Screening for Mitochondrial tRNA Mutations in 318 Patients with Dilated Cardiomyopathy

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Keywords

Dilated cardiomyopathy · Mitochondrial tRNA · Mutations · Mitochondrial dysfunction

Abstract

Objectives: Dilated cardiomyopathy (DCM) is a complex cardiovascular disease with unknown etiology. Although nuclear genes play active roles in DCM, mitochondrial dysfunction was believed to be involved in the pathogenesis of DCM. The objective of this study was to analyze the association between mitochondrial tRNA (mt-tRNA) mutations and DCM. **Materials and Methods:** We performed a mutational analysis of mt-tRNA genes in a cohort of 318 patients with DCM and 200 age- and gender-matched control subjects. To further assess their pathogenicity, phylogenetic analysis and mitochondrial functions including mtDNA copy number, ATP, and ROS were analyzed. **Results:** Seven possible pathogenic mutations, i.e., MT-TL1 3302A>G, MT-TI 4295A>G, MT-TM 4435A>G, MT-TA 5655T>C, MT-TH 12201T>C, MT-TE 14692A>G, and MT-TT 15927G>A, were identified in the DCM group but were absent in controls. These mutations occurred at extremely conserved nucleotides of corresponding tRNAs and led to the failure in tRNAs metabolism. Moreover, a significant reduction in ATP and mtDNA copy number and a marked increase in ROS level were observed in polymononuclear leukocytes (PMNs) derived from the DCM patients carrying these mt-tRNA mutations, suggesting that these

mutations may cause mitochondrial dysfunction that was responsible for DCM. **Conclusions:** Our data indicated that mt-tRNA mutations may be the molecular basis for DCM, which provides novel insights into the pathophysiology of DCM that manifested as mitochondrial dysfunction.

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Introduction

Dilated cardiomyopathy (DCM) is a nonischemic heart muscle disease with structural and functional myocardial abnormalities characterized by dilation of the ventricular chamber and impaired contraction [1]. Patients with DCM typically exhibit ventricular enlargement and subsequent cardiac dysfunction, which can be accompanied by arrhythmia, thromboembolism, or sudden death [2, 3]. The incidence of DCM is reported to be 5–7 cases of 100,000 people annually [4]. However, to date, the molecular mechanisms that trigger the development of DCM remain poorly understood.

Cardiac tissue requires a persistent production of energy in order to exert its pumping function [5, 6]. Therefore, maintenance of this function relies on mitochondria that represent the “powerhouse” of all cardiac activities [7]. In these cells, mitochondria occupy ~30% of the total

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volume of the cell and supply, through oxidative phosphorylation (OXPHOS), approximately 6 kg of ATP per day that is required to sustain cardiac function [8]. Indeed, human mitochondrial DNA (mtDNA) encodes 13 subunits of the respiratory chain complexes, 22 tRNAs, and 2 rRNAs; the replication, transcription, translation, and repair of mtDNA are controlled by proteins encoded by nuclear DNA (nDNA) [9]. Due to the lack of histone protection and a poor DNA repair system, mtDNA has a much higher mutation rate than nDNA [10]. Most recently, several mtDNA mutations have been reported to be associated with DCM, such as 3243A>G and 3303C>T mutations in MT-TL1 [11, 12], 15024G>A and 15482T>C mutations in MT-CYB gene [13], and 12338T>C mutation in MT-ND5 gene [14]. However, these studies reported only on relatively small-sized samples of pedigrees, while the spectrum and association of mutations in 22 mt-tRNA with DCM in large populations remain to be explored.

With this aim, in the current study, we first performed a mutational analysis of 22 mt-tRNA genes in a cohort of 318 subjects with DCM and 200 controls. These mutations were further assessed by phylogenetic evaluations as well as structural and mitochondrial functional analysis. As a result, we identified 7 possibly pathogenic mutations in mt-tRNA genes.

