LAMP2 Microdeletions in Patients With Danon Disease

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Background—Danon disease is an X-linked dominant disorder characterized by the clinical triad of hypertrophic cardiomyopathy, skeletal myopathy, and variable mental retardation. Pathologically, autophagic vacuoles are noted in both skeletal and cardiac muscle. It exhibits an X-linked dominant mode of inheritance, and male carriers are severely affected, whereas female carriers develop milder and later-onset cardiac symptoms. Danon disease has been associated with mutations in the lysosome-associated membrane glycoprotein 2 (LAMP2) gene located at Xq24, typically resulting in splicing defects or protein truncation affecting the LAMP2. Because of its rarity, the full spectrum of genetic mutation resulting in Danon disease has not been elucidated.

Methods and Results—We analyzed 3 male cases with clinical and pathological findings consistent with Danon disease. Comprehensive mutational analysis failed to yield detectable products for selected LAMP2 exons, and genomic DNA deletion was suspected. Genomic junction fragment polymerase chain reaction analysis in case 1 identified a novel Alu-mediated 34-kb microdeletion encompassing the entire 5′-untranslated region and exon 1 of LAMP2. In case 2 and 3, junctional polymerase chain reaction and Southern blot analyses mapped the breakpoint to an MIRb and (TA)n simple repeats present in intron 3, which determined a 64-kb and a 58-kb deletion, respectively, thereby ablating exons 4 to 10. Western blot analysis confirmed the absence of LAMP2 in protein extract from lymphocytes of index case 2.

Conclusion—This article is the first report of Danon disease caused by microdeletions at Xq24, which functionally ablate LAMP2. The microdeletion mechanism appears to involve 1 Alu-mediated unequal recombination and 2 chromosomal breakage points involving TA-rich repeat sequences. (Circ Cardiovasc Genet. 2010;3:129-137.)

Key Words: LAMP2 ▪ HCM ▪ Danon ▪ Alu ▪ (TA)n simple repeat ▪ hypertrophy ▪ cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is a complex cardiac disease with unique pathophysiological characteristics and a wide spectrum of morphological, functional, and clinical features. In patients with this condition, the interventricular septum and left ventricular posterior wall are thickened (usually asymmetrical septal hypertrophy), and hypercontractile systolic function and diastolic dysfunction are noted. Systolic anterior motion of the mitral valve and left ventricular outflow tract obstruction also may occur. Although HCM has been considered a relatively uncommon cardiac disease, the prevalence of echocardiographically defined HCM in a large cohort of apparently healthy young adults selected from a community-based general population was reported to be 1 in 500 persons. Although sporadic cases are common, familial disease with autosomal dominant inheritance predominates.

Most of the genes involved in HCM encode proteins of the sarcomere, which is a complex structure with an exact stoichiometry and multiple sites of protein-protein interactions. The encoded proteins include myofilament proteins β-MyHC (which was the first gene to be identified), α-MyHC, ventricular myosin essential light chain 1, and ventricular myosin regulatory light chain 2; thin filament proteins cardiac actin, cardiac troponin T, cardiac troponin I, cardiac troponin C, and α-tropomyosin; and myosin-binding proteins cMyBP-C and titin. These mutations have been described in detail in a number of reviews. Recently, mutations in the caveolar protein caveolin-3 and in several sarcomeric Z-line genes, such as cysteine and glycine-rich protein, telethonin, myozien 2, myopalladin, vinculin, α-actin 2, and LIM (Lin11, Isle-1, and Mec-3) domain-
binding protein 3, were identified in patients with HCM.\(^4\) In addition, mutations in junctophilin 2 and phospholamban, which modulate calcium ion release from the sarcoplasmic reticulum and calcium influx from the plasma membrane L-type calcium channels, have been reported to cause HCM.\(^4\)

Further adding to the spectrum of HCM-causing genes in human subjects, mitochondrial and metabolic abnormalities have been reported. In 2004, Van Driest et al.\(^8\) screened 389 unrelated patients with HCM for mutations in the 7 sarcolemic genes and identified potential mutations in <40%.

