



Original article

Early diagnosis of Danon disease: Flow cytometric detection of lysosome-associated membrane protein-2-negative leukocytes



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ABSTRACT

Introduction: Danon disease is an extremely rare X-linked dominant disorder characterized by progressive cardiomyopathy, muscle weakness, and mild mental retardation. Most cases harbor nonsense, frameshift, or splice-site mutations in *LAMP2* that result in lysosome-associated membrane protein-2 (*LAMP-2*) deficiency and lysosomal defects. The identification of *LAMP2* mutations makes it possible to detect female carriers with significant cardiomyopathy. Therefore, it is of paramount importance to develop useful carrier detection methods.

Methods: To screen for diminished *LAMP-2* expression among female patients with progressive cardiomyopathy, we developed a flow cytometric method to detect *LAMP-2*-deficient leukocytes.

Results: In healthy controls, all circulating leukocyte populations, including granulocytes, monocytes, and lymphocytes, expressed significant levels of *LAMP-2*. In contrast, cells from a male patient with Danon disease lacked detectable *LAMP-2*. His younger twin sisters showed reduced levels of *LAMP-2* expression with characteristic bimodal fluorescence intensity patterns. The percentage of *LAMP-2*-negative cells in the asymptomatic sibling was nearly the same as that in the symptomatic sibling.

Conclusion: We developed a flow cytometric assay for *LAMP-2* expression that can serve as a rapid primary screening method to detect carriers of *LAMP-2* deficiencies. This assay will narrow the target population before subjecting patients to more laborious and expensive gene mutation analysis.

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Introduction

Danon disease, an X-linked cardioskeletal myopathy, was originally reported as “lysosomal glycogen storage disease with normal acid maltase” by Danon et al. in 1981 [1]. The first two cases were unrelated young boys, and muscle biopsy led to the diagnosis of characteristic vacuolar myopathy in each case. In 2000, Nishino et al. reported that mutations in *LAMP2*, which resides on chromosome Xq24 and encodes the lysosome-associated membrane protein-2 (*LAMP-2*), are responsible for the primary defect in Danon disease [2]. *LAMP-2*, first identified in 1983, is a lysosomal membrane glycoprotein. It is critical for the process of autophagy, which involves lysosomal fusion to the autophagosome, maturation of autophagic vacuoles, and chaperon-mediated protein transport to

lysosomes. Autophagy is a cytoprotective pathway that eukaryotic cells use to degrade and recycle cytoplasmic contents and prevent starvation [3]. Under normal conditions, autophagy represents an important homeostatic mechanism for the maintenance of normal cardiovascular function and morphology [4]. Impaired autophagy leads to cardiac hypertrophy in *LAMP-2*-deficient mice [5].

Danon disease, which is clinically characterized by the triad of cardiomyopathy, skeletal myopathy, and mental retardation, exhibits a large spectrum of clinical characteristics. Dilated cardiomyopathy can coexist with hypertrophic cardiomyopathy within a family whose members have Danon disease. The clinical presentation of Danon disease is always more severe in males than in females because of its X-linked dominance [6]. In male patients, the clinical course is progressive and may lead to premature death from arrhythmia or severe heart failure by the end of the second or third decade of life. In affected females, the disorder sets in later than it does in males and predominantly involves the cardiac muscle. However, the cardiac phenotype can be severe even in some females [7,8]. Even among female patients, heart transplantation is the only effective treatment for advanced heart failure [9,10].

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A case of a symptomatic young female patient was recently reported [11]. Her family members were all healthy and did not show any laboratory abnormalities. The patient herself had a de novo mutation in *LAMP2*. Other family cases indicative of germline mutations have been reported [12]. Therefore, the absence of familial history should not exclude a diagnosis of Danon disease. Two studies strongly suggest that Danon disease occurs more frequently than has been reported. Arad et al. detected *LAMP2* mutations in 2 of 35 patients with hypertrophic cardiomyopathy [13]. Yang et al. identified *LAMP2* nonsense mutations in 2 of 50 pediatric hypertrophic cardiomyopathy cases [14]. Therefore, a diagnosis of Danon disease should be considered whenever a patient with unexplained ventricular hypertrophy is encountered.

The ability to diagnose Danon disease during its earliest stages is key to determine the most appropriate therapeutic intervention,

preventing sudden cardiac death, and improving patient prognosis. Broad diagnostic screening using a simple and easy method is necessary to diagnose more patients. Assaying *LAMP2* expression in the peripheral leukocytes of patients with Danon disease is minimally invasive and beneficial [15–17], but this method has not yet been adapted as a screening test for asymptomatic patients.