Materials and Methods

Study Population

The study population was composed of 318 patients (200 males and 118 females, aged 59 ± 15 years) with a clinical diagnosis of DCM, who were recruited from Tianjin Chest Hospital. All participants had Han ethnicity. The diagnosis of DCM was performed in the presence of left ventricular ejection fraction <50% at the time of diagnosis in the absence of any known possible cause of left ventricular dysfunction [15, 16]. Moreover, 200 healthy subjects (110 males and 90 females, aged 49 ± 13 years) were recruited from the Healthy Examination Center of our hospital. The inclusion criteria were as follows: healthy subjects without any diseases, no history of cardiovascular disease, no drug use, normal diet, and alcohol consumption <30 g/day. Exclusion criteria included a family history of mitochondrial disease, such as hearing loss, vision loss, neurological disease, or cancer; individuals who were taking regular medication were also excluded. All participants signed the informed consent prior to inclusion. The study was approved by the ethics committee of Tianjin Chest Hospital and conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice (approval number: 2020YS-030-01).

Mutational Analysis of mt-tRNAs

Blood samples from each subject were collected in the morning between 7:00 a.m. and 10:00 a.m. after an overnight fast. The ge-

nomic DNA was isolated from peripheral blood of all the subjects using Paxgene Blood DNA Isolation Kits (QIAGEN). The fragments spanning entire tRNA genes of 318 Han Chinese patients with DCM and 200 controls were PCR amplified by use of sets of the light-strand and the heavy-strand oligonucleotide primers, as described elsewhere [17] (Table 1). In individuals found to have potentially pathogenic mutations in tRNA genes, fragments that spanned the remainder of the mitochondrial genome were also amplified via PCR and were used to establish the mtDNA haplogroups of these individuals. The PCR reagents (all obtained from Takara Bio, Inc.) were as follows: 200 μ M dNTPs, 2 μ L 10 $\times$ PCR buffer (10 $\times$ 0.2 μ L Taq DNA polymerase, and 15 mmol/L Mg^{2+}). PCR was performed under the following thermocycling conditions: 95°C for 5 min, 94°C for 10 s, 60°C for 30 s, 30 cycles at 72°C for 60 s, and 72°C for 10 min for extension. Bidirectional sequencing in both directions was conducted in order to confirm amplicon sequences, after which sequences were compared with the revised Cambridge Reference sequence (rCRS, GenBank accession number: NC_012920.1) [18].

Phylogenetic Analysis

A total of 17 vertebrates mtDNA sequences were used in the interspecific analysis. These species were as follows: *Bos taurus*, *Cebus albifrons*, *Colobus guereza*, *Gorilla gorilla*, *Homo sapiens*, *Hylobates lar*, *Lemur catta*, *Macaca mulatta*, *Macaca sylvanus*, *Mus musculus*, *Nycticebus coucang*, *Pan paniscus*, *Pan troglodytes*, *Pongo pygmaeus*, *Pongo pygmaeus abelii*, *Tarsius bancanus*, and *Papio hamadryas*. The conservation index (CI) was calculated by comparing the human mtDNA mutations with other 16 vertebrates. A CI $\geq 75\%$ was regarded to have functional importance [19].

Haplogroup Classification

The entire mtDNA sequences of 9 subjects with putative pathogenic mt-tRNA mutations were assigned to the Asian mitochondrial haplogroups based on the PhyloTree database (<http://www.phyloree.org>) [20].

mtDNA Copy Number Analysis

The mtDNA copy number was assessed by real-time quantitative PCR using an Applied Biosystems 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to a previous report [21]. The following primers were used for real-time PCR analysis: for β -globin gene, forward: 5'-CTATGGGAC-GCTTGATGT-3', reverse: 5'-GCAATCATTCGTCTGTTT-3'; for mtDNA (mt-ND1 gene), forward: 5'-CACCAGCCTAACCA-GATTTTC-3', reverse: 5'-GGGTTGTATTGATGAGATTAGT-3'.

Isolation of Human Polymononuclear Leukocytes

The human polymononuclear leukocytes (PMNs) of the 9 subjects with putative pathogenic mt-tRNA mutations, as well as those of 9 healthy subjects, were isolated according to the protocol described previously [22]. Cells were grown in Hank's buffer salt solution before further analysis.

