical, Histopathological, and LAMP2 Molecular Data

<table>
<thead>
<tr>
<th>Patient, sex</th>
<th>Family history</th>
<th>Age and symptoms at muscle onset</th>
<th>Age and muscle involvement at last examination</th>
<th>CK level (units/L)*</th>
<th>Age at hepatopathy (years)</th>
<th>LAMP2 gene nucleotide change</th>
<th>Amino acid change</th>
<th>Tissue available for LAMP-2 protein test</th>
<th>Skeletal muscle pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, male†</td>
<td>+</td>
<td>20, easy fatigability</td>
<td>22, marked trunk and limb atrophy, severe girdle muscle weakness</td>
<td>283</td>
<td>22</td>
<td>+ 796–797insC</td>
<td>–</td>
<td>Muscle, fibroblasts</td>
<td>Diffuse atrophy and vacuoles in many fibers</td>
</tr>
<tr>
<td>2, male</td>
<td>+</td>
<td>22, difficulty in climbing stairs</td>
<td>22, diffuse hypotrophy, waddling gait, Gowers’ sign</td>
<td>558</td>
<td>9</td>
<td>– 680–701del</td>
<td>–</td>
<td>Muscle, heart</td>
<td>Vacuoles in many fibers</td>
</tr>
<tr>
<td>3, male†</td>
<td>+</td>
<td>18, easy fatigability</td>
<td>30, waddling gait, Gowers’ sign, moderate neck, facial, distal limb weakness</td>
<td>1094</td>
<td>12</td>
<td>+ 294G&gt;A</td>
<td>W98X</td>
<td>Muscle, leukocytes</td>
<td>Vacuoles in many fibers</td>
</tr>
<tr>
<td>4, female†</td>
<td>+</td>
<td>26, easy fatigability</td>
<td>54, myalgia, mild muscle weakness and distal atrophy</td>
<td>Normal</td>
<td>38</td>
<td>– 294G&gt;A</td>
<td>W98X</td>
<td>Muscle, leukocytes, fibroblasts</td>
<td>No vacuoles</td>
</tr>
</tbody>
</table>

*Normal values 0 to 190 units/L.
†Relatives (patient 4 is the mother of patient 3).
MR, mental retardation.

tional phase of the PCR for the two genes (from 25 to 39 cycles). The solution included 8 μl of template cDNA, 200 μmol/L dNTPs, 0.2 μmol/L of each primer for β-actin, 0.4 μmol/L of each primer for LAMP2 (to favor amplification), 1X Taq Platinum buffer, 1.5 mmol/L MgCl2, and 1 U Taq Platinum (Invitrogen). PCR parameters were as follows: 94°C for 2 minutes; 39 cycles of 94°C for 30 s, 55°C for LAMP2 gene and 61°C for β-actin gene for 30 s, and 72°C for 1 minute; with a final elongation step of 72°C for 10 minutes. The PCR products were analyzed by agarose gel electrophoresis. The level of each transcript expression was determined by densitometry (using ImageJ software v.1.34n) and expressed as percentage of control.

X-Chromosome Inactivation

The X-chromosome inactivation pattern was determined by PCR analysis of polymorphic CAG repeats in the first exon of the AR gene. DNA, extracted from blood leukocytes and muscle biopsy of heterozygote female patient, was digested using methylation-sensitive restriction enzymes (HpaII and CfoI). The PCR primers used for the AR locus were previously described. After digestion, DNA amplification occurred only in presence of methylated restriction sites (inactive allele). PCR products were separated on an ABI 377 automated sequencer and analyzed by GeneScan software (Applied Biosystems). Because the smaller allele amplifies more efficiently, a correction factor was generated using the undigested samples so that both alleles were represented equally in the calculations. All samples were analyzed in triplicate, and the average values were used in calculating the degree of X inactivation. The X inactivation pattern was classified as skewed when 90% or more of the cells preferentially used one X-chromosome.

