Multisystemic LAMP-2 defect in Danon disease

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Abstract

Danon disease is an X-linked dominant disorder due to mutations in the *LAMP2* gene, presenting 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 9 unrelated patients with hypertrophic cardiomyopathy and vacuolar myopathy. We identified 3 novel families 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 indicating that the biochemical diagnosis can be obtained on leukocytes and might be used for screening in male patients, and that the multiorgan protein deficiency would explain the multisystem clinical involvement.

In our female patient, muscle histopathology and LAMP-2 protein analysis was inconclusive, indicating that the diagnosis in females can be obtained only by mutation identification.

Key words: LAMP-2, Danon disease, hypertrophic cardiomyopathy, vacuolar myopathy.

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" [5] because the pathologic 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. By 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 [21]. In female patients, the disease predominantly involves the cardiac muscle and it 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 [19]. 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 [15]. LAMP-2a is distributed rather ubiquitously, while LAMP-2b is expressed predominantly in striated muscle and brain. By analogy with *LAMP2* knock-out mouse, other tissues 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 [3, 8, 12] and constitute a significant fraction of the total lysosomal membrane proteins. Though LAMP-2 and LAMP-1 have many structural and biochemical similarities, they differ in the expression level in skeletal muscle and they might have different roles in lysosomes [19,7,24]. 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 intra-luminal head, a transmembrane domain, and a small cytoplasmic tail containing a lysosomal membrane-targeting signal [11]. LAMP-2 is supposed to be involved both in the fusion

of lysosomes and with other membranes and in the maturation of autophagic vacuoles [24], and act as a receptor for proteins to be imported and degraded within lysosomes in chaperone-mediated autophagy [7, 4].

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 level, 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 tissues other than striated muscle, because the defect of LAMP-2 in

various cell types would explain the multisystem involvement observed at the clinical level, and could provide an easier and less invasive diagnostic tool in Danon disease.

Material and Methods

Patients

Our skeletal muscle biopsy tissue bank, which contains more than 6,000 specimens, was surveyed for patients affected with an unidentified form of vacuolar myopathy associated with hypertrophic cardio-myopathy. A total of 9 skeletal muscle biopsies matched our selection criteria and were selected for the screening of both LAMP-2 protein deficiency (by immuno-histochemical and western blot analysis) and *LAMP2* gene mutations. Three patients, in whom *LAMP2* gene mutations were identified, were the objects of the present study. Clinical data are summarized in Table 1.

Table 1. CLINICAL AND MOLECULAR DATA

Pt., sex	Family history	Age and symptoms at muscle onset	Age and muscle involvement at last examination	CK * level (U/L)	Mental Retar-dation	LAMP2 gene mutation	Skeletal muscle pathology
1, M	+	20, easy fatigability	22, marked trunk and limb atrophy, severe girdle muscle weakness	283	+	796-797insC	Diffuse atrophy and vacuoles
2, M	+	22, difficulty in climbing stairs	22, diffuse hypotrophy, waddling gait, Gowers' sign	558	-	680-701del	Vacuoles in many fibres
3, M°	+	18, easy fatigability	30, waddling gait, Gowers' sign, moderate neck, facial, distal limb weakness	1094	+	294G>A; W98X	Vacuoles in many fibres
4, F°	+	26, easy fatigability	54, myalgia, mild muscle weakness and distal atrophy	normal	-	294G>A; W98X	No vacuoles

*° : relatives (pt. 4 is the mother of pt. 3). * CK: Creatine Kinase (normal values 0-190 U/L).*

Skeletal muscle histopathology and immunohisto-chemistry

Sections of skeletal muscle biopsies from patients and controls were routinely stained to evaluate overall muscle morphology or used for immunohistochemistry. To study the plasma membrane we used antibodies against caveolin-3 (Transduction Lab., Lexington, KY). Sections were incubated for 1 hour with primary antibodies (diluted 1:100). After washes in PBS, sections were incubated for 30 minutes with anti-mouse cy-3 conjugated Ig (1:100) (Caltag, Burlingame, UK), and examined by fluorescence microscopy.

LAMP-2 Western blot analysis

Skeletal muscle biopsies, leukocytes and skin fibroblasts were dissolved in electrophoresis-loading buffer and processed as described [9] using LAMP-2

antibody (H4B4 luminal domain, Developmental Studies Hybridoma Bank, Iowa City) diluted 1:200. The protein quantity of each sample was normalised 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 analysis

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 #AC002476.1). PCR

reactions were performed under standard conditions. PCR products were purified by enzyme reaction (ExoSap-I, Amersham, UK) and directly sequenced using the Big Dye dideoxy-terminator cycle sequencing kit and the 377 ABI-PRISM automated sequencer, at the CRIBI Biotechnology Centre, University of Padova.