Analysis of ATP Production

The levels of ATP production from 9 subjects with putative pathogenic mt-tRNA mutations, together with those from 9 healthy subjects, were measured using the protocols described elsewhere [23].

Table 1. Primer sequences for amplification of 22 mt-tRNAs

Target gene	Primer name	Primer Sequence (5'-3')	T _m , °C	Product size
MT-TF	MT-1F MT-1R	CTCCTCAAAGCAATACACTG TGCTAAATCCACCTTCGACC	61	802 bp
MT-TV	MT-2F MT-2R	CGATCAACCTCACCACCTCT TGGACAACCAGCTATCACCA	58	802 bp
MT-TL1	MT-4F MT-4R	AAATCTTACCCCGCCTGTTT AGGAATGCCATTGCGATTAG	60	887 bp
MT-TI MT-TQ MT-TM	MT-6F MT-6R	TGG CTC CTT TAA CCT CTC CA AAG GAT TAT GGA TGC GGT TG	60	898 bp
MT-TA MT-TN MT-TC	MT-8F MT-8R	CTAACCGGCTTTTGGCC ACCTAGAAGGTTGCCTGGCT	60	814 bp
MT-TS1 MT-TD	MT-11F MT-11R	ACGCCAAAATCCATTTCACT CGGGAATTGCATCTGTTTT	58	987 bp
MT-TK	MT-12F MT-12R	ACG AGT ACA CCG ACT ACG GC TGG GTG GTT GGT GTA AAT GA	60	900 bp
MT-TG MT-TR	MT-15F MT-15R	TCTCCATCTATTGATGAGGGTCT AATTAGGCTGTGGGTGGTTG	60	891 bp
MT-TH MT-TS2 MT-TL2	MT-18F MT-18R	TATCACTCTCCTACTTACAG AGAAGGTTATAATTCCTACG	55	866 bp
MT-TE	MT-21F MT-21R	GCATAATTAACTTTACTTC AGAATATTGAGGCGCCATTG	55	938 bp
MT-TT MT-TP	MT-22F MT-22R	TGAAACTTCGGCTCACTCCT GAGTGGTTAATAGGGTGATAG	60	1,162 bp

T_m, annealing temperature.

ROS Analysis

The ROS level was analyzed by fluorometry. 2×10^6 cells were first incubated with the fluorescent probe 2',7'-dichlorodihydro-fluorescein (DCFH) for about 30 min, then the cells were analyzed by fluorescence plate reader, as described elsewhere [24].

Statistical Analysis

Student's *t* test was used to assess the statistical significance between unpaired samples. All analyses were performed using the SPSS software version 22.0. We regarded a $p < 0.05$ as statistically significant.

Results

Clinical Presentation

From January 2018 to January 2020, a total of 318 patients with a clinical diagnosis of DCM from Tianjin

Chest Hospital participated in this study. The ratio of males to females was 1.69:1. The age of these patients ranged from 42 to 78 years, with a median age of 55 years. The age at onset of DCM was 40–75 years, with an average of 59 years. In the DCM group, left ventricular ejection fraction was $31.55 \pm 9.55\%$ and left ventricular end diastole diameter was 6.76 ± 0.83 mm. Furthermore, 200 healthy Han Chinese subjects who did not have a family history of mitochondrial diseases were obtained from the same region. The age of these participants ranged from 35 to 55 years, with a median age of 42 years.

Mutational Screening for DCM-Associated mt-tRNA Mutations

We sequenced the complete mt-tRNA genes in a cohort of 318 affected subjects with DCM and 200 healthy controls. By comparing with the rCRS [18], 7 potentially