Results

Patients

Of the nine male patients who showed vacuolar myopathy and hypertrophic cardiomyopathy and who were screened for both LAMP-2 protein defect and LAMP2 gene mutations, three cases were affected by primary Danon disease. The latter showed a multiorgan deficiency of LAMP-2 protein and had null mutations in the LAMP2 gene (Table 1). Although all three patients had a positive family history (Figure 1), in only one family (family 3), the mother of a male patient (patient 4) was alive at the time of the survey and was included in this study. The onset of cardiac symptoms occurred in late adolescence (except in the female patient, who had onset in the third decade) with exertional dyspnea and easy fatigability. All patients had WPW syndrome (Figure 2; Table 2), supraventricular arrhythmias, or atrial fibrillation. In two patients, ICD was implanted for ventricular tachycardia or fibrillation. Echocardiography showed concentric left ventricular hypertrophy in patients 1 and 2 and asymmetric hypertrophy with a moderate left ventricular maximal hypertrophy in patient 3 (Figure 2; Table 2). Three of the four patients developed severe and progressive heart failure associated with end-stage systolic dysfunction; two male patients died of heart failure at age 22 and 29, and the female was transplanted at 52 years of age.

The severity of the cardiomyopathy did not match the severity of skeletal myopathy; all male patients presented skeletal myopathy of variable severity, which, however, never compromised ambulation. Muscle weakness appeared generalized and involved both proximal and distal limb girdles, trunk, neck, and facial muscles. The duration of muscle disease was not related to age at onset of symptoms. A mild mental retardation was present in two male patients. Hepatic involvement, which...
was present in all patients, had been clinically evident since childhood in two patients (Table 1). A milder involvement of organs other than striated muscles, such as kidney, spleen, and eye, was also observed. The female patient had mild myopathy and no mental retardation.

Skeletal and Cardiac Muscle Histopathology

Skeletal muscle histopathology showed extensive muscle fiber vacuolization and degeneration and focal storage of PAS-positive material. The degree of fiber vacuolization and of overall muscle architectural derangement was variable in the three male patients (Table 1; Figure 3). Patient 1 had generalized fiber atrophy and degeneration. In addition, the majority of fibers (46%) contained vacuoles that were sometimes large enough to replace all of the cytoplasm. Patients 2 and 3 had multiple, relatively small-sized vacuoles that were seen in a smaller proportion of fibers (24%). Female patient 4 had no fiber vacuolization. The accumulation of PAS-positive material and lysosomal acid phosphatase reaction were evident in small vacuoles of fibers undergoing degeneration. Cardiac muscle histopathology in patients 2 and 4 showed hypertrophic cardiomyocytes with enlarged and picnotic nuclei, empty cytoplasmic vacuoles, and myofibrillar disarray (Figure 3). Extensive replacement fibrosis associated with necrotic cardiomyocytes and collections of invading macrophages was observed in both patients. Increased acid phosphatase staining indicated active degeneration of cardiomyocytes.

Immunohistochemical Studies

Immunofluorescence analysis on both the skeletal and cardiac muscle biopsy from the male patients showed a complete absence of LAMP-2 protein when compared

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**Table 2.** Cardiological, Electrocardiographic, and Echocardiographic Data

<table>
<thead>
<tr>
<th>Patient, sex</th>
<th>Age at hypertrophic cardiomyopathy diagnosis</th>
<th>Symptoms at onset</th>
<th>ECG</th>
<th>Holter ECG</th>
<th>LAD (mm)*</th>
<th>LVEDD (mm)*</th>
<th>LVEF (%)*</th>
<th>Maximal LVWT (mm)*</th>
<th>PWT (mm)*</th>
<th>Pacemaker (years)</th>
<th>Age and cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, male</td>
<td>22</td>
<td>Syncope</td>
<td>WPW AF, AVB (pause 5350 ms)</td>
<td>58</td>
<td>63</td>
<td>20</td>
<td>19</td>
<td>18</td>
<td>Yes (22)</td>
<td>22, heart failure</td>
<td>22, heart failure</td>
</tr>
<tr>
<td>2, male</td>
<td>22</td>
<td>Syncope</td>
<td>WPW AF, SVT</td>
<td>38</td>
<td>60</td>
<td>46</td>
<td>25</td>
<td>22</td>
<td>ICD (24)</td>
<td>29, heart failure</td>
<td>29, heart failure</td>
</tr>
<tr>
<td>3, male</td>
<td>28</td>
<td>Fatigability</td>
<td>WPW SV arrhythmia</td>
<td>39</td>
<td>53</td>
<td>50</td>
<td>21</td>
<td>14</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4, female</td>
<td>36</td>
<td>Fatigability</td>
<td>WPW AF, NSVT</td>
<td>55</td>
<td>63</td>
<td>33</td>
<td>10</td>
<td>10</td>
<td>ICD (51)</td>
<td>52, heart transplant†</td>
<td>52, heart transplant†</td>
</tr>
</tbody>
</table>

