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A Mutational Hotspot in The *LAMP2* Gene: Unravelling Intrafamilial Phenotypic Variation and Global Distribution of The c.877C>T Variant: A Descriptive Study

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Abstract

Objective: Danon disease is defined by a clinical trio of cardiomyopathy, skeletal myopathy, and cognitive impairment. It results from the lysosomal-associated membrane protein-2 (*LAMP2*) gene variants. The aim of study is determination of genotype and phenotype of a newly diagnosed Iranian family with a unique phenotype due to a pathogenic variant of the *LAMP2* gene along with a phenotypic comparison of all reported patients.

Materials and Methods: In this descriptive study, we evaluated the demographic data, clinical features, management procedures, as well as genetic analysis of both patients in this newly diagnosed family. Whole genome sequencing (WGS) and in silico structural and functional predictions were applied. A comprehensive search of the c.877C>T variant in *LAMP2* was conducted using the PubMed, Google Scholar, VarSome, ClinVar, Human Gene Mutation Database (HGMD), and Franklin databases to identify any genotype-phenotype correlations.

Results: Nine patients were carriers of the c.877C>T variant. All patients were male, and displayed variable degrees of left ventricular hypertrophy (LVH) that ranged from mild to severe. All patients exhibited typical cardiac conduction abnormalities consistent with Danon disease. Four underwent heart transplants and survived. Skeletal muscle involvement and cognitive impairment were observed in four patients each. The mean age of onset was 14 years. The proband in this study exhibited an earlier onset of cardiac symptoms.

Conclusion: Genetic analysis is the preferred diagnosis approach for Danon disease and can assist families in managing affected patients, identify carriers, and assist with future family planning. This study highlights the intra-familial phenotypic variability of Danon disease. It is possible that variants of this gene may be frequent in Iran.

Keywords: Danon Disease, Next-Generation Sequencing, Cardiomyopathy

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Introduction

Danon disease (OMIM#300257) is a rare X-linked disorder that results from an abnormality in the lysosomal-associated membrane protein-2 (*LAMP2*) gene. While the prevalence of Danon disease is not accurately estimated, recent advancements in genetic testing have improved the diagnosis of disorders with substantial genetic components. The *LAMP2* gene (OMIM#309060) encodes a 410 amino acid protein. Its luminal domain is encoded by exons 1 to 8 and a portion of exon 9. The remainder of exon 9 contains the encoding for a transmembrane domain and a short cytoplasmic tail that contains the signal for targeting to the lysosomal membrane.

The LAMP2 protein is the principal regulator of autophagosome maturation, which maintains homeostasis by balancing the synthesis, degradation, and recycling of cellular substances. It facilitates the fusion and degradation of autophagosomes with lysosomes, resulting in autolysosome formation (1, 2).

Mutations in the *LAMP2* gene can hinder autophagy leading to impairments in lysosome maturation and biogenesis. Pathogenic variants in this gene may produce a nonfunctional or insufficient amount of the LAMP2 protein, which results in various problems depending on the affected cells. In Danon disease, the LAMP2 protein is

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often absent in muscle and heart cells, and leads to muscle weakness and heart abnormalities. Symptoms can vary widely among affected individuals even with the same variant; male patients are typically more severely affected and are often the proband of Danon disease. Although hemizygous males may indicate a loss of function, it is likely that the dominant effect of the mutations is primarily attributable to haploinsufficiency. This means that the protein produced by the unaffected, wild-type allele remains below the normal threshold level (3).

The classic clinical hallmark of Danon disease in males consists of severe cardiomyopathy, skeletal myopathy, and intellectual disability, whereas cardiac disease may be the most prominent symptom in females. Involvement of other organs, such as the liver, lungs, and retina, has also been reported in males with this multisystem disorder. Cardiomyopathy, particularly hypertrophic cardiomyopathy (HCM) in men and dilated cardiomyopathy (DCM) in women, are the most common and life-threatening symptoms. Electrocardiogram abnormalities are also observed in most patients, with ventricular pre-excitation in females and Wolf-Parkinson-White (WPW) syndrome primarily observed in males. Although cardiac symptoms typically appear during infancy, childhood, or adolescence, there is slower progression and later onset in women. Without a heart transplant, the condition rapidly advances to end-stage heart failure, and men typically die in their 30s and women in their 40s or 50s. Due to its similarity with sarcomeric HCM, an accurate diagnosis of Danon disease is critical to ensure appropriate management strategies (4, 5).

The diagnosis of Danon disease is gradually increasing due to advancements in genetic testing methods. Whole exome sequencing, as the most common genetic test, can identify genetic variants in a significant fraction of patients. However, whole genome sequencing (WGS), the most comprehensive genetic testing method, has a higher diagnostic sensitivity for identifying rare and common genetic variants in patients with HCM (6).

This study presents the genotype-phenotype relationship of well-characterised cases of Danon disease due to the c.877C>T variant. Also, a detailed genetic and clinical report on an Iranian family with two affected individuals, a 13-year-old boy and his mother, is also presented.

Materials and Methods

Data extraction

In this descriptive study, we conducted a comprehensive review of the c.877C>T (p.Arg293Ter) variant in PubMed and Google Scholar using the keywords: "LAMP2 gene", "c.877C>T", "p.Arg293Ter", "mutation", and "Danon disease". In addition, ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Leiden Open Variation Database (LOVD) (https://www.lovd.nl/), VarSome (https://varsome.com/), and the Human Gene Mutation Database (HGMD) (https://www.hgmd.cf.ac.uk/ac/index.php) databases were searched to identify all published papers that pertain to patients who carry this variant. The data from individuals with the p.Arg293Ter variant were extracted. Notably, a review article listed this variant in a table of LAMP2 gene variants reported in the last ten years (7); in one article, specific clinical details for the two families were not provided (8). We excluded these two articles from the clinical review of variant carriers. Characteristics extracted from the published articles included patient demographics, reporting countries, publication year, clinical characteristics (heart, skeletal muscle, cognitive function, and other organs), patient management procedures [implantable cardioverter-defibrillators (ICD) and heart transplants], as well as genetic analysis of both the patients and their families.