Table 2. Molecular features of DCM-associated mt-tRNA mutations

Genes	Alternations	Homoplasmy or heteroplasmy	CI, %	Watson-Crick base-pairing ^a	Number of nucleotide in tRNAs	Location	Patients, n (%) (n = 318)	Controls, n (%) (n = 200)	Disease association	Ref
MT-TL1	3302A>G	Heteroplasmy	100	T-A↓	71	Acceptor arm	1 (0.31)	0	PCOS, MELAS, myopathy	44, 45
MT-TI	4295A>G	Homoplasmy	100		37	Anticodon stem	1 (0.31)	0	Deafness, hypertension	47
MT-TM	4435A>G	Homoplasmy	100		37	Anticodon stem	2 (0.62)	0	LHON, deafness, hypertension	48
MT-TA	5655T>C	Homoplasmy	100	T-A↓	1	Acceptor arm	1 (0.31)	0	Deafness, hypertension	49–51
MT-TH	12201T>C	Homoplasmy	100	T-A↓	68	Acceptor arm	1 (0.31)	0	Deafness	52, 53
MT-TE	14692A>G	Homoplasmy	100		55	T loop	1 (0.31)	0	MIDD	54
MT-TT	15927G>A	Homoplasmy	100	C-G↓	42	Anticodon stem	2 (0.62)	0	Coronary heart disease, deafness, LHON	55, 56

CI, conservation index; PCOS, polycystic ovarian syndrome; MELAS, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; LHON, Leber's hereditary optic neuropathy; MIDD, maternally inherited diabetes and deafness. ^a Classic Watson-Crick (W-C) base pair: abolished (↓).

pathogenic mutations were identified: MT-TL1 3302A>G, MT-TI 4295A>G, MT-TM 4435A>G, MT-TA 5655T>C, MT-TH 12201T>C, MT-TE14692A>G, and MT-TT 15927G>A. Particularly, 2 patients carried the 4435A>G mutations, 2 patients harbored the 15927G>A mutations, while other pathogenic mutations, including 3302A>G, 4295A>G, 5655T>C, 12201T>C, and 14692A>G, were detected in 1 subject each (Table 2).

Assessment of the Pathogenicity

To further assess the pathogenicity of mt-tRNA mutations, we used the following criteria: first, the mutation itself occurred <1% in control subjects; second, the CI was ≥75%, as proposed by Ruiz-Pesini and Wallace [25]; third, the mutation may have a functional impact on mt-tRNA genes. For this purpose, we first performed the phylogenetic conservation analysis of these mt-tRNA mutations, as shown in Table 2. We noticed that all these tRNA mutations were well conserved from 17 species. In addition, the locations of these mutations within tRNA secondary structures are shown in Figure 1. Of these, 3 mutations occurred at the acceptor arm, 3 mutations were localized at the anticodon stem, and 1 mutation occurred at T loop. Moreover, 4 mutations (3302A>G, 5655T>C, 12201T>C, and 15927G>A) disrupted the highly conserved base-pairings and may have functional significance. In particular, as shown in Figure 2, sequence alignment of MT-TH gene revealed that the 12201T>C mutation was very conserved from different species (CI = 100%). Similarly, the CIs of other mutations (3302A>G, 5655T>C, 4435A>G, 14692A>G, and 15927G>A) were also evolutionary conserved. All these mutations were identified in the DCM group only, but were absent in 200 controls.

Clinical Features of 9 Probands Carrying 1 of Putative Pathogenic mt-tRNA Mutations

As shown in Table 3, 9 patients (4 males and 5 females) with DCM carried the pathogenic/likely pathogenic tRNA mutations, accounting for 2.8% of the cases in our cohort. The age at onset of DCM in these subjects ranged from 44 to 68 years, with a mean age of 57 years. Moreover, a comprehensive medical history showed that 4 probands had a family history of cardiovascular diseases, such as hypertension, coronary heart disease, and heart failure.

Analysis of the Complete mtDNA Genomes in 9 DCM Individuals Carrying 1 of the Pathogenic/Likely Pathogenic mt-tRNA Mutations

We further sequenced the whole mitochondrial genomes of 9 DCM patients harboring 1 of the potentially

Fig. 1. Cloverleaf structures of MT-TL1, MT-TI, MT-TM, MT-TA, MT-TH, MT-TE, and MT-TT. Arrows indicate the positions of DCM-associated mtDNA mutations.