*Echo values are referred to last control at our Department.
†Transplanted at age 52 and currently alive at age 55.
LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction (normal values = 53 to 63%); LVWT, left ventricular wall thickness; PWT, posterior wall thickness; AF, atrial fibrillation; AVB, atrio-ventricular block; SVT, supra-ventricular tachcardia; NSVT, not sustained ventricular tachcardia; LAD, left atrium diameter.
Figure 3. Routine histopathological stains of skeletal and cardiac muscle biopsy sections. A: Different degrees of skeletal muscle fibers involvement. Patient 1 had diffuse fiber atrophy, extensive fiber vacuolization and degeneration, accumulation of PAS-positive material, and positive acid phosphatase activity in some atrophic fibers. Patient 3 showed a milder degree of vacuolization and many fibers with apparently normal features. Patient 4 showed mild myopathic changes but no fiber vacuolization or degeneration. B: Vacuolization and autophagic degeneration of cardiomyocytes and extensive replacement fibrosis in cardiac muscle from patients 2 and 4. Collections of infiltrating macrophages and focal PAS-positive accumulation were also present. Microscope magnification, ×400.
with controls in which there was diffuse intracytoplasmic reaction (Figure 4). Conversely, in female patient 4, LAMP-2 immunolabeling was of intensity similar to controls. The labeling of proteins typical of plasma membrane and basal lamina (caveolin-3 [trans-membrane], dystrophin [intracellular], and laminin-A [basal lamina]) seemed to be increased in the cytoplasm because of the proliferation of lysosomes and vacuoles with their membranes. Lysosomal and vacuolar membranes were often in continuity with the plasma membrane (caveolin-3 and dystrophin) (Figure 4) but not with the nuclear membrane (lamin A/C and emerin). Muscle fiber regeneration and phagocytosis were not active. No proliferation of either Golgi apparatus or early endosomes was observed. Desmin was strongly expressed in the cytoplasm of vacuolated fibers. MHC class I molecules were expressed in the vacuolar membrane.

**Western Blot Analysis**

In the skeletal muscle from all male patients, LAMP-2 protein was virtually absent (Figure 5). On the contrary, the muscle from the female heterozygote patient showed LAMP-2 protein levels that were not significantly reduced compared with control. Fibroblasts and leukocytes showed absent LAMP-2 protein in male patients and nearly normal amounts of protein in the heterozygote female patient.

**Figure 4.** Cryostat sections of skeletal and cardiac muscle from patient 2 (PT) and control (CNTR), immunolabeled with antibodies against LAMP-2 and caveolin-3 (CAV-3). LAMP-2 reaction was absent in muscle and heart from the patient compared with the diffuse positive reaction observed in control. Caveolin-3 immunolabeling was positive in the plasma membrane of muscle fibers and cardiomyocytes of both patient and control. In the skeletal muscle from Danon disease, caveolin-3 labeling was observed also in the membrane of the lysosomes that delineate most vacuoles. The cellular membranes that make up both the plasmalemma and the lysosomes are often in continuity. Microscope magnification, ×400.

**Figure 5.** LAMP-2 and LAMP-1 Western blotting. **A:** Skeletal muscles from patients 1 through 4 and control (C) showed that all male patients (1 through 3) have virtually absent LAMP-2 protein, whereas the female heterozygote patient 4 showed nearly normal amounts of protein when compared with controls. Leukocytes and fibroblasts from male patients 3 and 1, respectively, showed virtually absent LAMP-2 protein, whereas the corresponding samples from the female patient 4 showed nearly normal amounts of protein when compared with controls. **B:** Skeletal muscle from control (C) and patient 3 loaded in duplicate and labeled with antibodies against LAMP-1 (right) and LAMP-2 (left). Control and patient samples are shown with different loading quantity of protein, as judged by MHC band in the Coomassie blue-stained gel.
To check the possibility that residual LAMP-2 immunolabeling in muscle tissue from male patients is attributable to a cross-reaction with the highly homologous LAMP-1 protein, we performed duplicated Western blotting for LAMP-1 and LAMP-2 that showed bands of similar molecular weight (Figure 5). A weak binding cross-reaction between these two proteins or between LAMP-2 and some unknown immunoreactive material cannot be excluded. In control muscle, LAMP-2 was considerably more highly expressed than LAMP-1 (Figure 5). LAMP-1 protein levels were similar in control and in patients’ muscle. LAMP-1 immunolabeling was similar in the leukocytes of patient and control.

