Identification of LAMP2 Mutations in Early-Onset Danon Disease With Hypertrophic Cardiomyopathy by Targeted Next-Generation Sequencing

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Danon disease is an X-linked disorder with the clinical triad of cardiomyopathy, skeletal myopathy, and mental retardation. Early diagnosis of this disease remains a challenge, especially in the pediatric population. In this study, we developed a targeted panel-based next generation sequencing pipeline to identify mutations by sequencing of selected candidate genes in 136 pediatric patients with either hypertrophic cardiomyopathy (HC) or idiopathic dilated cardiomyopathy (IDC). This led to the identification of lysosome-associated membrane protein 2 (LAMP2) mutations in 4 of the 64 (6%) probands with HC, including 3 novel nonsense mutations (p.Q240X, p.S250X, and p.G22X). No LAMP2 mutation was detected in the other 72 probands with IDC. All 4 probands and one additional affected family member (2 men and 3 women) had an early-onset age and presented either HC alone or combined with Wolff-Parkinson-White syndrome and skeletal myopathy. Immunofluorescence staining and Western blot analysis revealed absent LAMP2 expression in both cardiac and skeletal muscle samples of the first proband and severely decreased LAMP2 expression in the skeletal muscle samples of the second proband. In conclusion, cardiomyopathy in the patients with Danon disease may occur during early childhood and tend to be HC rather than IDC in both affected men and women. Therefore, Danon disease should be considered as one of the leading causes of unexplained ventricular hypertrophy in pediatric patients. The inclusion of LAMP2 gene in cardiomyopathy genetic screening panels may contribute to early diagnosis of Danon disease. © 2016 Elsevier Inc. All rights reserved. (Am J Cardiol 2016;118:888–894)

Danon disease (OMIM# 300257) is a rare X-linked dominant disease associated with the clinical triad of cardiomyopathy, skeletal myopathy, and mental retardation, which is caused by mutations in the lysosome-associated membrane protein 2 (LAMP2) gene located at Xq24.¹² Carcidual involvement is very common and is the most important cause of death in Danon disease.³ Early diagnosis is crucial for improving prognosis of the disease, but it may be difficult in the pediatric population where symptoms are often nonspecific. Genetic testing is now increasingly used as a means to confirm the specific diagnosis of cardiomyopathy but remains a challenge because of the large number of causative genes involved in its pathogenesis. One possible approach that has yet to be widely implemented is the use of high-throughput DNA sequencing techniques offered by next generation sequencing (NGS). In the present study, we developed a targeted panel-based NGS pipeline to identify mutations by sequencing of selected candidate genes in a large cohort of pediatric patients with either hypertrophic cardiomyopathy (HC) or idiopathic dilated cardiomyopathy (IDC). This led to the identification of LAMP2 mutations in 4 of the 64 (6%) probands with HC, including 3 novel nonsense mutations (p.Q240X, p.S250X, and p.G22X).

Methods

This study was approved by the Medical Ethics Committee of Shanghai Children’s Medical Center and complied with the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from the patient or legally responsible guardian.

During the period from 2012 to 2015, all patients with a clinical diagnosis of HC or IDC who were referred to our institution were enrolled in this study. IDC was defined as left ventricular ejection fraction <45% and left ventricular...
end-diastolic dimension >2 SDs above the normal mean for body surface area in the absence of known causes such as ischemic heart disease, primary valvular heart disease, or inflammatory cardiomyopathy; and HC was defined as left ventricular posterior and/or septal wall thickness >2 SDs above the normal mean for body surface area in the absence of an identifiable hemodynamic cause such as hypertension, congenital heart disease, or exposure to drugs known to cause cardiac hypertrophy.\(^5\) All patients were evaluated by a clinical history, physical examination, electrocardiography, and serum biochemical analyses. When available, cardiac magnetic resonance imaging was used to capture variants of 62 genes implicated in the causation of cardiomyopathy (Supplementary Table 1). All the variations were classified as benign or likely benign were not pursued for validation by Sanger sequencing. NM_002294.2 was used as the reference sequence for the coding regions of the LAMP2 gene. Cryostat sections of 8-μm thickness were used for routine histological and histochemical studies, including hematoxylin-eosin, Gomori modified trichrome, nicotinamide adenine dinucleotide-tetrazolium reductase, succinate dehydrogenase, adenosine triphosphatase, cytochrome c oxidase, and periodic acid-Schiff staining. Mouse monoclonal antibodies against dystrophin (DYS1: Rod domain and DYS3: N-terminus; Novocastra, Newcastle upon Tyne, United Kingdom), dysferlin (NCL-Hamlet; Novocastra), and sarcoglycan complex (NCL-g-SARC, NCL-a-SARC, NCL-b-SARC, NCL-d-SARC; Novocastra) were used in the immunohistochemical staining. The LAMP2 antibody (H4B4; Abcam, Cambridge, United Kingdom) was used for further immunofluorescence study.