In this study, we conducted a clinical and genetic examination of an Iranian family with two patients who referred to Rajaei Hospital Cardiovascular Medical and Research Centre, which represented the second reported family of this variant in Iran, as we previously reported the first family (9). The proband (case III-1) and mother (case II-5) underwent clinical evaluations that included laboratory tests and various cardiovascular examinations such as echocardiography, electrocardiography, Holter monitoring, electrophysiology study, paediatric percutaneous angiography, multi-slice computed tomography (CT) scan of the brain and thorax, and transabdominal ultrasound. The patients underwent cardioverter-defibrillator, ablation, cardioversion and drug therapy, as clinical interventions.

Genetic analysis

Whole genome sequencing and data processing

DNA samples were extracted from the patients' family members according to salting out standard protocols. High-quality DNA from the proband was assessed by WGS, achieving 30-fold coverage with ≥95% of bases sequenced at 8× or higher coverage using an Illumina NovaSeq 6000 sequencer (Dante Labs, Inc., L'Aquila, Italy). DRAGEN™ Bio-IT Platform was employed to generate raw data files, including FASTQ R1, FASTQ R2, BAM and VCF files of single nucleotide polymorphisms (SNPs), indels, and CNVs.

GATK variant recalibrator was employed to implement Variant Quality Score Recalibration (VQSR) for SNPs and indels at 99.5% and 99.0% sensitivity thresholds, with additional criteria of GQ (≥ 20X) and DP (≥ 10X). VCF files were annotated using the Ensembl Variant Effect Predictor (VEP) command line tool (https://github.com/Ensembl/ensembl-vep).

Potentially pathogenic SNP and indel variants were identified by considering the minor allele frequency (typically <0.01-1%), Combined Annotation Dependent Depletion (CADD) score >10, non-benign predictions in CLIN_SIG, and deleterious effects using IMPACT (categorised as high, moderate, modifier, and low) filtration. Subsequently, restricted analysis was carried out following American College of Medical

Genetics (ACMG) guidelines and integrated data from the HGMD, HPO, OMIM and PubMed databases, and scholarly articles. Significant CNVs were detected and genotyped through read-depth (RD) analysis using CNVnator.

Pathogenicity interpretation

In silico prediction scores were obtained as follows to study the pathogenicity of the different variants: MutationTaster (http://www.mutationtaster.org/) (10), Mutation Assessor (http://mutationassessor.org/) (11), CADD (https://cadd.gs.washington. edu/) (12), deleterious annotation of genetic variants using deleterious annotation of genetic variants using neural networks.

(DANN) (https://cbcl.ics.uci.edu/public_data/DANN/.) (13), Polymorphism Phenotyping v2 (PolyPhen2) (http://genetics.bwh.harvard.edu/pph2/) (14), Sorting Intolerant From Tolerant (SIFT) (https://sift.bii.a- star.edu.sg/) (15), Functional Analysis through Hidden Markov Models (FATHMM-MKL) (http://fathmm.biocompute.org.uk/fathmmMKL.htm) (16), likelihood-ratio test (LRT) (http://genetics.wustl.edu/jflab/lrt_query.html), (17) and BayesDel (https://fengbj-laboratory.org/BayesDel/BayesDel.html) (18).

Genomic conservation scores were obtained from following programs: Phylogenetic p-value from the Phylogenetic Analysis with Space/Time models (PHAST) package (http://compgen.cshl.edu/phast/) for multiple alignments of 99 vertebrate genomes to the human genome (phyloP100way_vertebrate) (19) and Genomic Evolutionary Rate Profiling (GERP) (http://mendel.stanford.edu/SidowLab/downloads/gerp/) (20).

The frequency of the variant in the population was evaluated by comparison with variants from the following databases: dbSNP (https://www.ncbi.nlm.nih.gov/snp/), Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/), and Iranome (http://www.iranome.ir/).

ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) were used to identify previously reported variants. Candidate variants were assessed for pathogenicity according to ACMG criteria (21).

Family screening and Sanger sequencing

In order to verify the candidate p.Arg293Ter variant, we designed a specific set of primers using Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/). The forward primer LAMP2-F (5′-ccttcagggtaatccacagtc-3′) and reverse primer LAMP2-R (5′-gcagtttattctaccgcatg-3′) were utilised to amplify target sequences that contained the variant. The Sanger sequencing protocol used in this study was obtained from our previous publication (22).

The obtained sequencing results were then analysed using 4Peaks and compared with the *LAMP2* gene sequence from the NCBI database (NM_002294.3).

In silico protein structure prediction and visualisation

The protein sequence of LAMP2 was aligned using UniProtKB/Swiss-Prot P13473. The Simple Modular Architecture Research Tool (SMART) (https://smart.embl.de/smart/show_motifs.pl?ID=P11532) and the protein families database (Pfam) (http://pfam.xfam.org/protein) were used to determine the LAMP2 protein domains.

There are only two experimental structures of the LAMP2 protein in the Protein Data Bank (PDB) that are related to the transmembrane domain. In the SWISS-MODEL repository, four models are available; however, none of them cover all 410 amino acids. The complete structure of the Lamp2 protein (AF-P13473-F1-model-v4) is available in the AlphaFold Protein Structure Database with high confidence.

Homology/analogy recognition engine V2.0 (Phyre2) (23) and Deep-learning based Iterative Threading ASSEmbly Refinement (D-I-TASSER) were utilised to determine the effect of the candidate variant on the protein structure and function predictions. The three-dimensional (3D) structure of the wild-type and mutant LAMP2 protein visualised by D-I-TASSER (24). Additionally, the conservational study was conducted via the ConSurf server (25).