**LAMP2 Gene Mutation Analysis**

LAMP2 gene mutations were identified in all three male patients who showed LAMP-2 protein deficiency and in the heterozygote female patient 4. Each patient showed a different and previously unreported mutation (Table 1; Figure 6): a 22-bp deletion in exon 5 (680–701del), causing a frame-shift (fsX8); a C nucleotide insertion in exon 6 (796–797insC), causing a frame-shift (fsX7); and a nucleotide substitution at position 294 in exon 3 (294G/H11022A), causing the change of a tryptophan to a stop codon at position 98 (W98X). The mutations found by sequence analysis were confirmed by allele-specific tests or by electrophoretic determination of amplicon size (Figure 6). All three mutations produce null alleles (nonsense or frame-shift mutations), which are predicted to prematurely truncate protein synthesis and result in the loss of the transmembrane domain (Figure 7).

**RNA Studies**

To study the transcriptional effect of premature truncating codon mutations observed at genomic level, we first analyzed LAMP2 mRNA in patients’ muscle by RT-PCR, using actin transcript as an internal standard for RNA quantity. Because LAMP2 mRNA was significantly reduced in all patients when compared with control, a semiquantitative RT-PCR was used to estimate the extent of transcript down-regulation. Aliquots were taken at different times during the exponential phase of the PCR. After β-actin normalization, LAMP2 mRNA levels were 65, 43, and 21% in patients 1, 2, and 3, respectively, compared with control (Figure 8). Considering all male pa-
tients in our study, the lowest LAMP2 mRNA expression was found in patient 3, who has the most proximal premature truncating codon gene mutation (Figure 7) and who presented the least severe phenotype.

**X-Chromosome Inactivation**

X-chromosome inactivation analysis was performed in three independent experiments on both skeletal muscle and leukocytes from the heterozygote female patient (no cardiac tissue was available for the study). After digestion, (CfoI+/HpaII+) PCR product is obtained only from the inactive X-chromosome. After compensation for unequal amplification of the two peaks caused by different product size, the inactivation ratio is calculated for the two CAG repeat alleles of the X-chromosomes. A random pattern of inactivation was found in both tissues analyzed: the mean skewing rate was 66% in skeletal muscle (62, 58, and 78% in the three independent experiments) and 60% in blood leukocytes (62, 48, and 69%) (Figure 9).

**Discussion**

About 30 families with Danon disease have been described in whom LAMP-2 protein deficiency on various cell types and/or LAMP2 gene mutations have been identified. We exploited the availability of a tissue bank with about 6000 skeletal muscle biopsies to select patients who presented vacuolar myopathy associated with hypertrophic cardiomyopathy for subsequent screening of both LAMP-2 protein deficiency and gene mutations. The study of nine patients with vacuolar myopathy and hypertrophic cardiomyopathy led to the identification of three novel families with Danon disease. Although Danon disease is considered very rare in the general population, its frequency is relevant (33%) among patients presenting with both vacuolar myopathy and hypertrophic cardiomyopathy, suggesting that the number of patients reported so far worldwide could be underestimated.

LAMP-2 protein deficiency was demonstrated in the explanted heart of affected patients, but Danon disease could be diagnosed by LAMP-2 immunofluorescence even from the very small tissue samples that are collected during heart catheterization. The search for LAMP-2 deficiency in endomycardial samples (when available) should be pursued in patients with hypertrophic cardiomyopathy especially when it is associated with skeletal myopathy (even with only high CK) and/or other organ impairment; moreover, a diagnostic skeletal muscle biopsy (less invasive and easier to obtain than myocardial biopsy) should be suggested in potential Danon disease. The identification of LAMP-2 protein deficiency in such tissue samples is very important because of the consequences of an early molecular diagnosis for both clinical evaluation (therapy and prognosis) and genetic counseling. In two of our male patients, the prognosis was poor for the rapid progression toward heart failure and death. The female patient was transplanted at age 52, and she is still alive.

In all of our male patients, we demonstrated a generalized LAMP-2 protein deficiency, which was detected in striated muscle, in fibroblasts, and in leukocytes. This result should be expected because LAMP-2 is a ubiquitous protein and because null gene mutations were found in our cases; nevertheless, few patients have been reported in which splice site mutations led to LAMP-2 protein synthesis in leukocytes.