In the present study, we describe a family with Danon disease that has no apparent inheritance. We employed immunohistochemical and flow cytometric analysis to detect *LAMP2* expression in the peripheral leukocytes of all family members and identified two preclinical heterozygous females (preclinical female carriers). To the best of our knowledge, this is the first report of fluorescence-activated cell sorting (FACS) detection of *LAMP2*-negative leukocytes being used to screen for Danon disease in females. FACS analysis of leukocytes can contribute to

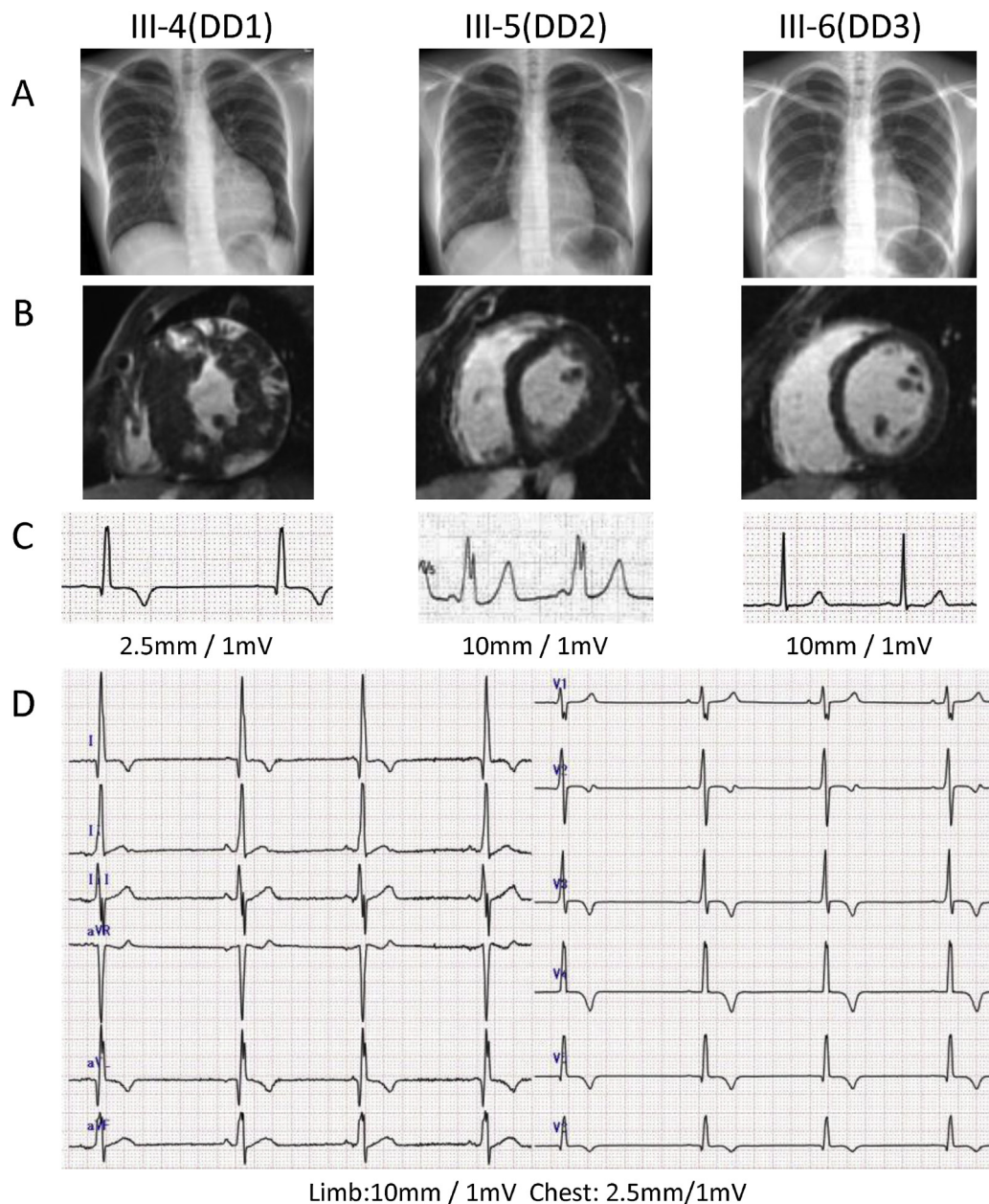


Fig. 1. Images of patients. (A) Chest X-ray. Enlargement of a central shadow is observed in DD1. (B) A short-axis view of gadolinium-enhanced cardiac magnetic resonance imaging. Images of DD1 show marked hypertrophy of the left ventricular wall and late gadolinium enhancement below the epithelium. Images of DD2 show milder hypertrophy than DD1 and no late gadolinium enhancement. Neither hypertrophy nor late gadolinium enhancement was observed on images of DD3. (C) V5-lead from a resting 12-lead electrocardiogram (ECG). ECG of DD1 shows prominent voltage and a giant negative T wave, whereas that of DD2 shows a short PR interval. DD3 has no ECG abnormalities. (D) Full resting 12-lead ECG of DD1.

Table 1

Clinical findings of patients. LVPWD, left ventricular end diastolic posterior wall dimension; IVSD, interventricular septal end diastolic dimension; ECG, electrocardiography; SVT, supraventricular tachycardia; WPW syndrome, Wolff–Parkinson–White syndrome; AVB, atrioventricular block; CK, creatine kinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; BNP, brain natriuretic peptide.

	DD1 (III-4)	DD2 (III-5)	DD3 (III-6)	Control (n=8)
Sex	Male	Female	Female	4 males/4 females
Subjective symptom	General fatigue	Palpitation	None	None
Age at diagnosis	14 years	11 years	11 years	10–14 years
Diastolic LVPWD (mm)	25.8	12.1	6.9	7.0 ± 0.3
Diastolic IVSD (mm)	21.7	10.8	7.0	6.7 ± 0.4
ECG abnormality	Giant negative T wave SVT	WPW syndrome AVB(II)	None	None
Muscle weakness	Upper extremities	None	None	None
Mental retardation	None	None	None	None
CK (IU/l)	567	156	125	134 ± 47
AST (IU/l)	185	67	19	24 ± 5
ALT (IU/l)	150	27	13	12 ± 4
LDH (IU/l)	924	516	179	199 ± 25
BNP (pg/ml)	439.5	22.6	12.2	4.9 ± 4

the early diagnosis of Danon disease not only in homozygous males but also in heterozygous females.

Materials and methods

Study subjects