Mutations in certain genes encoding metabolic and lysosomal storage proteins have been identified in a small proportion of patients, including the gene encoding the γ2 subunit of AMP-activated protein kinase\(^6,7\) and the α-galactosidase gene\(^8,9\) associated with Fabry disease. In addition to Fabry disease, another lysosomal storage disorder, known as Danon disease, is associated with the development of skeletal myopathy and variable mental retardation with intracytoplasmic cytoplasmic vacuoles containing autophagic material and glycogen in skeletal and cardiac muscle cells and presents with HCM.\(^10,11\)

Mutation in the lysosome-associated membrane glycoprotein 2 (LAMP2) gene, which encodes the lysosome-associated protein 2 and maps to Xq24, were initially identified as the cause of Danon disease.\(^12\) Subsequently, Danon disease was classified in the subgroup of autophagic vacuolar myopathies once it was recognized that sarcolemmal proteins and basal lamina are associated with the vacuolar membranes.\(^11,13\)

Affected male carriers usually present with HCM at puberty or even earlier, although most female carriers develop dilated cardiomyopathy rather than HCM during adulthood (as late as their 40s).\(^14–19\) Skeletal muscle biopsy usually reveals numerous glycogen containing (periodic acid-Schiff positive) cytoplasmic vacuoles.\(^10,11,15\) Mental retardation, although usually mild and of variable degree, has been noted in some patients.\(^10,14–19\) Female carriers also have skeletal myopathy and mental retardation less commonly than affected male carriers.\(^14–19\)

To date, the majority of reported mutations in the LAMP2 gene represent loss of function mutations (small insertions and deletions leading to frameshift and nonsense mutations).\(^12–27\) These mutations are predicted to result in complete absence of the protein through nonsense-mediated decay in which transcripts containing premature termination codons are targeted. This causes rapid degradation, protecting the organism from deleterious dominant-negative or gain-of-function effects of resulting C-terminal truncated proteins.\(^28\) Large genomic deletions generally are suspected in genes in which small loss of function mutations are common; however, they frequently are missed because of short-range polymerase chain reaction (PCR)-based mutation detection technologies, particularly for autosomal genes. Here, we report 3 patients with Danon disease who carry large genomic deletions involving the LAMP2 gene. To our knowledge, we are presenting the first evidence of chromosomal rearrangements affecting the LAMP2 genomic sequence through a homologous unequal recombination, an increasingly recognized mechanism in cardiac genetic diseases.\(^29\)

### Methods

#### Patient Evaluation

All patients were evaluated by physical examination, chest radiography, electrocardiography, echocardiography, and MRI. Left ventricular size and function were evaluated by M-mode, 2D Doppler, and color Doppler echocardiographic images, and cardiac arrhythmias were studied by 24-h Holter monitoring. Serum creatine kinase levels were measured to evaluate the association of skeletal myopathy.

#### Mutational Analysis

After informed consent, blood was obtained for lymphoblastoid cell line immortalization and DNA extraction,\(^30\) as regulated by the Baylor College of Medicine Institutional Review Board. Genomic DNA was amplified by PCR using primers designed to amplify the coding exons of the LAMP2 gene and the upstream and downstream genomic sequences encompassing the LAMP2 gene (primer sequences available on request). The PCR products were purified using exonuclease I and shrimp alkaline phosphatase. DNA sequence analysis was performed by using Big Dye terminator chemistry version 3.1 and an ABI 3730 genetic analyzer, as previously described.\(^30\)

#### Junction Fragment PCR and Sequencing

Sequential PCR using primers upstream and downstream of the LAMP2 gene was performed, and potential breakpoints were mapped. Long fragment PCR product was obtained by using primers flanking the deleted region. Amplified PCR product was purified and directly sequenced as described previously. Reference genomic DNA sequences were NM_013995 (National Center for Biotechnology Information) and Ensembl build 63 (www.ensembl.org).

#### Southern Blot Analysis

In brief, 15-μg genomic DNA was digested with SpeI endonuclease overnight at 37°C. The digested DNA was resolved on a 0.7% agarose gel and transferred to a 0.4-μm nylon membrane with 0.4 N NaOH solution after standard Southern blot transfer procedure. Twenty-five nanograms of purified probe (flanking LAMP2 exon 2 or 6) DNA (1 μL) was then labeled with 5 μL of 50-μCi \(^{32}\)P-dCTP using 4 μL High Prime solution, purified with Qiagen Nucleotide Removal Kit, and used to probe the Southern blot.