Pathogenic/likely pathogenic variants

Pathogenic/likely pathogenic variants in the *LAMP2* gene were systematically reviewed across multiple databases, including UniProt, ClinVar, VarSome, PubMed, and HGMD to identify all the published and unpublished variants up to March 2023. A literature review of *LAMP2* gene pathogenic variants in PubMed was performed using the keywords "Danon disease, *LAMP2*, variant, pathogenic, likely pathogenic, and gene". The mentioned databases were investigated, and duplicate records were excluded to avoid errors of overrepresentation. All variants were named according to the Human Genome Variation Database (HGVS). The ClinVar variation ID and submissions, as well as publication ID, were collected in the Table S1 (See Supplementary Online Information at www.celljournal.org).

Ethics approval and consent to participate

The study portion that involved human participants was carried out in accordance with the ethical standards set by the Ethics Committee of Tarbiat Modares University, Tehran, Iran (IR.MODARES.REC.1399.253). The study followed the ethical guidelines set forth in the latest update of the World Medical Association Declaration of Helsinki. Informed consent was acquired from all adult participants and,

for minors, consent was obtained from their parents or legal guardians for the genetic analysis. These procedures were carried out in accordance with national ethics regulations.

Results

Distribution of the p.Arg293Ter variant

We identified eight articles that pertained to the p.Arg293Ter variant. From these, six contained comprehensive clinical data and were selected for comparative analysis (Table 1). Nine patients carry the p.Arg293Ter variant, which is a germline pathogenic variant associated with Danon disease in the ClinVar database. The variant has been submitted to ClinVar five times (accession number: VCV000163812.12) between 2017 and 2020 by reputable submitters of ClinVar.

The cohort consisted of eight male patients and one female patient, consistent with the inheritance pattern and nature of Danon disease. Although the age of onset was not considered, the age range during the study was reported to be between 7 and 44 years for male patients and 35 years for the sole female patient. Three patients were from the United States of America, three cases were from Iran (the studied family), and the remaining individuals were from Italy, Greece, and Spain.

Iranian patients

Previously reported case

In 2018, Amin et al. (9) reported the case of a 30-year-old Iranian male who presented to Rajaie Hospital Cardiovascular Medical and Research Centre with exertional dyspnoea and a family history of cardiac failure in two older brothers. The patient had no notable musculoskeletal complaints, and normal mental health and speech. Electrocardiography revealed left axis deviation and left bundle branch block, whereas the echocardiography showed severe papillary muscle hypertrophy and mild left ventricular hypertrophy (LVH) with an ejection fraction (EF) of 45%. Initially, the patient received treatment for sarcomeric HCM, including an ICD. His condition deteriorated, with cardiomegaly and visible pulmonary congestion on chest X-ray. Progressive symptoms, such as peripheral oedema, ascites and muscle involvement, prompted genetic testing and muscle biopsy. Sequence analysis identified the p.Arg293Ter variant in the *LAMP2* gene, which confirmed Danon disease. Unfortunately, despite standard heart failure therapy, the patient succumbed to respiratory infection and cardiac pump failure.

Case (III-1), proband

A 9-year-old proband (III-1) male referred to Rajaie Cardiovascular Medical and Research Hospital due to recurrent chest pain, exertional dyspnoea, and palpitations. He had a history of myopia corrected with glasses and mild intellectual disability, including learning difficulties at school (not formally tested).

Physical examinations revealed normal tone, power, reflexes, and gait. However, his family reported mildly severe symptoms that consisted of difficulty walking, leg deformity, and proximal muscle weakness that occurred just before the patient's condition worsened in subsequent years. Clinical laboratory examinations that included blood counts, serum creatinine, and serum electrolyte levels were all within the normal range. Paediatric transthoracic echocardiography revealed severe LVH that caused a collapsed right ventricle (RV) due to interventricular septum hypertrophy (IVS >3 cm). Other findings included mild tricuspid regurgitation, severe diastolic dysfunction, and a left ventricular ejection fraction (LVEF) of 60%. HCM was diagnosed based on the echocardiogram and clinical indications, which led to treatment with a single-chamber ICD implantation.

Over the following years, despite standard heart failure treatments, the patient's condition deteriorated. Electrocardiogram abnormalities indicated LV hypertrophic remodelling, atrial rhythm issues, bi-atrial enlargement, and borderline long QT interval. Holter monitoring recorded various cardiac rhythms, including wide QRS tachycardia, supraventricular tachycardia, and premature beats. Subsequent echocardiography revealed severe atrial and ventricular enlargement, concentric LVH, severe right ventricular hypertrophy (RVH) with dysfunction, severe valves regurgitation and reduced LVEF (10-15%). The patient underwent two electrophysiology studies and ablation procedures. Despite interventions, the patient's condition worsened, and imaging studies showed cardiomegaly, hepatomegaly, and pulmonary abnormalities. Unfortunately, the patient passed away due to heart failure at the age of 14 despite medical efforts.

Case (II-5), mother

The mother of case III-1, a 35-year-old female, had a distinctly different heart condition, specifically DCM and left ventricular non-compaction cardiomyopathy (LVNC). Her echocardiogram indicated mildly enlarged LV (dilation), mildly reduced systolic function (EF: 35-40%) and global hypokinesia. She exhibited normal motor performance and muscle strength, with clinical laboratory test results within normal ranges. During 24-hour Holter monitoring, the patient experienced palpitations and dizziness, along with frequent premature ventricular contractions and short episodes of nonsustained ventricular tachycardia. External cardioversion was performed twice to restore normal sinus rhythm. Case II-5 experienced recurrent hospitalisations for decompensated heart failure over the years. She received an ICD and was in stable condition, having successfully delivered a child in the last three years.