A 13-year-old Japanese boy (proband DD1) was referred to our hospital in September 2008 with chronic jaundice and elevated liver enzyme levels. Three years earlier, elevated levels of transaminases [serum aspartate aminotransferase (AST)/alanine aminotransferase (ALT)] and creatine kinase (CK) had been incidentally detected during routine examinations. The enzyme levels did not return to normal; in fact, they had increased by July 2008. Physical examination revealed muscle weakness in the upper extremities and mild mental retardation. A systolic ejection murmur was heard at the second left interspace; therefore, we performed cardiac investigations. His chest X-ray revealed mild cardiomegaly (Fig. 1A), whereas ultrasound cardiography (UCG) revealed marked hypertrophy in his ventricular septum and left ventricular wall without outflow obstruction. The end-diastolic left ventricular dimension was 29.1 mm. Systolic and diastolic functions were almost normal. Magnetic resonance imaging (MRI) also revealed cardiac hypertrophy with late gadolinium enhancement (Fig. 1B). Electrocardiography (ECG) demonstrated distinct giant negative T waves in leads V3–6 and prominent voltages (Fig. 1C and D). Danon disease was suspected because of the following characteristic symptoms: unexplained left ventricular hypertrophy with a giant negative T wave, elevated CK and transaminase levels, and muscle weakness in the upper extremities (Table 1).

The family pedigree of DD1 is shown in Fig. 2. No parental consanguinity was present. To the best of our knowledge, no other family members showed symptoms of Danon disease at the time of initial DD1 presentation. To determine penetrance, we evaluated the parents, sisters, and the maternal uncle of DD1. We also investigated 8 healthy individuals (4 males and 4 females) with no signs of specific cardiac or systemic disorders, as controls.

The Human Research Committee of Kanazawa University Graduate School of Medical Science approved this study (No. 119), and informed consent was obtained from subjects according to the Declaration of Helsinki.

Cardiac function and laboratory data

All patients and controls underwent physical examinations, chest radiography, ECG, and UCG. Left ventricular size and function were evaluated by M-mode and 2-dimensional Doppler and color

Doppler imaging. Some patients were subjected to MRI studies. Serum levels of transaminases (AST/ALT), lactate dehydrogenase (LDH), CK, and brain natriuretic peptide were also determined.