pathogenic mt-tRNA mutations to examine if there were any other functional mtDNA mutations. As shown in Table 3, 6 mtDNA functional mutations were identified by Sanger sequencing. Of them, the MT-ND1 3398T>C mutation co-existed with the MT-TI 4295A>G mutation, the known CO1/MT-TS1 7444G>A co-existed with the MT-TM 4435A>G mutation, MT-ND6 14502T>C co-existed with MT-TA 5655T>C, and the MT-TH 12201T>C mutation was found in 1 patient carrying the MT-ND2 5178C>A mutation, MT-TE 14692A>G was identified in a patient with the MT-ND5 12811T>C mutation, and the MT-TT 15927G>A mutation occurred in a patient carrying the MT-RNR1 1095T>C mutation. Interestingly, the 3398T>C mutation has been found to contribute to mitochondrial disorders, such as mitochondriopathy [26], left ventricular noncompaction [27], and hearing loss

[28]. By molecular level, the 3398T>C mutation resulted in the substitution of a highly conserved methionine for a threonine at amino acid position 31 of the MT-ND1 gene. Biochemical analysis revealed that this mutation caused a reduction of complex I activity and impaired the OXPHOS function in defective mitochondrial respiratory chain complex hepatocytes [29]. Moreover, the 7444G>A mutation resulted in a read-through of the stop codon AGA of the MT-CO1 message, thereby adding 3 amino acids (Lys-Gln-Lys) to the C-terminal of the polypeptide. Thus, the mutated polypeptide may retain a partial function. Alternatively, the 7444G>A mutation was adjacent to the site of the 3'-end endonucleolytic processing of the L-strand RNA precursor, spanning MT-TS1 and ND6 mRNA [30]. A previous study suggested that the 7444G>A mutation may modulate the clinical expression

12201

Organism	Acc-stem	D-stem	D-loop	D-stem	Ac-stem	Anticd-loop	Ac-stem	V-region	T-stem	T-loop	T-stem	Acc-stem			
	1	9	10	15	22	26	27	32	39	49	58	61	66 68	73	
<i>Bos taurus</i>	GTAAATA	TA	GTTT	AACAA	AAAC	A	TTAGA	TTGTGAA	TCTAA	CAAT	AGAAA	CTCATTAC	CTTCT	TATTTAC	C
<i>Cebus albifrons</i>	GTAGCTA	TA	GTTT	AATTA	AAAC	A	TTAGA	TTGTGAA	TCTAA	TAAT	AGAAG	ACTACAA	CTTCT	TATCTAC	C
<i>Colobus guereza</i>	GTAAATA	TA	ATTT	AACTA	AAAT	A	CTAGA	TTGTGAA	CCTAG	ACAT	AGAAG	CTTACCG	CTTCT	TATTTAC	C
<i>Gorilla gorilla</i>	GTAAATA	TA	GTTT	AACCA	AAAC	A	TCAGA	TTGTGAA	TCTGA	TAAC	AGAGG	CTCACAA	CCCCT	TATTTAC	C
<i>Homo sapiens</i>	GTAAATA	TA	GTTT	AACCA	AAAC	A	TCAGA	TTGTGAA	TCTGA	CAAC	AGAGG	CTTACGA	CCCCT	TATTTAC	C
<i>Hylobates lar</i>	GTAAACA	TA	GTTT	AATCA	AAAC	A	TTAGA	TTGTGAA	TCTAA	CAAT	AGAGG	CTCGAAA	CCTCT	TGTTTAC	C
<i>Lemur catta</i>	GTAAATA	TA	GTTT	AAAA	AAAC	A	CTAGA	TTGTGAA	TCCAG	AAAT	AGAAG	CTCAAAAC	CTTCT	TATTTAC	C
<i>Macaca mulatta</i>	GTAGATA	TA	GTTT	AACTA	AAAC	A	TTAGA	TTGTGAA	TCTAA	CCAC	AGAGA	CTTACCA	CCTCT	TATTTAC	C
<i>Macaca sylvanus</i>	GTAAATA	TA	GTTT	AATTA	AAAC	A	TTAGA	CTGTGAA	TCTAA	CTAT	AGAAG	CTTACCA	CTTCT	TATTTAC	C
<i>Mus musculus</i>	GTGAATA	TA	GTTT	ACAAA	AAAC	A	TTAGA	CTGTGAA	TCTGA	CAAC	AGGAA	ATAAAC	CTCCT	TATTCAC	C
<i>Nycticebus coucang</i>	GTAAATA	TA	GTTT	AACAA	AAAC	A	TTAGA	TTGTGAA	TCTAA	CAAT	AGAAG	ACTTAAC	CCTCT	TATTTAC	C
<i>Pan paniscus</i>	GTAAATA	TA	GTTT	AACCA	AAAC	A	TCAGA	TTGTGAA	TCTGA	CAAC	AGAGG	CTCACGA	CCCCT	TATTTAC	C
<i>Pan troglodytes</i>	GTAAATA	TA	GTTT	AACCA	AAAC	A	TCAGA	TTGTGAA	TCTGA	CAAC	AGAGG	CTCACGA	CCCCT	TATTTAC	C
<i>Pongo pygmaeus abelii</i>	GTAAATA	TA	GTTT	AACCA	AAAC	A	TCAGA	TTGTGAA	TCTAA	TAAT	AGGGC	CCACAA	CCCCT	TATTTAC	C
<i>Tarsius bancanus</i>	GTAAATA	TA	GTTT	AAACA	AAAC	A	TTAGA	TTGTGAG	TCTAA	CAAT	AGAAG	CCAAAAA	TTTCT	TATTTAC	C
<i>Papio hamadryas</i>	GTAGACA	TA	GTTT	AATCA	AAAC	G	TTAGA	CTGTGAA	TCTAA	CTAT	AGAAG	CCCACCA	CTTCT	TGTTTAC	C

