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Do mitochondria contribute to left ventricular non-compaction cardiomyopathy? New findings from myocardium of patients with left ventricular non-compaction cardiomyopathy

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ABSTRACT

Background: Left ventricular non-compaction cardiomyopathy (LVNC) is a rare congenital cardiomyopathy that is associated with mutations in mitochondrial DNA (mtDNA), however, no study of myocardium mtDNA of LVNC patients has been reported. To identify novel candidate mtDNA variants that may be responsible for the pathogenesis of LVNC, myocardial specimens were examined to investigate pathogenic mtDNA variants.

Materials and methods: Samples from six patients who were diagnosed with LVNC and underwent heart transplantation were analyzed. The sequence and copy number of mtDNA from these samples were determined by Sanger sequencing and fluorescence-based quantitative polymerase chain reaction, respectively.

Results: Myocardial mtDNA sequences analysis revealed 227 substitution variants, including 157 coding variants and 70 non-coding variants. An m.9856T>C (Ile217Thr) mutation in *MT-CO3* from one LVNC patient was found to be a non-haplogroup associated variant, and was rare in the mtDB Human Mitochondrial Genome Database, suggesting that the variant may be pathogenic. And there was statistically significant difference in mtDNA copy number between LVNC patients and normal control subjects. Electron microscopy (EM) of left ventricular myocardium showed abnormality in mitochondrial morphology and disordered sarcomeric organization.

Conclusion: The identification of mtDNA sequence variants in myocardial specimens may be helpful for further investigation of the underlying pathogenic implications of myocardial mtDNA mutations in LVNC. However, measurement of mtDNA copy number showed that there was lower mtDNA content in myocardium of LVNC patients than in normal controls ($P<0.01$). Lower mtDNA copy number and morphological abnormalities of mitochondria suggested mitochondrial dysfunction that may be associated with etiology of LVNC.

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1. Introduction

Left ventricular non-compaction cardiomyopathy (LVNC, OMIM: #604169) is a rare congenital cardiomyopathy characterized by excessive and unusual hypertrabeculation of the myocardium. It is generally considered to be an arrest of myocardial morphogenesis during the later stages of the compact myocardium development, although the exact mechanisms of LVNC pathogenesis are still unclear [1]. Some evidence suggests that mutations in mitochondrial DNA (mtDNA) may contribute to LVNC [2]. The mitochondrial genome contains a control region and a coding region of 37 genes including two rRNAs, 22 tRNAs, and 13 polypeptides that are critical for oxidative phosphorylation. Mitochondria are involved in cell apoptosis, proliferation and heme metabolism, and play an important role in cardiomyocyte differentiation and development [3,4]. MtDNA defects may cause mitochondrial complex defects, mitochondrial malfunction, and ultimately impair embryonic heart development.

Pathogenic mtDNA mutations can have a wide range of manifestations, but usually start as a single organ disease, often affecting organs with high energy demands such as the brain, heart, eyes, and skeletal muscles [4]. Because of the high energy requirements of the heart, mtDNA mutations contribute to several cardiac disorders, such as biventricular hypertrophic cardiomyopathy, rhythm abnormalities and congestive heart failure [5]. Point mutations in the MT-ND1, MT-TL1, MT-ATP6, MT-ATP8 and MT-CYB genes were identified in some non-compaction patients [2], suggesting that mitochondrial dysfunction caused by mtDNA mutations might be involved in the pathogenesis of LVNC.

Abbreviations: LVNC, left ventricular non-compaction cardiomyopathy; mtDNA, mitochondrial DNA; rCRS, revised Cambridge Reference Sequence; CI, conversation index; *MT-CO3*, cytochrome c oxidase subunit 3 gene; *MT-ATP6*, ATPase subunit 6 gene; *MT-ND5*, NADH dehydrogenase subunit 5 gene.

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One cardiac myocyte contains thousands of mitochondria with hundreds of copies of mtDNA in each. The state of mtDNA copy number was more stable in myocardial tissue than in other tissues, and this was essential for normal cellular functions [6]. Certain mtDNA mutations may result in a replicative advantage and defective mtDNA appear more readily that may be aberrant mitochondrial biogenesis [7]. An increase in mtDNA copy number was recognized as a compensatory effect of defective mtDNA [8]. Study in transgenic mouse model showed that reduced mtDNA copy number was associated with cardiomyopathy [9]. Furthermore, mitochondrial abnormalities were observed in samples from skeletal muscles or from cardiac biopsy of LVNC patients [1]. Therefore, we hypothesized that this complex myocardium was associated with aberrant mtDNA replication and abnormal mtDNA copy number.

Previous studies of mtDNA mutations in LVNC patients were mostly performed by sequencing mtDNA from blood samples; however, mtDNA heteroplasmy between different tissues in same individual is ubiquitous [10]. Therefore, mtDNA in blood cells do not mirror the mtDNA in myocardial cells. Therefore, we speculated that mtDNA sequencing of myocardial tissues from six patients with pathologically characteristic LVNC may unveil novel candidate mtDNA variants or mutations responsible for this congenital cardiomyopathy. We also measured mtDNA copy number in myocardium samples from LVNC patients and normal control subjects to identify the differences between these groups.