Genetic analysis

Genomic DNA was extracted from whole blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Primers were designed to amplify all coding exons of *LAMP2*, including adjacent exon–intron boundaries [2]. Polymerase chain reaction (PCR) fragments were purified using the QIAquick PCR purification kit (Qiagen). Sequences were determined in the forward and reverse directions using BigDye[®] Terminator v3.1 and v1.1 cycle sequencing kits (Applied Biosystems, Foster City, CA, USA).

Immunohistological detection of LAMP-2

Peripheral blood mononuclear cells (PBMCs) were isolated from patients and normal controls by Ficoll–Hypaque gradient centrifugation. Slides with PBMC cytospin preparations were air dried and fixed in cold acetone. LAMP-2 expression was examined by immunohistochemistry using an anti-LAMP-2 antibody (BD Biosciences Pharmingen[™], Tokyo, Japan).

Flow cytometric analysis of leukocytes

Whole blood was used to simplify the screening method. After incubation with lysis buffer, intracellular staining with monoclonal antibodies was performed according to standard procedures. In brief, 1×10^6 leukocytes were incubated with 100 ml of Fix

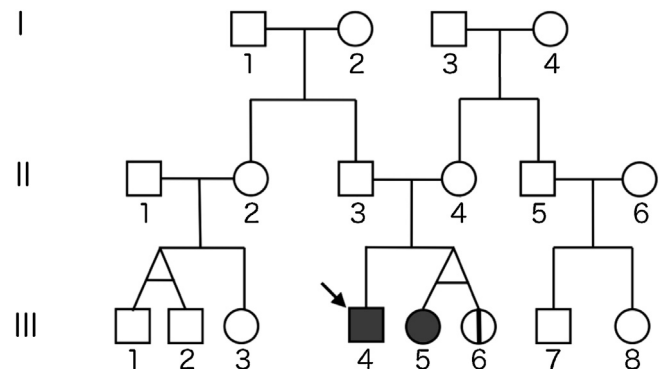


Fig. 2. Pedigree of DD1. III-4 (DD1) is the proband and III-5 (DD2) and III-6 (DD3) are monozygotic twins. DD2 is a symptomatic female, and DD3 is an asymptomatic female carrier.

solution (BD Cytofix/Cytoperm™ plus fixation/permeabilization solution; BD Biosciences Pharmingen) for 20 min at room temperature. After two washes with this solution, the cells were resuspended in 100 μ l of permeabilization solution, and 4 μ l of a primary monoclonal antibody directed against LAMP-1/LAMP-2 (BD Biosciences Pharmingen) or isotype-matched monoclonal antibodies (BD Biosciences Pharmingen) were added. LAMP-1 and LAMP-2 are homologous lysosomal membrane proteins encoded by distinct genes (*LAMP1* is located on 13q34) [18]. LAMP-1 was used as a control for the integrity of the preparation. After incubation for 20 min, cells were washed twice in the permeabilization solution, resuspended in 200 μ l of phosphate-buffered saline, and analyzed on the FACS Calibur flow cytometer supported by cellquest software (Beckton Dickinson, San Diego, CA, USA).

Sensitivity of flow cytometric analysis for detecting LAMP-2 in leukocytes

We mixed varying numbers of granulocytes obtained from a healthy male control and DD1 and determined the percentage of LAMP-2-negative cells by flow cytometry. Intracellular staining was performed as described above.

Results

Clinical characterization of female carriers

The sisters of DD1 (DD2 and DD3) were monozygotic twins. One developed mild cardiomyopathy, whereas the other did not. No abnormalities were observed in DD2 during her school medical check-up (which included ECG) at the age of 6. She was 11 years old when we examined her. Although she did not exhibit any obvious symptoms of cardiac disease, mild left ventricular hypertrophy was observed by UCG. No late gadolinium enhancement was observed on MRI (Fig. 1B). Her ECG demonstrated intermittent Wolff–Parkinson–White syndrome (Fig. 1C) and grade 2 atrioventricular block. Her AST and LDH levels were slightly elevated (Table 1), and she did not exhibit skeletal myopathy or mental

retardation, thus making her family history of Danon disease the only clue to an accurate diagnosis.