#### Western Blot Analysis

Lymphoblastoid cell lines were homogenized in lysis buffer containing 1% Triton X-100.\(^31\) The lysates were clarified by centrifugation at 20 000g for 30 min, and the protein concentration was determined by bicinchoninic acid protein assay kit; equal amounts of protein were subjected to SDS-PAGE followed by transfer to nitrocellulose membranes by electrotransfer, as previously described.\(^31\) Proteins were detected as previously described.\(^31\) Monoclonal antibodies against LAMP2 (antibody H4B4) and LAMP1 (H4A3) were obtained from the Developmental Studies Hybridoma Bank, University of Iowa.

### Results

#### Clinical Characterization

**Clinical Presentation of Case 1 (0014)**

A 12-year-old white boy presented with recurrent rest and exertional syncopal episodes from 4 years of age. He was in special classes for reading and math because of visual and auditory processing problems but otherwise was without other health issues, including hypertension. His family history revealed no cardiac or skeletal muscle disease or sudden death. Echocardiography demonstrated moderate concentric left ventricular hypertrophy without left ventricular outflow tract obstruction (Figure 1A). His ECG demonstrated sinus bradycardia,
a short PR interval (100 to 110 ms) with possible preexcitation, an interventricular conduction delay (QRSd, 110 to 118 ms), and marked left lateral lead ST segment depression and was suggestive of severe left ventricular hypertrophy (Figure 1B). Electrophysiological testing demonstrated a fasciculoventricular fiber as manifested by constant short HV interval during atrial extrastimulus testing (Figure 1C). Electron microscopic examination of right ventricular apicooseptal endomyocardial biopsy demonstrated rare, slightly hypertrophic cardiomyocytes and punctate periodic acid-Schiff-positive inclusions in multiple cells that were generally perinuclear and diastase resistant. These were not associated with identifiable vacuoles. E, Ultrastructural evaluation showed glycogen present within the cytoplasm and in some vacuoles. The vacuoles were of mild to moderate density and few in number. Multiple myocytes exhibited the perinuclear myeloid bodies lamellated lysosomes that indicate myocyte injury and degeneration.

Clinical Presentation of Case 2 (00732)
A 12-year-old white boy presented with gastrointestinal problems. Skeletal muscle weakness was noted on examination, and laboratory evaluation revealed a high level of creatine kinase. Muscle biopsy was performed, and the specimen showed dystrophic features that along with the clinical findings of a mild proximal weakness and an increased creatine kinase could suggest the diagnosis of limb-girdle muscular dystrophy despite the presence of basophilic cytoplasmic granular inclusions. Of note, preexcitation was observed on the preoperative ECG. The family history was unremarkable for any members with known neuromuscular disorders. Two years later, the patient underwent Holter
monitoring and stress test, which identified premature ventricular contractions with variable coupling intervals before, during, and after exercise, although ventricular preexcitation persisted throughout the test (Figure 2A).

At 17 years of age, the patient was referred for ophthalmologic evaluation, after suspicion of Danon disease, and despite no decline in visual function, he presented with a peripheral pigmented retinopathy consistent with the disease. He also presented with mild learning delays necessitating special educational classes and vocational training instead of college-level course work. On examination, a progressive skeletal myopathy was appreciated. Echocardiogram demonstrated concentric left ventricular hypertrophy. After \textit{LAMP2} gene mutational analysis, the patient was given a diagnosis of Danon disease. Because the ECG continued to show evidence of ventricular preexcitation, consistent with Wolff-Parkinson-White syndrome, he underwent radiofrequency ablation of 3 of the 5 mapped accessory atrioventricular connections.