Table 1: Reported clinical characteristics of individuals that carry the p.Arg293Ter variant

Origin	Gender	Age (Y)	First refer	Echocardiography results	Creatine kinase (U/L)	Cardiac conduction impairments	CMR	ICD	HTx	Extracardiac manifestations	Histopathology	Variant status	Reference
Italy	Male	12	Heart failure	Dilated LV Concentric hypertrophy Impaired systolic function	Increased (638 U/L)	-	-	-	Yes	-	Yes	De novo	(26)
USA	Male	19	Dyspnoea Chest pain	-	Increased (500 U/L)	-	-	--	Yes	Myopathy Cognitive impairment	Yes	Mother's death due to heart failure	(27)
USA	-	-	-	-	-	-	-	-	-	-	-	-	(8)
Spain	Male	44	-	Myocardial fibrosis EF 30%	-	Yes	-	-	-	-	-	De novo	(28)
USA	Male	15	Syncope Muscle weakness after HTx	Dilated LV Concentric hypertrophy EF 25%	Increased (660 U/L)	Yes	-	Yes	Yes	Myopathy Seizure Cognitive impairment	Yes	Mother's death due to heart failure (age 30 years)	(29)
Iran	Male	30	Dyspnoea	Severe hypertrophy Valves regurgitation EF 45%.	-	Yes	Cardiomegaly Prominent right heart Pulmonary congestion	Yes	-	Myopathy	-	2 older brothers died from heart failure	(9)
Greece	Male	-	Syncope Dyspnoea Chest pain	Concentric hypertrophy	Increased (267 U/L)	Yes	Extensive LGE of all myocardial walls sparing only the IVS	Yes	Yes	Cognitive impairment	-	Sister sudden death (age 1 year)	(30)
Iran	Male	7	Chest pain Dyspnoea Palpitations	Severe LVH Severe IVS hypertrophy Collapsed RV Valves regurgitation Impaired diastolic function EF 60% ---------- Bilateral/ biventricular enlargement Concentric hypertrophy Severe IVS hypertrophy Severe RVH Impaired systolic/ diastolic function Valves regurgitation Pulmonary insufficiency EF 10%–15%	-	Yes	Cardiomegaly Pulmonary congestion	Yes	-	Following walking difficulty, leg deformity, and proximal muscle weakness (mild severity) Mild cognitive impairment	-	Mother's cardiac symptoms	Case (III-1)*
Iran	Female	35	DCM LVNC	LV dilation Impaired systolic function Valves regurgitation Global hypokinesia EF: 35%–40% ---------- LV enlargement Impaired systolic/ diastolic function Mild RV enlargement Valves regurgitation Pulmonary/aortic insufficiency Mild to moderate pulmonic/aortic insufficiency Global hypokinesia EF: 20%	-	Yes	-	Yes	-	-	-	Son's death due to heart failure	Case (II-5)*

The results of echocardiography are presented for both the initial visit and the most recent examination. ICD; Implantable cardioverter-defibrillator, HTx; Heart transplantation, LGE; Late gadolinium enhancement, DCM; Dilated cardiomyopathy, LVNC; Left ventricular non-compaction cardiomyopathy, EF; Ejection fraction, LV; Left ventricle, RV; Right ventricle, IVS; Interventricular septum, LVH; Left ventricular hypertrophy, RVH; Right ventricular hypertrophy, and *; Shows the variant carriers within the family we investigated in this study.

Genotype-phenotype architecture

Patients with the p.Arg293Ter variant were systematically categorised according to the following parameters: publication source, patient demographics, referral concerns, cardiac assessments, laboratory values, conduction abnormalities, imaging reports, interventions, clinical manifestations, histopathology, genetic test results, family screening, and mortality details (Table 1).

Nine patients harboured the c.877C>T variant, with no reported ethnic associations, except for the two patients from Iran. The mean age of onset was approximately 14 years, consistent with previous findings for male patients with *LAMP2* variants. However, the proband in this study exhibited an earlier onset of cardiac symptoms, with a reported age of onset of seven years. Clinical presentations, cardiac exam results, and age at mortality differed between the two Iranian patients who had the same variant. One patient died in their 30s, while the proband passed away at age 14. All previously reported patients were male and presented with varying degrees of concentric LVH and the typical cardiac conduction abnormalities seen in Danon disease.

Of the nine patients, four underwent heart transplants and survived. Despite receiving preventive treatments, the two male patients from Iran ultimately experienced heart failure and were unable to receive a heart transplant, resulting in fatal outcomes.

Four male patients showed elevated serum creatine kinase levels and skeletal myopathy in their proximal and distal muscles, which was not age-related. Notably, three cases did not present noticeable skeletal myopathy symptoms at disease onset. The p.Arg293Ter variant was associated with a range of neurological symptoms, with varying severities. Four cases reported cognitive impairment, while one reported seizures. Typically, these neurological symptoms were mild, as observed in the proband in this study (Table 1).

Cardiac features

Dyspnoea was the most common cardiac complication in four patients at their initial visit, whereas chest pain was the most common cardiac complication reported by three patients at their first visit. Furthermore, two cases of episodic syncope and one case of early-onset heart failure were the first referral symptoms. Additionally, a female patient exhibited symptoms of DCM and LVNC. Cardiac hypertrophy patterns included four cases of concentric LVH, and ranged from mild to severe. LVEF ranged from 10 to 60%, and varied according to gender, age, and disease progression.

Systolic dysfunction was noted in three patients and two patients exhibited diastolic dysfunction. The proband and mother in the studied family displayed concurrent impairments in both systolic and diastolic function.

Cardiovascular guidance abnormalities were found in six patients, and the reported values were consistent with previous articles and guidelines. However, each patient exhibited a distinct phenotypic pattern (Table 1).

Cardiac imaging tools, such as MRI/chest X-rays revealed cardiomegaly and pulmonary congestion in two patients, and one patient exhibited a specific Danon-related pattern of extensive late gadolinium enhancement (LGE) that involved all myocardial walls except the interventricular septum.

Treatment involved the use of an ICD in five patients, while four patients underwent cardiac transplantation as the most aggressive intervention.

Regarding genetic status, two patients had de novo variants, while six had suspected family histories that included five with heart failure and one case of sudden death in first-degree relatives (Table 1).

Muscular and neurological involvement

Elevated creatine kinase levels were detected in four male patients, but not all cases were associated with myopathy symptoms. Nonetheless, the involvement of skeletal muscles was evident from the disease onset and continued late into disease progression in four patients. Skeletal disorders were characterised by diffuse myopathy in proximal and distal muscles, difficulty walking, leg deformities, and proximal muscle weakness.