Fig. 2. Alignment of MT-TH gene from different species. The arrow indicates the position 68, corresponding to the m.12201T>C mutation.

Table 3. mtDNA genetic background and clinical characterization of 9 probands carrying 1 of pathogenic/likely pathogenic DCM-associated mt-tRNA variants

Gene	Mutations	Probands	Sex	Age at onset, years	Age at test, years	Family history	Other functional mtDNA variants	Haplogroup
MT-TL1	3302A>G	DCM-066	Male	67	70	No	None	F2
MT-TI	4295A>G	DCM-102	Female	55	60	No	MT-ND1 3398T>C	D4
MT-TM	4435A>G	DCM-179	Male	68	72	Mother and brother have hypertension, coronary heart disease	None	G2a
	4435A>G	DCM-128	Female	49	51	No	CO1/MT-TS1 7444G>A	D4
MT-TA	5655T>C	DCM-095	Male	59	61	Mother and sister have coronary heart disease	MT-ND6 14502T>C	M22b
MT-TH	12201T>C	DCM-228	Male	44	47	No	MT-ND2 5178C>A	Z3
MT-TE	14692A>G	DCM-300	Female	58	58	Mother has coronary heart disease	MT-ND5 12811T>C	G1a1
MT-TT	15927G>A	DCM-153	Female	53	54	No	MT-RNR1 1095T>C	B5
	15927G>A	DCM-231	Female	59	62	Mother died of heart failure	None	B5b2

Fig. 3. Analysis of mitochondrial functions in DCM patients carrying putative pathogenic mt-tRNA mutations and controls. **a** mtDNA copy number. **b** ATP qualification. **c** ROS level.

of deafness-associated MT-RNR1 1555A>G mutation [31]. While the homoplasmic 14502T>C mutation caused the substitution of a highly conserved isoleucine for valine at position 58 in MT-ND6 [32], this mutation was regarded to play a synergistic role in increasing the penetrance and expressivity of Leber's hereditary optic neuropathy (LHON)-associated MT-ND4 11778G>A mutation [33]. On the other hand, the MT-ND2 5178C>A mutation has been associated with longevity and type 2 diabetes mellitus [22, 34], and the 12811T>C mutation is believed to affect the clinical expression of LHON-related 11778G>A mutation in a Han Chinese population [35], while the 1095T>C mutation is a risk factor for hearing impairment [36]. Thus, we proposed that the combination of these secondary and primary mtDNA mutations may be involved in the pathogenesis of DCM.