At 21 years of age, routine MRI using a 3.0 Tesla magnet and an 8-channel phased-array cardiac coil confirmed persistent concentric left ventricular hypertrophy without obstruction. There was depressed systolic dysfunction (ejection fraction [EF], 37%; Z score, –5.9). Gadolinium delayed enhancement examination demonstrated severe left ventricular myocardial free wall and inferoseptal nonperfusion and fibrosis (Figure 2B). The MRI findings were similar to those described by Piotrowska-Kownacka et al\textsuperscript{32} of a case of Danon disease. Echocardiography confirmed the severe concentric left ventricular hypertrophy with no left ventricular outflow tract obstruction, mild to moderately depressed biventricular systolic function, and trivial to mild mitral regurgitation (Figure 2C). Routine ECG evaluation identified sinus rhythm and ventricular preexcitation with short PR interval (96 ms) and wide QRS complexes (194 ms), premature ventricular complexes, and prolonged QT interval (QTc, 499 ms). Holter monitoring showed frequent premature atrial contractions and premature ventricular contractions and a short run of tachycardia (24 beats) (Figure 2D). While out shopping 2 months later, the patient experienced a generalized seizure and was unresponsive, followed by flaccid right-side paresis. In the emergency department, he had runs of ventricular tachycardia lasting 3 h. Neuroimaging demonstrated the presence of an extensive central nervous system infarction. The patient was declared brain dead, and support was withdrawn.

**Clinical Presentation of Case 3 (0013)**

A 25-year-old white man with no evidence of skeletal muscle weakness but with developmental delay and learning disabilities underwent full clinical evaluation, and the echocardiogram demonstrated left ventricular hypertrophy, whereas muscle biopsy demonstrated myopathy, glycogenosis, and intracytoplasmatic vacuoles. Baseline ECG analysis demonstrated third-degree AV-block, and the patient underwent implantation of a pacemaker. Metabolic studies revealed increased serum levels of creatine kinase, alanine transaminase, and aspartate transaminase.

**Molecular Characterization**

**Molecular Analysis of Case 1 (0014)**

The DNA was sent to our laboratory to confirm the result of a previously performed \textit{LAMP2} gene mutational test, which had identified a possible genetic deletion of exon 1. We confirmed that the PCR analysis failed to amplify exon 1 of the \textit{LAMP2} gene. Primers were designed to perform long-fragment PCR (available on request) followed by sequence analysis, which located the breakpoints $\approx 25.5$ kb upstream of the start codon (within the 5'-untranslated region [UTR]) and $\approx 8.9$ kb downstream of start codon (within intron 1) of the \textit{LAMP2} transcript (ENST00000371345) of the \textit{LAMP2} gene (Figure 3A). This $\approx 34.4$-kb deleted region includes the first coding exon of the \textit{LAMP2} gene and $\approx 9$ kb of the first intron; no other known genes are present in this deleted region.
RepeatMasker analysis of the LAMP2 genomic sequence encompassing the aforementioned breakpoints identified Alu-repeat elements of distinct subfamilies in both 5’ and intron 1 regions of LAMP2 (Figure 3A). The breakpoint sequence identified a 45-bp core motif with 100% homology to an AluSx-repeat element at the 5’-UTR and 1 AluSx1-repeat element in intron 1 of LAMP2 (Figure 3B). Overall, the 2 Alu-repeat elements shared 88% identity.

Molecular Analysis of Case 2 (00732)

Comprehensive open reading frame/splice site LAMP2 gene mutational analysis followed by direct DNA sequencing was performed on the patient genomic DNA. However, we were unable to amplify exons 4 to 10 of the LAMP2 gene. Therefore, a significant genomic DNA deletion was suspected, and therefore, sequential PCR primers were designed to identify the possible chromosomal deletion. Using long-fragment PCR, the breakpoints were localized \(\approx 14\) kb and \(\approx 72\) kb downstream of the transcript LAMP2-201 (ENST00000371345) of the LAMP2 gene, deleting intron 3, exons 4 to 10, and part of the 3’-UTR (Figure 3A and 3C). Computational analysis of the LAMP2 genomic sequence identified a \((\text{TA})_6\) simple repeat element at the 5’ breakpoint and an MER21B repeat element highly rich in TA (62%) at the 3’ breakpoint in our index case 2. Southern blot analysis using the available family members confirmed the deletion, which was present only in index case 2, consistent with a de novo mutation (Figure 3D). We found that, as expected, a probe against LAMP2 exon 2 detected an \(\approx 3\)-kb band in the patient sample as opposed to a 7-kb band in normal family samples (Figure 3D). Furthermore, a probe against LAMP2 exon 6 detected no band in the patient sample as opposed to an \(\approx 14\)-kb band in normal family sample controls (Figure 3D). To determine whether this deletion affects the translation of the LAMP2 gene, we also performed Western blot analysis for LAMP2 and LAMP1 (another lysosomal membrane protein) proteins, using GAPDH as a loading control. LAMP2 protein was undetectable in the index case 2 sample,

