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ORIGINAL ARTICLE

Mutational analysis of mitochondrial tRNA genes in 138 patients with Leber's hereditary optic neuropathy

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Abstract

Introduction Mutations in mitochondrial DNA (mtDNA) are the most important causes for Leber's hereditary optic neuropathy (LHON). Of these, three primary mtDNA mutations account for more than 90% cases of this disease. However, to date, little is known regarding the relationship between mitochondrial tRNA (mt-tRNA) variants and LHON.

Aim In this study, we aimed to investigate the association between mt-tRNA variants and LHON.

Methodology One hundred thirty-eight LHON patients lacking three primary mutations (*ND1* 3460G > A, *ND4* 11778Gxs > A, and *ND6* 14484 T > C), as well as 266 controls were enrolled in this study. PCR-Sanger sequencing was performed to screen the mt-tRNA variants. Moreover, the phylogenetic analysis, pathogenicity scoring system, as well as mitochondrial functions were performed.

Results We identified 8 possible pathogenic variants: $tRNA^{Phe}$ 593 T > C, $tRNA^{Leu(UUR)}$ 3275C > T, $tRNA^{Gln}$ 4363 T > C, $tRNA^{Met}$ 4435A > G, $tRNA^{Ala}$ 5587 T > C, $tRNA^{Glu}$ 14693A > G, $tRNA^{Thr}$ 15927G > A, and 15951A > G, which may change the structural and functional impact on the corresponding tRNAs, and subsequently lead to a failure in tRNA metabolism. Furthermore, significant reductions in mitochondrial ATP and MMP levels and an overproduction of ROS were observed in cybrid cells containing these mt-tRNA variants, suggesting that these variants may lead to mitochondrial dysfunction which was responsible for LHON.

Conclusion Our study indicated that mt-tRNA variants were associated with LHON, and screening for mt-tRNA variants were recommended for early detection, diagnosis, and prevention of maternally inherited LHON.

Keywords LHON · Mitochondrial dysfunction · Mt-tRNA · Pathogenic · Variants

Abbreviations

mtDNA	Mitochondrial DNA
LHON	Leber's hereditary optic neuropathy
mt-tRNA	Mitochondrial tRNA
RGCs	Retinal ganglion cells
OXPHOS	Oxidative phosphorylation
rCRS	Revised Cambridge Reference Sequence
CI	Conservation index
FBS	Fetal bovine serum
DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
MMP	Mitochondrial membrane potential
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
ETC	Electron transport chain
PCOS	Polycystic ovary syndrome

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Introduction

Leber's hereditary optic neuropathy (LHON) was a typical mitochondrial disorder, which was associated with a rapid, painless, acute, or subacute bilateral visual loss in young adults [1, 2]. It has been reported that 1:8500 individuals harbored a primary LHON-causing mutation and 1:31,000 experienced visual loss as a result of LHON in the North East of England [3]. The typical features in this disorder included the primary degeneration of retinal ganglion cells (RGCs) accompanied by ascending optic atrophy [4, 5]. Mutations in mitochondrial DNA (mtDNA) had

been identified to contribute to the pathogenesis of LHON though to varying degrees [6–8]; in particular, three primary mutations—*ND1* 3460G > A, *ND4* 11778G > A, and *ND6* 14484 T > C—had been reported to account for more than 90% of LHON cases [9]. Incomplete penetrances and gender bias were two features of LHON, but the underlying molecular mechanisms for the onset of these two features had not been fully understood.

Human mitochondrial tRNA (mt-tRNA) was a short, non-coding RNA, which constituted approximately 4 ~ 10% of all nuclear RNAs [10]. In fact, human mitochondrial genome encoded 13 peptides for oxidative phosphorylation (OXPHOS) system, as well as 22 tRNAs for mitochondrial translation [11]. Variations in mt-tRNAs, either in a sporadic status or maternally inherited, constituted the most common mtDNA alternations that were associated with human disorders [12]. Most recently, several LHON-associated mt-tRNA variants have been reported, such as $tRNA^{Met}$ 4435A > G [13], $tRNA^{Thr}$ 15927G > A [14], and $tRNA^{Ala}$ 5601C > T [15]. However, the pathophysiology of mt-tRNA variants in LHON was not fully understood.