DD3 was screened simultaneously with DD2. She also did not exhibit definitive symptoms. Compared with DD2, her chest X-ray, UCG, MRI, and ECG results were normal (Fig. 1). Transaminase levels were not elevated and no muscle weakness was detected (Table 1). The parents of DD1 exhibited no overt clinical symptoms. No abnormalities were identified in their ECG, UCG, or blood enzyme levels. The ECG and UCG findings of DD1's maternal uncle were also normal.

Molecular characterization

No *LAMP2* mutations were detected in DD1's parents (II-4 and II-3). In contrast, we identified a 4-bp deletion in *LAMP2* at the intron 6 splice site (IVS6+1_4delGTGA) in DD1 (Fig. 3). DD2 and DD3 were found to be heterozygotes with one copy each of the wild type (upper sequence) and IVS6+1_4delGTGA (lower sequence) sequences. *LAMP2* mutations were not detected in any of the control samples.

Immunohistochemistry

Representative LAMP-2 expression profiles in PBMCs are shown in Fig. 4. All control monocytes (open arrow heads) and a significant portion of the control lymphocytes expressed LAMP-2. Some lymphocytes had less cytoplasm, making it difficult to judge the intracellular LAMP-2 expression microscopically. Therefore, we analyzed LAMP-2 expression in monocytes. DD1's monocytes lacked detectable LAMP-2 immunoreactivity (arrows). The heterozygote females (DD2 and DD3) possessed both LAMP-2-positive (open arrow heads) and LAMP-2-negative monocytes (arrows). This chimerism was confirmed by FACS.

FACS analysis of leukocytes

We first quantified intracellular LAMP-2 expression in each leukocyte subpopulation obtained from the peripheral blood samples of normal controls (Fig. 5). LAMP-2 was expressed in all

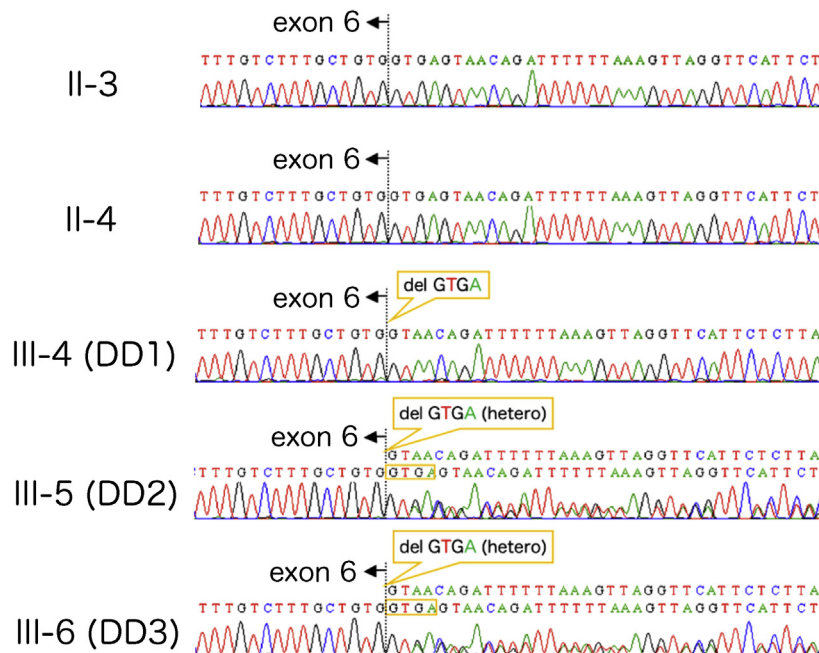


Fig. 3. Mutation detection in the family. Deletion of GTGA at the 3' end of exon 6 is demonstrated in the genomic DNA of DD1 (hemizygous) and of DD2 and DD3 (heterozygous) but not in that of the parents (II-3 and II-4).