Neurological manifestations were observed in four male patients, including cognitive impairment in three cases and rare occurrences of seizures and mental retardation reported in one patient.

Variant validation and familial segregation

Case (III-1), proband

The *LAMP2:*c.877C>T (p.Arg293Ter) variant was validated by Sanger sequencing and confirmed the hemizygous state and X-linked dominant inheritance. This variant led to a null variant (stop gain, nonsense) in the *LAMP2* gene. Loss-of-function is a well-established mechanism of this disease (Fig.1).

Case (II-5)

Familial segregation analysis indicated that the *LAMP2:*c.877C>T (p.Arg293Ter) variant originated from the proband's mother. The X-linked dominant inheritance was also confirmed by the heterozygous state of the mother, which corresponds to her phenotypic pattern.

The absence of *p.Arg293Ter* variant in clinically unaffected family members (father and sister) was confirmed by Sanger sequencing (Fig.1).

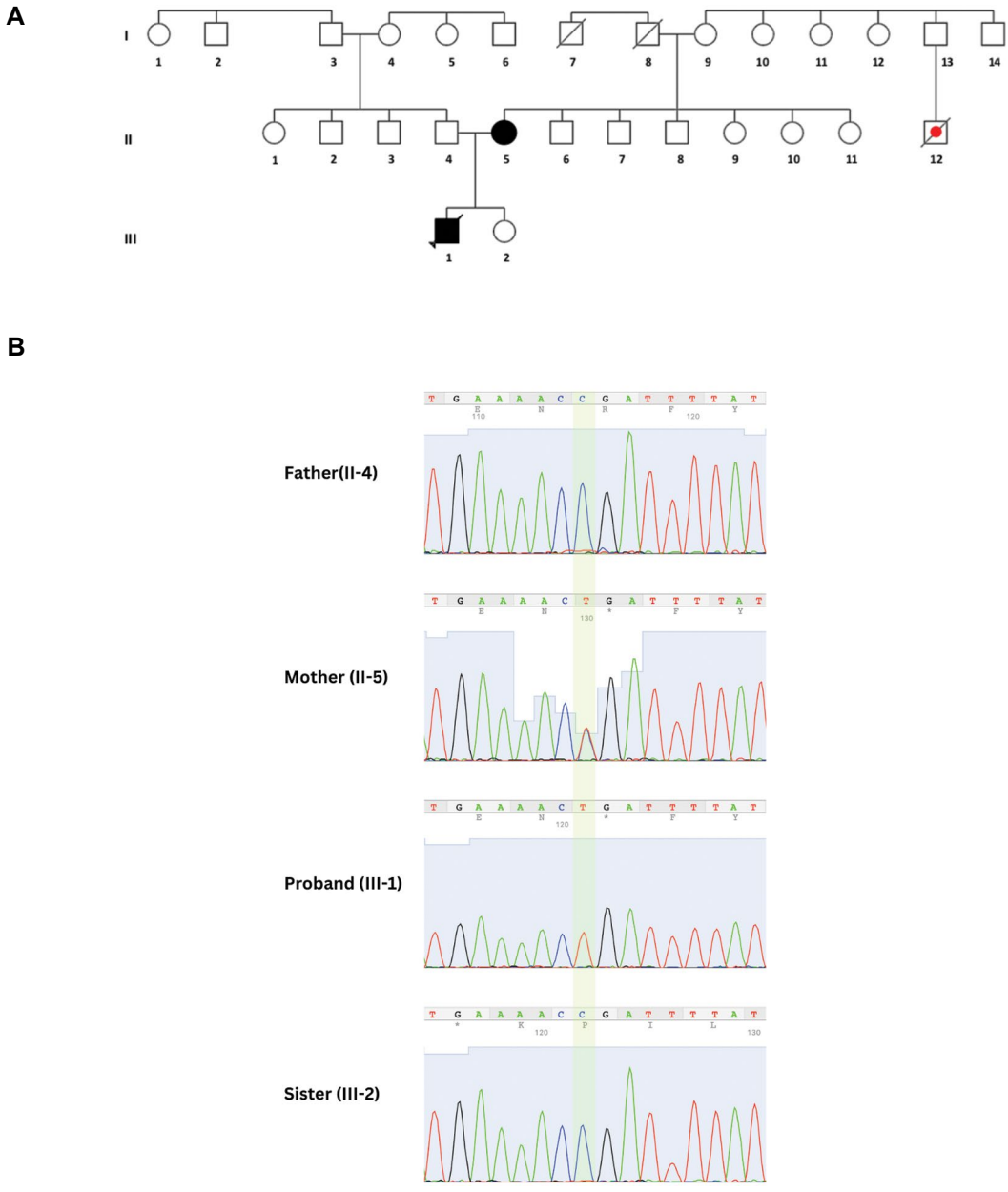

Fig.1: Family pedigree and genetic analysis results. **A.** Pedigree of the family with the lysosomal-associated membrane protein-2 (LAMP2) variant. The proband (III-1) is indicated by the arrow. Slashed symbols indicate the deceased members. **B.** The nonsense variant, c.877C>T in exon 7 of the *LAMP2* gene, is located at the hemizygous state in proband (III-1) and the heterozygous state in his mother (II-5). The proband's father and sister carry a normal allele. Circles; Females, Squares; Males, Black-filled shapes; Danon disease phenotype-positive subjects, Empty shapes; Unaffected subjects, and Red dot; Cardiac disorders.

Bioinformatic analysis p.Arg293Ter

Conservation analysis utilising PhyloP100way (value 3.7), showed conservation of the p.Arg293Ter variant. However, bioinformatics analysis of the p.Arg293Ter variant was predicted the variant to be damaging by BayesDel (addAF and noAF), FATHMM-MKL, GenoCanyon and MutationTaster, while other tools indicated varied interpretations. CADD predicted a PHRED score of 43, which indicated that these variants rank in the top 1% for deleteriousness in the human genome (Table 2). The ACMG guidelines (21) showed the candidate variant to be classifiable as "pathogenic" based on PVS1, PP5 and PM2 criteria.