In addition, the complete mitochondrial genomes of 9 subjects carrying 1 of putative pathogenic mt-tRNA mutations have been assigned to East Asian mitochondrial haplogroups based on the PhyloTree database (<http://www.phylotree.org/>) [20]. According to their distinct sets of polymorphisms, the mtDNA of these 9 subjects belonged to East Asian mitochondrial haplogroups F2, D4, G2a, D4, M22b, Z3, G1a1, B5, and B5b2, respectively [37].

Analysis of mtDNA Content

As shown in Figure 3a, we found that patients carrying these putative pathogenic mt-tRNA mutations had a lower level of mtDNA copy numbers when compared to the controls ($p = 0.013$).

Decrease in ATP Production

A defect in ATP production is an important cause for mitochondrial dysfunction. For this purpose, the ATP level was examined by luciferin/luciferase assay, as shown in Figure 3b. We noticed that DCM patients with these

mt-tRNA mutations had a much lower level of ATP when compared to the healthy subjects ($p = 0.0134$).

ROS Increased Significantly

Since mitochondria generate ATP via OXPHOS and release ROS as a toxic byproduct, overproduction of ROS may cause mitochondrial dysfunction [38]. For this purpose, the ROS levels in DCM patients with putative pathogenic mt-tRNA mutations and controls were analyzed, as displayed in Figure 3c. The ROS levels in the DCM group were significantly higher than in control subjects ($p = 0.0127$).

Discussion

In the present study, we analyzed the frequencies of mt-tRNA mutations in a cohort of 318 patients with DCM and 200 age- and gender-matched controls. To the best of our knowledge, this is the first report regarding the association of mt-tRNA mutations with DCM. In fact, mitochondria are most abundant in cells with high energy demands, notably striated muscles. Moreover, the heart is the most metabolically active organ in the body and possesses the highest content of mitochondria of any tissue [39], comprising about 25% of cell volume in the human myocardium [40]. Mutations in mtDNA have the potential to affect OXPHOS and in turn cellular death, but the mechanisms whereby these mutations cause DCM are largely unknown [41].

Through PCR and direct sequencing analysis, we identified 7 potentially pathogenic mutations: MT-TL1 3302A>G, MT-TI 4295A>G, MT-TM 4435A>G, MT-TA 5655T>C, MT-TH 12201T>C, MT-TE 14692A>G, and MT-TT 15927G>A. Of these, the 3302A>G mutation was located 2 nucleotides away from the 5' terminal MT-TL1,

which was highly conserved from different species. Recent experimental studies indicated that the 3302A>G mutation caused the severe respiratory chain complex I deficiency and lowered complex IV activity [42]. Moreover, a significant reduction in the steady-state level of MT-TL1 was observed in cells carrying this mutation [43]. Clinically, the 3302A>G mutation was found to be associated with polycystic ovary syndrome [44] and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like symptoms (MELAS) [45]. In addition, the 4295A>G and 4435A>G mutations affected a highly conserved adenosine at position 37 in the anticodon stem of MT-TI and MT-TM, respectively. Notably, both of them introduced a tRNA methyltransferase 5 (TRMT5)-catalyzed m¹G37 modification of the corresponding tRNAs [46]. Functional analysis of cybrid cells containing these mutations showed significant impairment in the steady-state level, as well as the aminoacylation ability of tRNAs; the failure in tRNA metabolism led to the impairment of mitochondrial translation, ATP, and increasing the ROS production [47, 48]. Therefore, the 4295A>G and 4435A>G mutations may cause mitochondrial dysfunction that was responsible for DCM, while the 5655T>C mutation disrupted a very conserved base-pairing (1A-72U) at the acceptor stem of ME-TA. An in vitro processing analysis showed that the 5655T>C mutation reduced the efficiency of ME-TA precursor 5' end cleavage catalyzed by RNase P [49]. Moreover, ~41% reduction in the steady-state level of ME-TA was observed in cell lines carrying the 5655T>C mutation [50, 51]. Similarly, the heteroplasmic 12201T>C mutation destabilized a conservative base-pairing (5A-68U) on the acceptor stem of MT-TH. tRNA northern analysis revealed that the 12201T>C mutation causes ~75% reduction in the steady-state level of MT-TH. An in vivo protein labeling analysis showed an ~47% reduction in the rate of mitochondrial translation in cells carrying the 12201T>C mutation [52, 53]. Furthermore, the 14692A>G mutation caused loss of Ψ55 modification and increased angiogenin-mediated endonucleolytic cleavage in mutant MT-TE. An approximately 65% decrease in the steady-state level of MT-TE was found in mutant cells as compared to control cells [54]. A failure in MT-TE metabolism impaired mitochondrial translation, especially for polypeptides with a high proportion of glutamic acid codons, such as ND1, ND6, and CO2, in mutant cells, whereas the 15927G>A mutation affected a highly conserved guanine at position 42 at the anticodon stem of MT-TT, destabilizing the conservative base-pairing (28C-42G) [55]. Northern blot analysis revealed a 60% decrease in the steady-state level of MT-TT