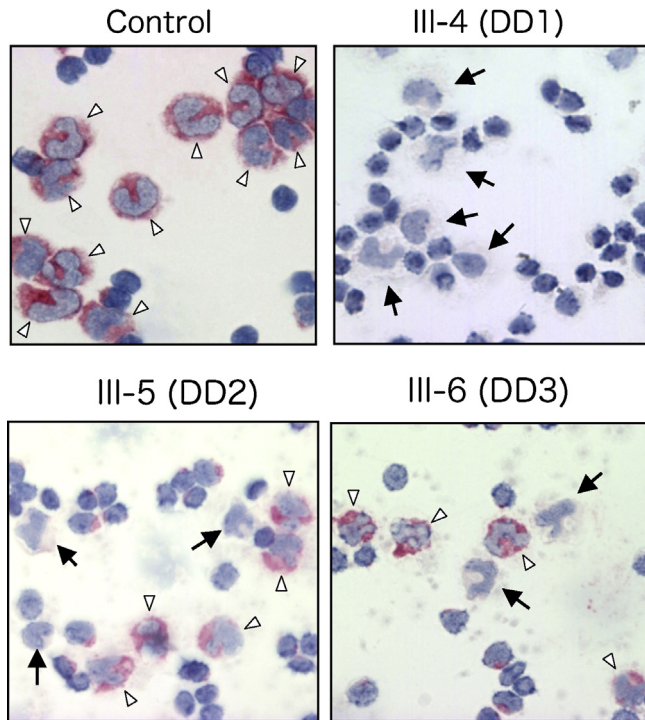


Fig. 4. Immunoreactivity in peripheral blood mononuclear cells. The control shows lysosome-associated membrane protein-2 (LAMP-2) expression in all peripheral blood mononuclear cells. DD1 has no detectable LAMP-2 expression, and both DD2 and DD3 show chimerism, i.e. LAMP-2-positive and LAMP-2-negative cells. Δ , LAMP-2-positive monocyte; \rightarrow , LAMP-2-negative monocyte.

cell types, but the signal was more intense in granulocytes, monocytes, and CD56⁺ NK cells than in CD20⁺, CD4⁺, or CD8⁺ lymphocytes. Monocytes expressed LAMP-2 as intensely as granulocytes. Because large leukocytes could have been included in the monocyte region of the plot, we chose to use granulocytes for patient analysis and gated the granulocyte region.

An investigation of the sensitivity of the FACS data showed that we could detect very small percentages (0.78%) of LAMP-2-negative cells precisely (Fig. 6).

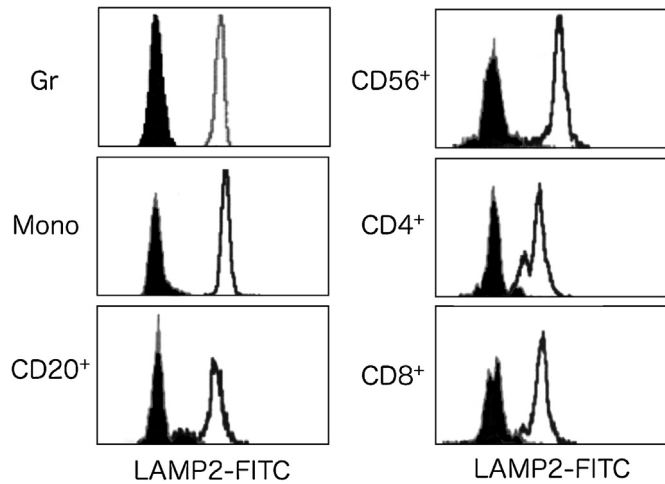


Fig. 5. Intracellular lysosome-associated membrane protein-2 (LAMP-2) expression in leukocyte subpopulations. LAMP-2 expression is more intense in granulocytes (Gr), monocytes (Mono), and CD56⁺ NK cells (CD56⁺) than in lymphocytes (CD4⁺, CD8⁺, and CD20⁺). FITC, fluorescein isothiocyanate.