Western blot was performed on muscle homogenates as described.\(^6\) Briefly, muscle specimens (30 mg of wet weight for each) were homogenized in 20 volumes of lysis buffer and boiled at 100°C for 5 minutes. After centrifugation, the supernatant was subjected to SDS-PAGE, followed by transfer to nitrocellulose membrane. Mouse monoclonal antibodies against LAMP2 and glyceraldehyde-3-phosphate dehydrogenase were used as primary antibodies.

### Results

A total of 136 unrelated Chinese pediatric patients clinically diagnosed with HC or IDC were enrolled in this study. Among the index cases, there were 64 patients with HC (39 men and 25 women; age at diagnosis: 2 months to 14 years), and 72 patients with IDC (44 men and 28 women). A total of 136 unrelated Chinese pediatric patients clinically diagnosed with HC or IDC were enrolled in this study. Among the index cases, there were 64 patients with HC (39 men and 25 women; age at diagnosis: 2 months to 14 years), and 72 patients with IDC (44 men and 28 women).
women; age at diagnosis: 3 months to 15 years). We performed targeted NGS on all these patients and identified 3 novel and one previously reported *LAMP2* nonsense mutations in 4 of 64 probands with HC. No *LAMP2* mutation was detected in the other 72 probands with IDC. The results were validated by Sanger sequencing in probands and family members. The clinical features and laboratory test results pertinent to the 4 probands and their family members

Figure 1. Pedigree and genetic analysis of family 1. Sanger sequencing showed that the proband (II: 1) was hemizygous for a novel mutation (c.718 C > T, p.Q240X) in exon 5 of the *LAMP2* gene; his mother (I: 1) and sister (II: 2) were heterozygous for the mutation; and his father (I: 2) did not present the mutation. *Diagonal symbols* indicate affected subjects; *white symbol* indicates unaffected subject. The proband is indicated by an *arrow*.

Figure 2. Patient DD1. (A) 12-lead electrocardiogram showing sinus rhythm for the Wolff-Parkinson-White pattern and LVH. *(B,C)* Parasternal long-axis view and M-mode echocardiography showed left ventricular hypertrophy (septum 19.4 mm, posterior wall 16.5 mm) with dilatation of the left ventricle (57.2 mm at end diastole) and severe reduction of left ventricular ejection fraction (22%).
are described in the following section and summarized in Table 1.

**Family 1:** The proband (DD1 in Table 1; II: 1 in Figure 1) in this family was a 13-year-old man who presented with a 3-month history of short of breath and edema of lower extremities. He was born of an uneventful full-term pregnancy and apparently had normal growth and developmental milestones. On admission, he presented with mild skeletal weakness but no learning difficulties. Laboratory tests showed elevated creatine kinase and alanine aminotransferase. The aminoterminal probrain natriuretic peptide was strikingly elevated at 18,759 ng/L. His electrocardiography revealed Wolff-Parkinson-White pattern and left ventricular hypertrophy (LVH; Figure 2). Echocardiography revealed LVH with dilatation of the left ventricle and severe systolic dysfunction, a finding compatible with the end-stage phase of HC (Figure 2). The patient died of progressive heart failure 6 months after his initial diagnosis despite maximal medical management and treatment of the condition.