2. Materials and methods

2.1. Patients

Six patients with definitive LVNC diagnoses, who underwent heart transplantation in our institution, were recruited for this study. Diagnostic criteria for LVNC were based on the following: (1) absence of other cardiac abnormalities; (2) color Doppler evidence of deeply perfused intertrabecular recesses; (3) two myocardial layers (outer untrabeculated [C] and an inner trabeculated [NC] region) measured in end-systole meeting quantitative criterion: NC:C ≥ 2 [5]; (4) pathological examination of the left ventricular myocardium revealed numerous apical trabeculations and a ratio of NC:C > 1.5. All patients met the diagnostic criteria for LVNC, and had no other clinical manifestation of mitochondrial disorder. All exons of the TAZ-tafazzin gene, which is essential for the structural integrity and bioenergetic function of mitochondria, were sequenced and no mutations were found. TAZ mutations lead to cardiomyopathy characterized by myocardial non-compaction [11,12]. One of the patients (#5) was diagnosed with LVNC and Ebstein's anomaly, a common type of congenital heart diseases usually associated with LVNC in adults [13]. Normal control hearts came from autopsies or donors with no history of heart disease who died in accidents. All participants gave informed written consent for this investigation, which was approved by the Institutional Ethical Review Board of Fuwai Hospital (Beijing, China). The investigation conforms to the principles outlined in the Declaration of Helsinki. The major clinical information of six LVNC patients is shown in Table 1.

2.2. Sanger sequencing of myocardial mtDNA

Genomic DNA was extracted from left ventricular myocardium of LVNC patients using a DNA extraction kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The entire length of the mtDNA genome was amplified using nine PCR primer pairs, and then sequenced with an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA) using 62 primers, according to a method described previously [14]. The mtDNA sequencing data of each patient was assembled using ContigExpress software [15].

Table 1
Clinical information of six LVNC patients.

Patient	Age[a]	Sex	Pathological description				Other symptoms
			NC:C	LV dilated	Thrombus	RV affected	
#1	15	F	3:1	No	No	Yes	
#2	14	M	3:1	Yes	Little	No	
#3	28	M	3:1	No	Little	No	
#4	15	M	2:1	Yes	No	No	
#5	13	M	2:1	Yes	No	No	Ebstein's syndrome
#6	35	M	2:1	Yes	No	No	Sinus arrhythmia

[a] Age of patients at heart transplantation operation.

2.3. Sequence analyses using online databases

We compared the complete mtDNA sequences of each patient with the revised Cambridge Reference Sequence (rCRS, GenBank accession NC_012920) to determine mtDNA variants and evolutionary conservation. We further determined the mtDNA haplogroups using MitoTool [16], MitoMap [17] and PhyloTree [18]. The frequencies of mtDNA variants were evaluated using the Human Mitochondrial Genome Database [19] and MitoMap. We excluded haplogroup-associated variants, while the remaining variants with allele frequencies <0.5% were defined as rare and those with frequencies of ≥0.5% were defined as common [20]. Exclusion of haplogroup-associated variants was an important screening criterion for finding potential mtDNA mutations. Specific haplogroup-associated variants are the result of ancient polymorphisms associated with distinct ethnic or geographic maternal lineages, and occur frequently in different populations [21]. Therefore, haplogroup-associated normal variants should not be interpreted as potentially pathogenic. The pathogenic potential of rare and novel mtDNA variants was evaluated based on the conservation index (CI) of the altered amino acid and by MitoTool. Relevant publications in the literature were also reviewed to evaluate the pathogenic possibility of mtDNA variants.

MtDNA variants that could potentially affect protein function and contribute to LVNC pathogenesis were defined as mutations. Additionally, rare variants that were associated with sub-haplogroups and were reportedly associated with human diseases were noted and analyzed by assessment of CI, the effect of the amino acid substitution, functional analysis, and family screening, prior to being excluded.

2.4. Quantitative PCR analysis of the mtDNA content

To compare the mtDNA copy number of LVNC patients and normal control subjects, genomic DNA was extracted from left ventricular myocardium of six LVNC patients and twenty normal control subjects as described above. Total mtDNA copy number was determined by fluorescence-based quantitative polymerase chain reaction (qPCR), using the estimation of threshold cycle number of the mitochondrial gene *MT-ND1*. The single-copy nuclear gene *HBB* was used for normalization [22]. Gene-specific real-time PCR primers were designed using Primer3 and verified for base complementarity [23]. The primer sequences were as follows: ND1, 5′-CAC CAG CCT AAC CAG ATT TC-3′, and 5′-GGG TTG TAT TGA TGA GATT AGT-3′. HBB, 5′-GCA AGG TGA ACG TGG ATG AA-3′, and 5′-TAA CAG CAT CAG GAG TGG ACA GA-3′. PCRs were performed using a DNA Master SYBR green I kit (Roche Diagnostics, Mannheim, Germany) and an Applied Biosystems 7300 Fast Real-Time PCR System (Foster City, CA, USA) following the manufacturer's instructions.

Purified DNA was quantified on NanoDrop ND-2000 UV-Vis spectrophotometer. The DNA samples were serially diluted down to a final concentration of 0.25 ng/μl in double-distilled water. Standard curves of both fragments were generated and their respective amplification efficiencies were calculated. Each sample DNA was amplified with MT-ND1 and nuclear gene HBB primer pairs in triplicate in separate

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