![Figure 2. Cardiac evaluation in subject 00732. A, ECG of the proband demonstrating ventricular preexcitation consistent with Wolff-Parkinson-White syndrome. B, Steady-state free precession MRI short-axis image (right) shows severe concentric left ventricular hypertrophy. Gadolinium delayed contrast MRI 4-chamber image (left) indicates presence of severe left ventricular free wall and inferior septal fibrosis (arrowheads). C, Echocardiographic parasternal short-axis (right) and long-axis views (left) of the index case demonstrates severe concentric left ventricular hypertrophy without left ventricular outflow tract obstruction. The echocardiogram also demonstrates depressed biventricular systolic function and mild mitral regurgitation. D, ECG demonstrating ventricular preexcitation, short PR interval, wide QRS, premature complexes, and prolonged QT interval consistent with Wolff-Parkinson-White syndrome.](http://circgenetics.ahajournals.org/
although its expression was similar among all other family members (Figure 3E). In contrast, there was no difference in the expression of LAMP1 protein in the patient and his family members (Figure 3E). We also performed microsatellite analysis and confirmed that the proband and his relatives are from the same family (data not shown), which supports the concept that this is a de novo deletion in the LAMP2 gene of this patient that is not present in other family members.

Molecular Analysis of Case 3 (0013)

The DNA sample for case 3 was sent to our laboratory to confirm the previously performed LAMP2 gene mutational test, which had identified a possible genetic deletion of exons 4 to 10 of LAMP2. No parental DNA samples were available for analysis. We confirmed that the PCR analysis failed to amplify exons 4 to 10 of the LAMP2 gene, and similar to case 2, we located the breakpoints at \( \approx 14 \) kb downstream of the start codon (within intron 3) and \( \approx 78 \) kb downstream of start codon (within 3′-UTR) of the LAMP2 gene (Figure 3A). The deleted region contained only the LAMP2 gene, and no other known genes were involved. Computational analysis of the LAMP2 genomic sequence encompassing the breakpoints identified in index case 3 revealed an MIRb repeat element at the 5′ breakpoint and the LIMA4A repeat element at the 3′ breakpoint families of LAMP2, both rich in TA (68.5%). The Southern blot analysis was performed using the DNA from the index case 3, and we compared it with a control individual from the family of case 2. The analysis detected differentially sized bands in the patient compared to the control sample, using a probe against LAMP2 exon 2 (Figure 3F). With a probe against LAMP2 exon 6, we were able to detect a band in the control sample but not in the case 3 sample (Figure 3F).

Discussion

Danon disease is an X-linked disorder that affects multiple organs and should be strongly considered in persons with HCM and skeletal myopathy. The LAMP2 gene, located on chromosome Xq24, is the only gene known to cause Danon disease, and the majority of the identified missense and nonsense mutations as well as small insertions and deletions result in impaired mRNA splicing.\(^{12–27}\) In particular, even missense mutations, such as the V310I that has been identified in multiple patients, were found to affect RNA processing, resulting in a frameshift.\(^{15,27}\) Although LAMP2 has been cloned from humans for >2 decades,\(^{33}\) the role the gene plays in human disease still is uncertain. The need for a functionally normal LAMP2 in the cell also was recently suggested by studies using the monoclonal antibody H4B4, which could block human LAMP2 and induce apoptosis in human microvascular endothelium in vitro.\(^{34}\)

To our knowledge, we present the first evidence of LAMP2 microdeletions involving repetitive sequence motifs such as Alu-mediated or TA-rich repeat elements and predicting the ablation of a functional LAMP2 product. These chromosomal rearrangements caused the deletion of critical portions of the gene, thus preventing it from yielding a functional protein. In particular, the Alu-mediated microdeletion in case 1 caused the ablation of the entire 5′-UTR regulatory sequence, the first exon, and a large part of the first intron. This microdeletion predicts the absence of a functional transcript as it occurs in mutations causing altered splicing that affect the first exon and intron boundaries.\(^{21}\) The microdeletions in cases 2 and 3, mediated by TA-rich repetitive sequences, ablate exons 4 to 10, although their breakpoints differ slightly from each other.