In silico protein analyses of p.Arg293Ter

The human *LAMP2* gene spans 1233 nucleotides and is divided into 9 exons, encoding a 410 amino acid protein. Structurally, LAMP2 consists of three domains. The first is the lysosome-luminal domain, which includes exons 1 to 8 and a portion of exon 9. These domains are interconnected by a hinge region notable for its high proline content. There are two conserved disulfide bonds in each of the duplicated domains. The second is a transmembrane region (the remaining part of exon 9

encodes the transmembrane domain). The third is a short cytoplasmic tail at the C-terminal extremity.

The p.Arg293Ter variant occurs within the second luminal domain, specifically at position 229-375 of the LAMP2 protein.

Table 2: Bioinformatics tools and prediction scores of the *LAMP2:* c.877C>T (p.Arg293Ter) variant

Bioinformatics tools	Prediction	Score
BayesDel addAF	Damaging	0.618
BayesDel noAF	Damaging	0.649
CADD	Pathogenic	43
DANN	Uncertain	0.9982
FATHMM-MKL	Damaging	0.8429
Frequency in gnomAD	Not found	Not found
GenoCanyon	Deleterious	1
GERP	Uncertain	5.3
Iranome	Not found	Not found
LRT	Neutral	0.002944
Mutation Assessor	N/A	-
Mutation Taster	Disease causing	1
PhyloP100way	Conserve	9.5
PolyPhen2	N/A	-
SIFT	N/A	-
Splice AI	Splice-altering/moderate	0.21
SNP ID	rs727503118	-

LAMP2; Lysosomal-associated membrane protein-2, CADD; Combined annotation dependent depletion, DANN; Deleterious annotation of genetic variants using neural networks, FATHMM-MKL; Functional analysis through hidden markov models, GERP; Genomic evolutionary rate profiling, gnomAD; Genome aggregation database, LRT; Likelihood-ratio test, PolyPhen2; Polymorphism phenotyping v2, SIFT; Sorting intolerant from tolerant, and SNP; Single nucleotide polymorphism.

Using the Phyre2 web portal, LAMP2 was modelled based on the PDB entry 5gv0(A), and it represents the local structural environment of the Arg293 residue with 34% identity and 100% confidence. Secondary structure analysis revealed that the β-strand at amino acid 293 was predicted to be disordered and altered with a high score, which indicated reduced flexibility, dynamics, and extension. According to secondary structure model analysis, the p.Arg293Ter variant was predicted to alter the N-terminal β-strands, causing destruction of the second luminal domain ends, and the cytoplasmic tail of LAMP2 led to drastic conformational change.

The D-I-TASSER server ranked 5gv0A as the optimal output model, primarily due to the Arg293 location. The secondary structure for the normal sequence was predicted to be a strand, whereas it was predicted to be a coil in the Arg293 mutant, which resulted from the stop codon. The solvent accessibility prediction changed from a score of 3 (normal) to 8 (mutant), and indicated that the mutated protein at this position is more exposed than the normal sequence. The scores range from 0 to 8, with higher values indicative of greater exposure. The B-factor profile values showed a significant change, from -0.07 for the normal sequence at Arg293 (indicating a strand and being exposed) to 2.14 in the mutant sequence, which was predicted to be unstable, coiled, and highly exposed.

The enzyme function remained unchanged, with a C-scoreEC of 0.060 (scores range from 0 to 1, with higher values indicative of more reliable predictions). Predicted active site residues were not identified by D-I-TASSER analysis. Gene ontology (GO) values (C-scoreGO) showed slight changes in biological processes, molecular functions, and cellular components (Data not shown). Additionally, downstream residues of exon 7 were found to be highly conserved using the ConSurf web server. Mutant and wild-type 3D structures, generated by D-I-TASSER, exhibited significant structural alterations in line with the secondary structure analysis from Phyre2 (Fig.2).

LAMP2 is associated with Danon disease through 137 documented pathogenic LOF variants, which indicates a well-established loss-of-function mechanism. Among these variants, Arg293Ter, found in exon 7, significantly impacts the 'second lumenal domain' of the UniProt protein LAMP2_HUMAN. This alteration is predicted to cause nonsense mediated decay. Exon 7 contains seven pathogenic variants, while the truncated region includes 39 pathogenic variants, which supports a pathogenic criterion (PVS1) for this particular variant.

According to Uniport information, Arg293 is a monomethylation site identified in vivo (epithelial cells) via mass spectrometry. Sites modified by arginine monomethylation, like Arg293Ter, are known hotspots for disease-related mutations. Additionally, downstream of Arg293, there are N-Glycosylation sites at positions 300, 307, 312, 317, and 356, a disulfide

bond at position 331-368, and Phosphorylation sites at 304, 309, and 407. Disruption of these elements significantly impacts the protein's 3D conformation. LAMP2 functions as a receptor in the lysosomal membrane, and facilitates the degradation of substrate proteins like *GAPDH*, *NLRP3*, and *MLLT11*. Notably, four amino acids (GLKHHHAGYEQF) in the cytosolic tail are essential for binding to substrate proteins (Fig.2). The Arg293Ter variant disrupts this critical binding site, which results in the loss of LAMP2's proper cellular functionality and the development of Danon disease.