In this proband, we identified a hemizygous c.718 C > T mutation in exon 5 of the *LAMP2* gene, resulting in a premature stop codon (p.Q240X). His mother (I: 1 in Figure 1) carried the same mutation but had no overt clinical symptoms. She also presented with a normal electrocardiography and echocardiography. The 2-year-old sister (DD2 in Table 1, II: 2 in Figure 1) of the proband was found to be heterozygous for the same mutation. She did not exhibit skeletal myopathy or mental retardation. Her serum creatine kinase was slightly elevated. Electrocardiography revealed LVH without ventricular preexcitation. Echocardiogram demonstrated mild LVH, but there was no evidence of left ventricular outflow tract obstruction.

**Family 2:** The proband (DD3 in Table 1) was a 10-year-old woman, presenting a 4-month history of exertional dyspnea and easy fatigability. She was born uneventfully, had normal growth, and did not exhibit skeletal myopathy or mental retardation. Serum creatine kinase was slightly elevated. The aminoterminal probrain natriuretic peptide was elevated at 2,855 ng/L. Her electrocardiography showed markedly increased voltages with a deeply inverted T-wave in both standard lead and left precordial lead, but no ventricular preexcitation was observed. Cardiac ultrasound demonstrated severe LVH and left ventricular outflow tract obstruction, with a peak instantaneous gradient of 50 mm Hg. Her cardiac magnetic resonance imaging also showed LVH with patchy late gadolinium enhancement (Figure 3).

In this proband, we identified a novel heterozygous c.749 C > G mutation in exon 6 of the *LAMP2* gene, resulting in a premature stop codon (p.S250X). This mutation was not found in her healthy parents, indicating that the mutation had arisen de novo.

**Family 3:** The proband (DD4 in Table 1) was a 3-month-old man who was diagnosed with HC after the detection of a heart murmur. He was born of an uneventful full-term pregnancy and had normal growth. In addition, there was no documented muscle weakness in the limbs or mental retardation. Blood tests revealed elevated creatine kinase and alanine aminotransferase. His electrocardiography revealed LVH without ventricular preexcitation. Echocardiography exhibited severe LVH but no left ventricular outflow tract obstruction at the time of initial diagnosis. At present, he is 17-month-old and remains asymptomatic with normal developmental milestones; however, his echocardiogram demonstrated left ventricular outflow tract obstruction with a peak instantaneous gradient of 66 mm Hg.

In this proband, we identified a reported hemizygous c.467 T > G mutation in exon 4 of the *LAMP2* gene, resulting in a premature stop codon (p.L156X). Neither of his healthy parents carried this mutation. Therefore, it is considered a de novo mutation.

**Family 4:** The proband (DD5 in Table 1) was a 2-year-old woman who was diagnosed with HC after the detection of a heart murmur. She was born of an uneventful full-term pregnancy and apparently had normal growth and developmental milestones. On admission, she presented with mild skeletal weakness but no learning difficulties. Laboratory tests showed elevated creatine kinase and alanine aminotransferase. The aminoterminal probrain natriuretic peptide was strikingly elevated at 18,759 ng/L. Her electrocardiography revealed Wolff-Parkinson-White pattern and left ventricular hypertrophy (LVH; Figure 2). Echocardiography revealed LVH with dilatation of the left ventricle and severe systolic dysfunction, a finding compatible with the end-stage phase of HC (Figure 2). The patient died of progressive heart failure 6 months after his initial diagnosis despite maximal medical management and treatment of the condition.

In this proband, we identified a reported hemizygous c.467 T > G mutation in exon 4 of the *LAMP2* gene, resulting in a premature stop codon (p.L156X). Neither of his healthy parents carried this mutation. Therefore, it is considered a de novo mutation.
pregnancy and had normal growth. A neurological examination revealed normal muscle strength and normal deep tendon reflexes. No mental retardation was observed. Serum creatine kinase was slightly elevated. Her electrocardiography revealed LVH and Wolff-Parkinson-White pattern. Echocardiogram demonstrated severe LVH and left ventricular outflow tract obstruction, with a peak instantaneous gradient of 42 mm Hg. At present, she is 3-year-old and remains asymptomatic.