The (TA)\(_n\) and MER21B elements at the breakpoint involved in the deletion in case 2 shared 45.4% identity, whereas the MIRb and LIMA4A elements present at the breakpoint in case 3 microdeletion shared 46.3% identity. Because of the low identity rate, the exact mechanism leading to the microdeletions in cases 2 and 3 remains elusive. Breakpoints occurring within TA-rich segments have been previously reported to encompass complex chromosomal rearrangements due to inexact or illegitimate homologous recombination mechanisms.\(^{35,36}\) However, elements rich in TA also have been involved in fragile site-based genomic rearrangements.\(^{37}\) At this stage, we do not have sufficient elements to dissect the detailed nature of the mechanism leading to LAMP2 microdeletions in cases 2 and 3.

Nonetheless, the absence of exons 4 to 10 causes the lack of any canonical stop codon and predicts the deficiency of functional transcripts, as predicted by nonsense-mediated decay, although cryptic termination sites could have occurred. Therefore, to test this hypothesis, we also performed
Western blot analysis, which confirmed that the microdeletion caused a functional knockout in the affected case but did not influence LAMP1 expression.

Although all microdeletions identified in this report affect different regions of the LAMP2 gene, they probably originate from a similar homologous unequal recombination mechanism. However, the sequence identity of the repeated elements causing microdeletions in LAMP2 appears to be low compared with the canonical repeat sequence-mediated genomic rearrangements, typically sharing >90% identity. This low sequence identity perhaps explains why LAMP2 large deletions have been encountered less commonly than small mutations. Nonetheless, homologous unequal recombination is a well-described mechanism on the X chromosome, as we recently demonstrated in a subject with Barth syndrome presenting with an Alu-mediated mechanism totally ablating TAZ and functionally ablating DNLIL. In addition, our findings confirm that the Xq24 region is prone to chromosomal rearrangements as recently demonstrated in premature ovarian failure.

Genomic deletions often are observed in genes for which small loss-of-function mutations are common. These deletions can be difficult to detect because resequencing usually is performed by using amplicons that are smaller than the deleted interval. Heterozygous deletions on autosomes therefore are masked by the presence of the wild-type allele, which raises the
question of whether some forms of dilated cardiomyopathy (DCM) and HCM also may stem from the deletion of 1 of the 2 autosomal alleles that are generally required to exert a normal function, leading to a copy number variation in these subjects. Because the LAMP2 gene is located in the X chromosome, hemizygous mutations and large deletions can be detected through common techniques such as PCR in male patients.

In conclusion, this report is the first, to our knowledge, to describe LAMP2 microdeletions in patients with Danon disease, cardiac hypertrophy, and cardiac rhythm disturbance. These findings broaden the range of genomic alterations observed in Danon disease, focusing attention on the increasing complexity of genetic testing for these patients.

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Disclosures

None.

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Danon disease is an X-linked autophagic vacuolar myopathy in which patients present with hypertrophic cardiomyopathy, skeletal myopathy, and variable mental retardation. Male carriers usually present with a severe disease, whereas female carriers can develop milder and later-onset cardiac symptoms. Thus far, lysosome-associated membrane glycoprotein 2 (LAMP2) gene located at Xq24 is the only gene identified to cause Danon disease, and the spectrum of genetic mutations includes missense mutations, small insertions, or deletions leading to splicing defects or protein truncation affecting the LAMP2. In this article, we identified submicroscopic chromosomal rearrangements ranging from 34 to 64 kb, all causing the functional ablation of LAMP2 in 3 patients with Danon disease. The molecular mechanism leading to these microdeletions was due to a novel Alu-mediated homologous unequal recombination or subsequent to chromosomal breakage mediated by interspersed TA-rich repetitive elements, which are among the mechanisms leading to genomic copy number variation. Our observation in 3 patients with Danon disease suggests that this mechanism may have been underestimated in select cardiovascular disorders. The X-linked nature of Danon disease allowed us to detect LAMP2 gene microdeletion in male patients through commonly used techniques such as polymerase chain reaction. However, if microdeletions occur in autosomal dominant diseases, the standard mutational analysis may fail to detect the lack of 1 of the 2 wild-type alleles, thus leading to a false-negative mutational screening and an undetected copy number variation in these patients. Therefore, our findings suggest that copy number variation analysis in cardiomyopathies and generally in cardiovascular diseases represents an uncharted territory that must be comprehensively investigated in future studies.
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