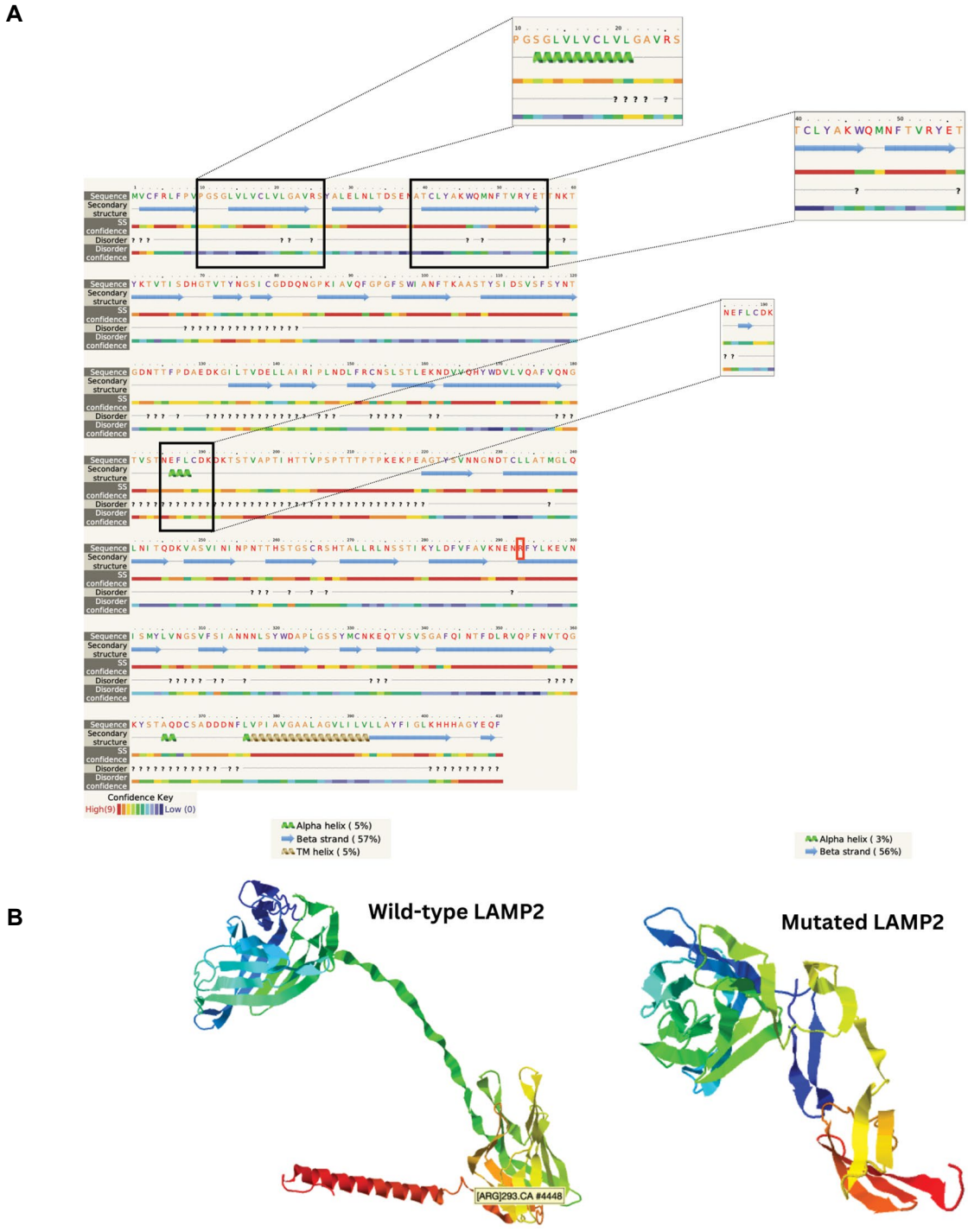

Fig.2: In-silico structural modeling of the identified mutation. **A.** Secondary structure model analysis of the lysosomal-associated membrane protein-2 (LAMP2) protein using Homology/analogY Recognition Engine V2.0 (PHYRE2). The left column indicates the protein sequence, secondary structure, and disorder for each line of the wild-type LAMP2 protein (left) and the mutated (p.Arg293Ter) protein (right). The confidence value score for each item is determined by the confidence key colour palette. Destruction of the end of the second luminal region, the cytoplasmic tail, and the alternation of the N-terminal β-strands lead to visible secondary structure changes. The boxes on the right side show the altered regions of the protein after the variant. **B.** Three-dimensional (3D) structure of wild-type (left) and mutated (right) LAMP2 as generated by Deep-learning based Iterative Threading ASSEmbly Refinement (D-I-TASSER) software.

Pattern of the *LAMP2* pathogenic/likely pathogenic variant

At the time of this consideration, 145 pathogenic/likely pathogenic *LAMP2* variants have been reported based on published and unpublished databases (Table S1, See Supplementary Online Information at www.celljournal.org).

Although pathogenic variants occur in all exons, they are most frequent in the last exon. Frameshift indels followed by nonsense variants are the majority (~85%) of pathogenic truncating variants that are predicted to cause the loss of the transmembrane region and cytoplasmic tail, both of which are critical domains for LAMP2 protein structure. Large pathogenic deletions are mainly found in exon 8, followed by exons 1 and 6, while one pathogenic duplication, 199bp, has been reported in exon 4. The splicing variants, either in the splice site or not within 2 bp of a splicing junction region, that cause LAMP2 deficiency have also been described in most of the exons. The five missense variants are only identified in exons 7 and 8, with the most common pathogenic variant belonging to p.Val310 (Table S1, See Supplementary Online Information at www.celljournal.org). It has been reported as pathogenic in nine submissions in ClinVar and various reports in the LOVD databases, as well as in nine articles published in PubMed until February 2023.

Discussion

Diagnosis and management of cardiac diseases can be difficult due to multiple non-cardiac symptoms and variable clinical presentations. Danon disease is a prime example of this challenge, as an accurate diagnosis is essential to prevent potentially severe complications. The significant heterogeneity in clinical manifestations and genetic variants associated with this disease further complicates its diagnosis and management. Herein, we report the first well-characterised family with Danon disease in Iran. In this study, bioinformatic analysis revealed that the p.Arg293Ter variant creates a premature translational stop signal, disrupts the consensus splice site in the *LAMP2* gene through changes in RNA splicing and ultimately affects protein structure, which results in an absent or disrupted protein product. Prior research has indicated that most *LAMP2* gene variants result in protein deficiency, including nonsense mutations, frame-shift deletions/insertions, and splicing site variants. In contrast, missense mutations and large rearrangements that impact one or more exons are infrequent. Notably, missense variants have a lower incidence of cardiomyopathy, while truncation variants have the earliest onset, followed by splicing and missense variants. While there is no established genotype-phenotype correlation in Danon disease, null variants lead to non-functional proteins, while other variants impact protein function (8, 31).