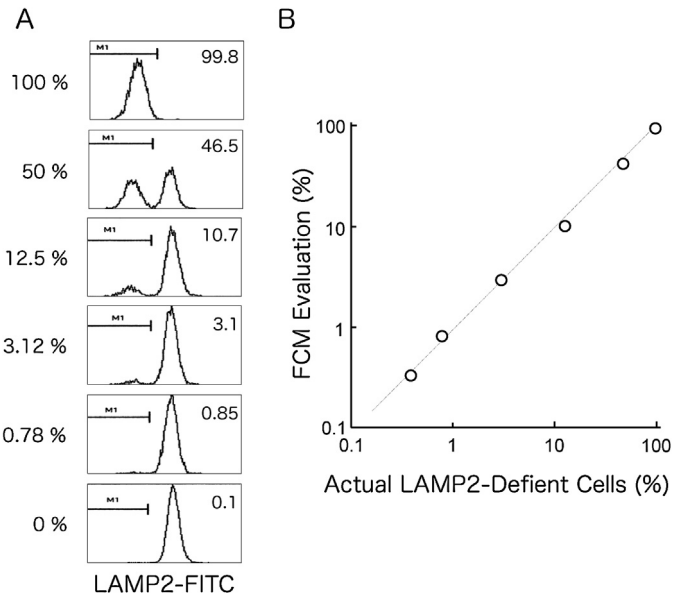


Fig. 6. Quantitative analysis of lysosome-associated membrane protein-2 (LAMP-2)-negative cells. (A) Actual percentages of LAMP-2-negative cells are shown on left side of each panel, and measured percentages are shown in each panel. (B) Measured percentages of LAMP-2-negative cells are consistent with actual percentages. FITC, fluorescein isothiocyanate; FCM, flow cytometry.

Healthy controls showed a single LAMP-1-positive peak and a single LAMP-2-positive peak (Fig. 7). Only representative data are shown in Fig. 7. Approximately 99.5–99.9% (mean 99.7%) of the cells in healthy control samples were LAMP-2 positive. DD1

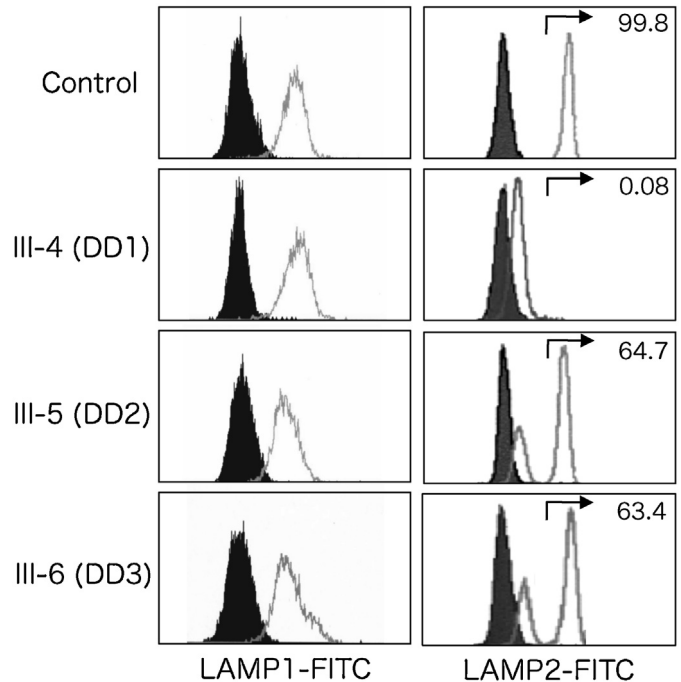


Fig. 7. LAMP-1 and LAMP-2 expressions by granulocytes. DD1 has no detectable LAMP-2 expression but expresses normal LAMP-1 levels. DD2 and DD3 show two peaks of LAMP-2 expression. The lower peak is of the same intensity as that for DD1. Therefore, this peak can be attributed to LAMP-2-negative cells. The higher peak shows the same LAMP-2 signal intensity as that shown by healthy controls. These data indicate that granulocytes of female patients contain LAMP-2-negative and LAMP-2-positive cells. The ratio of LAMP-2-positive to LAMP-2-negative cells exhibited by DD2 is similar to that displayed by DD3. LAMP, lysosome-associated membrane protein; FITC, fluorescein isothiocyanate.

showed a single LAMP-2-negative peak and a LAMP-1-positive peak, indicating a deficit of LAMP-2 in all of DD1's leukocyte subpopulations. In contrast, the LAMP-1-positive peak displayed the same intensity as that in the healthy controls.

The two heterozygous females (DD2 and DD3) showed identical patterns of double peaks. The fluorescence intensities of their LAMP-2-negative peaks were the same as those for DD1, indicating a deficit in LAMP-2 expression in these cells. The intensities of their LAMP-2-positive peaks, however, were the same as those of the healthy controls, indicating normal LAMP-2 expression in these cells. Although DD3 was asymptomatic, the percentage of her LAMP-2-negative cells was similar to that in symptomatic DD2. To prove that the LAMP-2 negative cells contained no lymphocytes, we purified the granulocytes by Ficol-Hypaque gradient centrifugation and analyzed them by the same FACS method. The double peaks reappeared, revealing that the granulocytes of the female patients consisted of either LAMP-2-positive or LAMP-2-negative cells. In summary, a single LAMP-2-negative peak in male patients (no LAMP-2 expression) and double peaks in female patients characterized Danon disease.