Our first conclusion is that the collection of leukocytes is much less invasive than skeletal and myocardial biopsy but it is equally useful for LAMP-2 protein diagnosis in males. LAMP2 gene mutation analysis ensures complete sensitivity, whereas LAMP-2 immunoblot could fail to identify the patients with a cardiac-predominant phenotype due to partially functional mutant proteins. However, clinicians should consider leukocyte immunoblot analysis as a diagnostic screening option when suspecting Danon disease in males for these reasons: 1) it is expected to have high sensitivity because LAMP-2 protein deficiency was found in different tissues of the large majority of mutant patients (present study 2,3,19,21,22,24–27); 2) it should have high specificity because there are no reports...
of LAMP-2 protein deficiency in other disorders; and 3) it is much less expensive and time consuming than mutation screening.

The second conclusion is that the detection of LAMP-2 deficiency in a variety of cells/tissues supports the clinical evidence that Danon disease is a multisystemic disorder. Accurate clinical history collection revealed that hepatic involvement was present in all of our patients (in two cases, it was the first clinical sign), indicating that also in the human disease, as in the LAMP-2 knockout mouse, different organs other than striated muscles may suffer from LAMP-2 deficiency.

Another important result from our study is that female patients with Danon disease might escape the diagnosis unless mutation identification is obtained: in our heterozygote patient, muscle pathology and LAMP-2 protein analysis was inconclusive, and molecular diagnosis was pursued because of her affected son. The only other female patient so far analyzed showed a reduction of LAMP-2 protein levels in skeletal muscle; the discrepancy with our results might originate from a different X-chromosome inactivation pattern, a different gene mutation, or other unknown modulating factors. It is thus conceivable that a number of female patients with Danon disease (especially isolated cases) could remain under-diagnosed.

The most prominent histopathological feature of Danon disease is the vacuolization of muscle fibers. We confirm that the extent of these changes is related to the degree of clinical muscle involvement, suggesting that the accumulation of autophagic material within muscle fibers correlates with disease progression. We observed that the vacuolar membrane occasionally merged with the sarcolemma and was delineated by the basal lamina. The expression of sarcolemmal-specific proteins in the vacuolar membrane could be related to their function as a mechanical reinforcement. As in other disorders, one could speculate that mutant LAMP-2 protein could not be correctly targeted to the lysosomal membrane and could be retained in the Golgi complex or endoplasmic reticulum, but we showed that this is not the case.

The mechanism leading from LAMP2 mutations to clinical phenotype is an intriguing aspect in the study of Danon disease that requires further studies. One could speculate that, depending on different types of mutations, either mutant LAMP-2 proteins could be degraded or their synthesis could be abolished by the nonsense-mediated RNA decay mechanism in the nucleus. Although the demonstration that nonsense-mediated RNA decay mechanism is at play in this disease is beyond the purpose of our study, an eventual synthesis of low levels of prematurely truncated proteins in our cases would be followed by cytoplasmic protein degradation because of the lack of the transmembrane domain.

Although the clinical triad of hypertrophic cardiomyopathy, skeletal myopathy, and mental retardation typically characterizes the disease, a different degree of skeletal and cardiac muscle involvement can be observed between inter- and intrafamilial patients. Although the cardiac involvement associated with a high CK level of skeletal muscle origin is a constant feature in male patients, in females, the possible involvement of different organs other than heart (muscle and brain) is variable. Such unpredictability can hardly be attributed to skewed X-chromosome inactivation, because we showed that X-chromosome inactivation occurred at random in different tissues in our female patient. An alternative hypothesis is that brain and striated muscles have high-energy requirements, high protein turnover, and low regenerative capacity and are therefore likely to be more prone to damage. However, because LAMP-2 protein function is not fully understood, the reason why specific tissues are more vulnerable in Danon disease remains unclear.

It is not known how LAMP2 gene mutations produce a dominant effect. A model involving a dominant-negative effect of mutation is incompatible with X-linked inheritance where female patients express about 50% of protein level. Although the absence of mutant protein in hemizygote male patients indicates a loss-of-function, the phenotype in heterozygote females could originate either from skewed inactivation of X-chromosomes or from haploinsufficiency. Because preferential X-chromosome inactivation of wild-type allele could not explain why the disease is clinically manifest in all female heterozygotes (at least in the heart), the dominant inheritance is likely to be due to haploinsufficiency, where the normal protein product of the wild-type allele does not reach the threshold level necessary for normal function.

The pathogenetic mechanism underlying the disease is still unclear. An unsolved issue is whether the LAMP-2 protein deficiency might cause structural or functional lysosomal impairment. One hypothesis is that the lysosomal membrane could be structurally normal but that abnormal LAMP-2 function might cause increased lysosomal storage, which, in turn, could trigger the rupture of membrane with the consequent release of acidic hydrolases into the sarcoplasm.

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