Our research contributes significantly to our understanding by assessing the prevalence of all pathogenic variants in the *LAMP2* gene, notably the p.Arg293Ter variant, which serves as a mutational hotspot linked to a range of clinical phenotypes. Among the more than 145 pathogenic variants identified in the *LAMP2* gene, c.926G>A and c.877C>T are two of the most frequently observed.

Significant disparities in disease outcomes and symptoms have been noted among different patients (5). The proband's mother in this study (case II-5) is the first female with the variant that has both LVNC and DCM. This specific cardiac phenotype, also known as dilated LVNC, has been previously found in disorders associated with the Titin (TTN), Lamin A/C (LMNA), and RNA binding motif protein 20 (RBM20) genes (32). The presence of non-compaction cardiomyopathy in Danon disease is unusual, with limited previous reports; to the best of our knowledge, this is the first documented instance of both DCM and LVNC that occur together (29, 33).

Clinical heterogeneity between genders could stem from the varying extent of X-chromosome inactivation, including random and skewed, which occur in female cells (34). Furthermore, it could also be due to the effect of oestrogen on autophagy and the lysosomal pathway (35). The phenotype of Danon disease in female patients remains inadequately elucidated relative to males, and leads to many undiagnosed cases in females. Lack of early diagnosis can cause uncompensable costs and lost opportunities for heart transplantation (HTx).

Female patients typically have milder symptoms and a later onset, with an average onset age of 19 years (8). Cardiac disease is an isolated clinical feature in 73% of female patients, but its severity is equal to that of males. HCM is the most commonly observed type of cardiomyopathy in both males and females. However, it is noteworthy that females have a lower prevalence of HCM and a higher prevalence of DCM compared to males (5).

Males with Danon disease experience a multisystem disorder that presents with cardiomyopathy, cognitive impairment, and skeletal myopathy, usually occurring around the age of 13 years. Other organs, including the retina and liver, may also be affected. Studies have indicated that 80-100% of male patients experience skeletal myopathy. However, the symptoms may not always be obvious; in some cases, the disease may progress and result in severe manifestations (36). In a review of patients with the p.Arg293Ter variant, four male patients showed a significant increase in serum creatine kinase and skeletal myopathy. However, no significant symptoms of skeletal myopathy were noted at the onset of the disease in three cases, including the two Iranian patients and a 44-year-old patient with end-stage HCM.

Studies have shown that 70-100% of patients have mild to moderate cognitive disabilities. Paediatric patients have

reported mild speech delay, attention deficits, autism, and behavioural problems. Brain CT scans and MRI imaging studies have indicated the involvement of the central nervous system (37). The p.Arg293Ter variant is linked to a range of neurological symptoms that are often mild and may only be noticeable to close family members, as seen in the case under examination in this study. The c.877C>T variant may arise from an ancestry in our population such as variants in other genes; however, further studies are required to prove a founder effect about this variant (38, 39).

An isolated cardiac phenotype has been reported in 18% of male cases, with HCM in 96% of affected patients. Conduction abnormalities have also been observed in up to 80% of cases. The most common electrocardiography finding is pre-excitation, specifically WPW syndrome, which occurs in approximately 48% of affected male (5, 8).

Echocardiography often reveals concentric LVH, especially in childhood-onset cases and this necessitates a heart transplant. In males, there is a rapid progression towards end-stage heart failure in their third decade of life, while females may experience this in their forties to fifties if the condition is not treated. Four patients with the p.Arg293Ter variant underwent heart transplants and survived. Despite preventive treatments, two patients from Iran eventually experienced heart failure and died.

A prevalence of 60% of Danon disease patients have ocular involvement that include strabismus, myopia, and retinopathy. Hepatomegaly has been observed in 36% of cases, often accompanied by elevated liver enzymes since childhood (40). Gastrointestinal complaints have been reported by 77% of individuals (8). The proband in this study had myopia and hepatomegaly, which aligns with these clinical findings. Advancements in genetic testing have led to a precise diagnosis of Danon disease, which is often initially misdiagnosed in HCM. Next-generation sequencing technology and bioinformatics have enabled the development of cost-effective and accurate diagnostic tools for genetic disorders such as cardiomyopathies. WGS analysis of intronic regions and the mitochondrial genome may also help identify pathogenic variants in 9% of HCM patients with a familial history and no causative variant identified through initial genetic testing (6).

The discovery prompted a genetic evaluation and cascade screening for the patient's family, which revealed the patient's mother as a carrier with compatible clinical manifestations. On the other hand, the intergender phenotypic variability in Danon disease underscores the importance of precise genetic analysis to prevent false negative results. An accurate phenotype characterisation that incorporates WGS along with complimentary copy number variation genetic analysis can offer the best possible care for affected families and enable family planning for their offspring.

Our inability to perform histopathological examinations of the proband's skeletal muscle and heart in our case series, given that the proband was deceased, is recognized as a limitation of this study.

Conclusion

This study provides important insights into the diagnosis and genetic analysis of Danon disease in Iran. Through a detailed case report and bioinformatic analysis, we shed light on the impact of the p.Arg293Ter variant in the *LAMP2* gene on protein structure and function. Our findings also highlight the high frequency of this variant among individuals with Danon disease. Overall, this study enhances our understanding of the disease and may help improve the diagnosis and management of affected individuals.

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Authors' Contributions

M.N., N.M.; Conceptualisation, Study design, Critical supervision of the manuscript, Data analysis, and Interpretation validation. S.K.; Experiment investigation, Performed formal analysis, Interpretation of data, and Writing- original draft preparation. M.D.; Surveyed the patients clinically and Participated in collecting clinical data of the patients. B.R.; Counselling and Participated in collecting clinical data of the patients. M.B.; Counselling and Research plan management. All authors read and approved the final manuscript.

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