Discussion

Since 2000, Danon disease has been diagnosed by PCR-based mutation detection technologies and Western blot analysis of LAMP-2 expression. Almost all reported *LAMP2* mutations lead to LAMP-2 protein loss through frameshift or nonsense mutation. However, in 2010, Yang discovered *LAMP2* microdeletions in patients with Danon disease [19]. In these cases, LAMP-2 was undetectable, but short range PCR-based mutation detection technologies missed these patients, providing a compelling argument for employing both genetic and protein expression analyses to diagnose Danon disease.

Here we describe the development of a flow cytometric assay that greatly facilitates the detection of females heterozygous for Danon disease. The assay is based on the detection of the lysosomal protein LAMP-2 in peripheral blood leukocytes. In normal controls, all circulating leukocyte populations, including granulocytes, monocytes, and lymphocytes, expressed significant levels of LAMP-2. This assay can be used for any patient with or without symptoms of Danon disease; it takes less time and is less expensive than genetic analysis. Accordingly, it is more suitable as an initial screening process.

Males have one copy of the X chromosome; thus, all cells from male patients with Danon disease lacked detectable LAMP-2. A potential imbalance of gene expression from the two X chromosomes in females is resolved by inactivating one X chromosome. X-chromosome inactivation is a stochastic event that occurs during the early stages of embryonic development. In each cell, a choice is made independently. Therefore, there are two possible patterns of LAMP-2 expression in female patients with Danon disease: positive expression by normal paternal X chromosomes and negative expression by affected maternal X chromosomes.

Primary immunodeficiency diseases such as X-linked agammaglobulinemia and Wiskott–Aldrich syndrome are diagnosed by protein expression analysis using FACS. In these X-linked inherited diseases, heterozygous females usually do not exhibit clinical symptoms, with the exception of some rare cases. Protein levels in the tissues of affected females can be reduced because of skewed X-chromosome inactivation. Fanin et al. reported an X-chromosome inactivation analysis in three female patients of Danon disease and found a random pattern of inactivation in leukocytes. The mean skewing rate in blood leukocytes was 60% [17].

We believe that our analysis can demonstrate chimerism, even if some female patients with Danon disease show small percentages of LAMP-2-negative cells by skewed X-chromosome

inactivation, because we could detect as few as 0.8% of cells lacking LAMP-2 (Fig. 6). The sensitivity of flow cytometric assay, which can detect the fluorescent intensity of each cell, is much higher than that of Western blot analysis. On the other hand, LAMP-2 is expressed in all leukocytes in healthy males and females; therefore, LAMP-2-negative cells could not be detected by flow cytometric assay. This indicates that both the sensitivity and specificity of this assay are extremely high. Nevertheless, confirmation by genetic analysis is necessary.

We used our assay to detect LAMP-2 expression in a family that has a male member who suffers from Danon disease. There was no apparent inheritance, although we were able to identify two young heterozygous females (DD2 and DD3) without the clinical onset of Danon disease. The clinical courses of DD2 and DD3 should prove to be highly informative about the early stages of Danon disease. DD2 and DD3 are monozygotic twins with identical leukocyte LAMP-2 expression and *LAMP2* mutations; DD2 was symptomatic, whereas DD3 was asymptomatic. In addition, there were no differences in their lifestyles. We were therefore unable to account for the differences in their clinical findings. Using our flow cytometric assay, we can investigate LAMP-2 expression not only in leukocytes but also other cells and tissues. Such a study could explain the complex organ-specific symptoms and different severity among patients with Danon disease.

Inherited cardiomyopathies are genetically heterogeneous [20]. A majority of childhood-onset isolated cardiac hypertrophies are caused by genetic mutations for which adults are routinely screened [21,22]. However, in some young patients, nonsarcomeric causes such as inborn errors of metabolism, mitochondrial dysfunction, and neuromuscular conditions have been increasingly recognized [23], and the outcomes depend largely on the cause [24]. Among all causes of ventricular hypertrophy, the clinical course of Danon disease is the most aggressive. Early diagnosis of Danon disease should be highly informative of prognosis, and this disease should always be ruled out in patients presenting with unexplained cardiomyopathy.

Our present study demonstrates that flow cytometric analysis of LAMP-2 expression by leukocytes provides a novel, rapid, simple, and highly sensitive screening method for the early detection of Danon disease.

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Conflict of interest

The authors have declared no conflicts of interest.

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