Results

Patients

Of the 9 male patients who showed vacuolar myopathy and hypertrophic cardiomyopathy and who were screened for both LAMP-2 protein defect and *LAMP2* gene mutations, 3 cases resulted 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). Though all 3 patients had a positive family history, in only one family (family 3) the mother of a male patient (pt. 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 dyspnoea and easy fatigability. All patients had WPW syndrome, supraventricular arrhythmias or atrial fibrillation. In 2 patients, implanted cardioverter defribillator was implanted for ventricular tachycardia or fibrillation. Echocardiography showed concentric left ventricular hypertrophy in pts. 1 and 2, and asymmetric hypertrophy with a moderate left ventricular maximal hypertrophy in pt. 3. Three of the 4 patients developed severe and progressive heart failure associated with end-stage systolic dysfunction; 2 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 2 male patients. Hepatic involvement, which was present in all patients, had been clinically evident since childhood in 2 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 muscle histopathology and immuno-histochemistry

Skeletal muscle histopathology showed extensive muscle fibre vacuolisation and degeneration, and focal storage of PAS-positive material. The degree of fibre vacuolisation and of overall muscle architectural derangement was variable in the 3 male patients (Figure 1): pt. 1 had generalized fibre atrophy and degeneration;

vacuoles, which were sometimes large enough to replace all the cytoplasm, were seen in the majority of fibres (46%); pts. 2 and 3 had multiple and relatively small-sized vacuoles that were seen in a smaller proportion of fibres (24%). Female pt. 4 had no fibre vacuolisation. The accumulation of PAS-positive material and lysosomal acid phosphatase reaction were evident in small vacuoles of fibres undergoing degeneration.
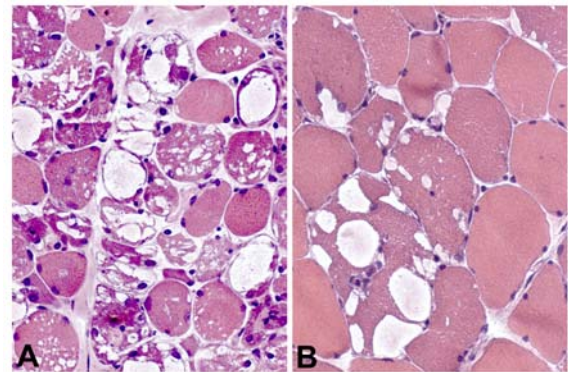
Figure 1. *Skeletal muscle biopsy sections stained for Hematoxylin-Eosin showing different degrees of muscle fibres involvement in 2 different patients: pt. 1 (A) had diffuse fibre atrophy and extensive fibre vacuolisation and degeneration; pt. 3 (B) showed a milder degree of vacuolisation and many fibres with apparently normal features. Microscope magnification x 200.*

Immunofluorescence analysis on skeletal muscle biopsy from the male patients showed a complete absence of LAMP-2 protein when compared to controls in which there was diffuse intracytoplasmic reaction. Conversely, in female pt. 4, LAMP-2 immunolabelling was of intensity similar to controls. Lysosomal and vacuolar membranes were often in continuity with the plasma membrane (caveolin-3) but not with the nuclear membrane.

LAMP-2 western blot analysis

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

A weak binding cross-reaction between these two proteins or between LAMP-2 and some unknown immunoreactive material cannot be ruled out.

LAMP2 gene mutation analysis

LAMP2 gene mutations were identified in all 3 male patients who showed LAMP-2 protein deficiency, as

well as in the heterozygote female pt. 4. Each patient showed a different and previously unreported mutation (Table 1). All three mutations produce null alleles (nonsense or frame-shifting mutations), which are predicted to prematurely truncate protein synthesis and result in the loss of the transmembrane domain.

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 were identified [1, 2, 14, 16-23, 25]. 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 9 patients with vacuolar myopathy and hypertrophic cardiomyopathy led to the identification of 3 novel families with Danon disease. Though 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 [19], but Danon disease could be diagnosed by LAMP-2 immuno-fluorescence even from the very small tissue samples that are collected during heart catheterization. The search for LAMP-2 deficiency in endomyocardial 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 at both clinical level (therapy and prognosis) and genetic counselling.

In all our male patients we demonstrated a generalized LAMP-2 protein deficiency, which was detected in striated muscle, in fibroblasts and 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 [1]. 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 and [2, 16-22, 25]); 2) it should have high specificity because there are no reports of LAMP-2 protein deficiency in other disorders; 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 indeed a multisystemic disorder [21, 16]. Accurate clinical history collection revealed that hepatic involvement was present in all our patients (in 2 cases it was the first clinical sign), indicating that also in the human disease, as in *LAMP2* knockout mouse [20, 24], different organs other than striated muscles may suffer from LAMP-2 deficiency [21, 16].

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 most prominent histopathological feature of Danon disease is the vacuolisation of muscle fibres. We confirm that the extent of these changes is related to the degree of clinical muscle involvement [22], suggesting that the accumulation of autophagic material within muscle fibres 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 due to their function as a mechanical reinforcement [6].

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, mutant LAMP-2 proteins could either be degraded or their synthesis could be abolished by the nonsense mediated RNA decay mechanism (NMD) in the nucleus [10, 13, 26]. Though the demonstration that NMD 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.

Concluding remarks

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 LAMP-2 abnormal 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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