in the mutant cell lines. Western blot analysis showed the variable reductions of 4 mtDNA encoding proteins, especially for marked decrease of MT-ND1 and MT-CYTB observed in mutant cell lines [56].

To see whether these mt-tRNA mutations caused the mitochondrial dysfunction that was responsible for DCM, we analyzed the mtDNA copy number, ATP, and ROS in patients carrying these pathogenic mtDNA mutations. Consequently, we found that patients carrying these mutations had a lower level of mtDNA copy number when compared to the controls. In fact, levels of mtDNA copy number were directly correlated with energy reserves, oxidative stress, and changes in mitochondrial membrane potential [57]. According to the most accepted theory, reductions in mtDNA copy number were associated with downregulation of mitochondrial transcription and decreased levels of proteins involved in OXPHOS [58]. Thus, it can be speculated that the reduction in respiratory capacity induced by reduced mtDNA copy number levels may have important pathogenic consequences. Moreover, compared to the controls, there was an approximately 35% decrease in ATP production in the mutant cell lines, whereas ~42% enhancement of ROS production was observed in this study. In fact, the impairment of OXPHOS will increase the production of ROS in mutant cells carrying these pathogenic mt-tRNA mutations. The overproduction of ROS can establish a vicious cycle of oxidative stress in mitochondria [59]. As a result, these biochemical defects can cause dysfunction or apoptosis in cardiac muscle cells, thereby producing the clinical phenotype.

Based on these observations, we propose that the possible molecular mechanisms underlying mt-tRNA mutations in the clinical expression of DCM may be as follows: first, the mutation itself disrupts the secondary structure of mt-tRNA, thus causing a failure in mt-tRNA metabolism, such as CCA addition, post-transcriptional modification, or aminoacylation [60]. Failures in mt-tRNA metabolism caused by these mutations would impair mitochondrial protein synthesis and respiration [61]. Second, abnormal mitochondrial respiration causes oxidative stress and uncoupling of oxidative pathways for ATP synthesis, which causes apoptosis or necrosis in the endothelial cells and contributes to DCM.

In summary, our study provided evidence that mitochondrial genetic defects may lead to DCM. In particular, MT-TL1 3302A>G, MT-TI 4295A>G, MT-TM 4435A>G, MT-TA 5655T>C, MT-TH 12201T>C, MT-TE 14692A>G, and MT-TT 15927G>A mutations altered the structure and function of their tRNAs, thereby causing mitochon-

drial dysfunctions and long-standing increase of ROS in cardiovascular cells. These 7 mutations may be the inherited risk factors for DCM. Thus, our findings may provide new insights into the understanding of the pathophysiology and valuable information for the management and treatment of DCM.

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Statement of Ethics

The study protocol was reviewed and approved by the ethics committee of Tianjin Chest Hospital (approval number: 2020YS-030-01), and the study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. Written informed consent was obtained from all subjects enrolled in this study.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

P.L. designed the study, Y.Q. and Z.W. performed the genetic screening of mt-tRNA genes, Y.B. and Y.J. enrolled the blood samples of DCM and healthy controls, Y.Q. and P.L. wrote the paper. All authors have approved the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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