In this proband, we identified a novel heterozygous c.64 G > T variant in exon 1 of the LAMP2 gene, resulting in a premature stop codon (p.G22X). This mutation was not
Muscle biopsies were performed in the biceps brachii and heart within 2 hours after death for patient DD1. The skeletal muscle biopsy showed mild variation in fiber size and a few cytoplasmic vacuoles with positive reactions to antisarcosomal protein staining. The cardiac biopsy of the patient showed hypertrophic cardiomyocytes, severe interstitial fibrosis with small cytoplasmic vacuoles in several fibers. Immunohistochemical staining with dystrophin and sarcoglycan antibodies disclosed increased immunoactivity on the rim of the small vacuoles. Immunofluorescence staining (Figure 4) and Western blot analysis (Figure 5) revealed a complete absence of LAMP2 expression in both cardiac and skeletal muscle samples.

For patient DD3, a muscle biopsy of the biceps brachii showed a slight variation in fiber size with no visible cytoplasmic vacuoles. Immunohistochemical studies with antibodies against dystrophin and sarcoglycan antibodies were normal. Immunofluorescence staining (Figure 4) and Western blot analysis (Figure 5) showed a severe reduction of LAMP2 in her skeletal muscle samples.

**Discussion**

Danon disease is an X-linked dominant disorder predominantly affecting cardiac muscle. HC is the most commonly described cardiac phenotype in affected men, whereas the IDC phenotype has been less widely described in male patients and may account for roughly 10% of cases. Cardiac involvement in female carriers presents with a broad spectrum of clinical features. Previously, the IDC phenotype was considered more prevalent in affected women; however, a recent registry study revealed nearly equal rates of IDC versus HC in women. In the present study, we evaluated the prevalence of LAMP2 mutation among a large cohort of pediatric patients with HC and identified mutations in 4 index patients, which included 2 men and 2 women. The prevalence in the total pediatric HC population was 6% (4 of 64) of all index cases in this study, which was higher than 4% reported in another pediatric HC group by Yang et al. Moreover, we evaluated the prevalence of LAMP2 mutation among a large cohort of pediatric patients with IDC, but no LAMP2 mutation was found in the 72 patients with IDC. Our observation suggests that HC appears to be the more common cardiac phenotype in children with Danon disease not only in hemizygous men but also in heterozygous women.

The age at presentation of Danon disease can range from infancy to the second decade in men, whereas female carriers of LAMP2 mutations most often present with late-onset cardiomyopathy and slow disease progress. Until now, only a few female carriers have been described with childhood onset and all of them presented as HC (Table 2). In the present study, we identified a LAMP2 mutation in 3 female patients with early-onset HC, which were much younger than previously reported female cases.

The mechanism underlying the early-onset cardiomyopathy in children with Danon disease is not well understood but could be related to a variable genotype-phenotype relation of different mutations. D’Souza et al. suggested that nonsense, frameshift and large deletion/duplication mutations were associated with an early onset of Danon disease, whereas splicing and missense mutations showed a trend of a later disease onset. In the present study, we identified 3 novel and one previously reported LAMP2 mutations in 4 unrelated probands with early-onset HC. All these mutations were predicted to truncate the LAMP2 protein, resulting in loss of the transmembrane and cytoplasmic domains and likely disabling its function as a lysosomal membrane protein. It is therefore likely that truncating mutations tend to cause an early disease onset and more severe phenotype, which is consistent with the findings by D’Souza et al.

Although men are usually severely affected, clinical presentation in female patients with Danon disease may be more variable, ranging from asymptomatic to equally as severe as in male patients. In the first family of this study, the sister of the proband (DD2 in Table 1, II: 2 in Figure 1) presented ventricular hypertrophy as early as 2 years old. Her mother (I: 1 in Figure 1) carried the same c.718 C > T mutation but with no overt clinical symptoms, as well as a normal electrocardiography and echocardiography. The wide spectrum of clinical manifestations in heterozygous women is not clear but might originate from a different X chromosome inactivation pattern, and skewed X chromosome inactivation favoring expression of the LAMP2-mutated X chromosome might account for severe early-onset cardiomyopathy in women with Danon disease.

The early diagnosis of Danon disease is important but remains a challenge in the pediatric population. The cardiac involvement associated with a high level of creatine kinase is a constant feature in male patients. Therefore, the search for Danon disease in patients with HC should be considered, especially when it is associated with unexplained elevation of serum creatine kinase. Ventricular preexcitation on electrocardiography is a common observation in patients with Danon disease. However, the absence of preexcitation in 3 of the 5 patients in our series suggests that electrocardiography would not be a reliable tool alone in predicting Danon disease. Moreover, the potential for de novo mutation in the LAMP2 gene, as demonstrated in 3 of 4 probands in this study, indicated that a lack of familial history should not rule out the presence of Danon disease.
Compared with male patients, female patients with Danon disease have a lower incidence of skeletal muscle and cognitive impairment although cardiac problems are a common manifestation in affected women. In the absence of a family history, it may be a challenge for early clinical diagnosis in female patients. In the present study, no women had clinically significant neurologic disease, and none had overt skeletal muscle weakness or myopathic symptoms although serum creatine kinase and hepatic enzyme levels were normal to slightly elevated. It may be difficult to distinguish Danon disease from sarcomere-associated HC in these patients based on clinical presentations alone.

The diagnosis of Danon disease is usually based on the demonstration of LAMP2 protein deficiency in skeletal or cardiac muscle and/or the identification of LAMP2 gene mutations. However, muscle biopsies are not routinely recommended in patients with cardiomyopathy and might be nonspecific in some female patients. Therefore, it is conceivable that a number of patients with Danon disease might miss the diagnosis unless they obtain mutation identification. The present study demonstrates that target NGS provides a novel, rapid, simple, and highly sensitive screening method for the early detection of Danon disease.

Supplementary Data
Supplementary associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ajmcard.2016.06.037.

Disclosures
The authors have no conflicts of interest to disclose.


Table 2
LAMP2 mutations identified in this study and previous studies in childhood-onset female patients with Danon disease and HC phenotype

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at onset (years)</th>
<th>Clinical presentation</th>
<th>Nucleotide Change</th>
<th>Amino acid Alteration</th>
<th>Other features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>No symptoms</td>
<td>c.1075 C&gt;T</td>
<td>p.Q359X</td>
<td>No</td>
<td>Yang et al</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>Heart murmur</td>
<td>IVS6–2 A&gt;G</td>
<td>Nonsense decay</td>
<td>No</td>
<td>Maron et al</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>Unexplained elevation of serum creatinine kinase</td>
<td>c.241 delG</td>
<td>p.D81fsX7</td>
<td>Skeletal myopathy</td>
<td>Kim et al</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>Near-syncopal episode, palpitations and shortness of breath</td>
<td>Unspecified</td>
<td>Unspecified</td>
<td>Persistent ventricular tachycardia</td>
<td>Dara et al</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>Transient palpitations</td>
<td>c.294 G&gt;A</td>
<td>p.W98X</td>
<td>Retinal microhemorrhages</td>
<td>Miani et al</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>Heart murmur, palpitations</td>
<td>c.973 delC</td>
<td>p.L325fsX21</td>
<td>No</td>
<td>Hedberg Oldfors et al</td>
</tr>
<tr>
<td>7</td>
<td>13</td>
<td>Palpitations, chest discomfort and breathlessness on physical exertion</td>
<td>c.865-3 C&gt;G</td>
<td>p.K289fsX36</td>
<td>No</td>
<td>Hedberg Oldfors et al</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>No symptoms</td>
<td>c.718 C&gt;T</td>
<td>p.Q240X</td>
<td>No</td>
<td>This study</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>Exertional dyspnea</td>
<td>c.749 C&gt;G</td>
<td>p.S250X</td>
<td>No</td>
<td>This study</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>Heart murmur</td>
<td>c.64 G&gt;T</td>
<td>p.G22X</td>
<td>No</td>
<td>This study</td>
</tr>
</tbody>
</table>

LAMP2 = lysosome-associated membrane protein 2; HC = hypertrophic cardiomyopathy.