To investigate the role of mitochondrial genetic defects in the pathogenesis of LHON, we recently carried out a systematic and extended mutational screening of 22 mt-tRNA genes in a cohort of 138 patients with LHON lacking three primary mutations and 266 control subjects. PCR-Sanger sequencing identified 27 nucleotide alternations in 13 mt-tRNA genes. Through the application of phylogenetic conservation analysis and tRNA structural-function prediction, 8 mt-tRNA variants were found to be pathogenic. To see the contributions of mtDNA genetic background to mt-tRNA variants, we sequenced the entire mitochondrial genomes of the probands carrying one of these pathogenic/likely pathogenic mt-tRNA variants. In addition, the trans-mitochondrial cybrid cells were used to analyze the mitochondrial function.

Materials and methods

Study population

A total of 138 genetically unrelated LHON patients lacking the known LHON-associated mtDNA mutations (*ND1* 3460G > A, *ND4* 11778G > A, and *ND6* 14484 T > C) participated in this study; these subjects were recruited from the Department of Ophthalmology, the First Affiliated Hospital of Nanjing Medical University. Moreover, 266 healthy subjects, including 130 males and 136 females, were obtained from the Healthy Examination Center of the First Affiliated Hospital of Nanjing Medical University. The inclusion criteria of patients for this study, included acute or subacute visual loss in both eyes simultaneously or sequentially within 1 year, clinical evidence of relatively symmetric optic neuropathies with central visual loss, and age less than 50 years at onset of visual symptoms. Patients who had hearing loss, cardiovascular or muscle diseases will be excluded. Furthermore, 266 controls were healthy individuals, without any clinical diseases; the subjects who have a family history of mitochondrial diseases will be excluded.

This study was in compliance with the Declaration of Helsinki, and informed consent, blood samples, and clinical evaluations were obtained from all participating subjects, under the protocol approved by the First Affiliated Hospital of Nanjing Medical University.

Clinical examinations

All patients and control subjects were received the following ophthalmic examinations including the visual acuity, visual field examination, visually evoked potentials, and fundus photography. The degree of visual impairment was defined according the following standard: normal, > 0.3; mild, 0.3 to 0.1; moderate, < 0.1 to 0.05; severe, < 0.05 to 0.02; and profound, < 0.02.

DNA preparation, PCR amplification of mt-tRNA genes, and sequencing

The DNA was extracted from 2 mL peripheral blood using a QIAamp DNA Blood Minikit (Qiagen Chins Co., Ltd, China). The DNA concentrations > 1.0 ng/μl were employed for the next experiments.

For screening the mt-tRNA variants, subjects' DNA (138 LHON patients and 266 controls) fragments spanning 22 mt-tRNA genes were amplified by PCR using the primers as described in Table 1. The PCR reagents (Takara Bio, Inc.) were as follows: 200 μM dNTPs, 2 μl 10 × PCR buffer (10 × 0.2 μl Taq DNA polymerase and 15 mmol/l Mg^{2+}). Subsequently, the PCR products were purified and sequenced using the ABI PRISM™ 3700 machine (Applied Biosystems; Thermo Fisher Scientific, Inc.), as previously described [16]. The sequence data was handled by the DNASTAR program (DNASTAR Inc., Madison, USA). Mt-tRNA variants were scored relative to the revised Cambridge Reference Sequence (rCRS, GenBank Accessible No. NC_012920.1) [17].

Phylogenetic analysis

We carried out a phylogenetic conservation analysis for the identified mt-tRNA variants, as described previously [18]. The conservation index (CI) was then calculated by comparing the human nucleotide variants with the other 14 vertebrates. These species were as follows: *Mus musculus*, *Rattus norvegicus*, *Cebus albifrons*, *Pongo pygmaeus*, *Bos taurus*, *Sus scrofa*, *Phoca vitulina*, *Kogia breviceps*, *Gorilla gorilla*,

Table 1 Primer sequences for amplification of 22 mt-tRNAs

Target gene	Primer name	Primer sequence (5′–3′)	Tm (°C)[a]	Product size
$tRNA^{Phe}$	MT-1F MT-1R	CTCCTCAAAGCAATACACTG TGCTAAATCCACCTTCGACC	61	802 bp
$tRNA^{Val}$	MT-2F MT-2R	CGATCAACCTCACCACCTCT TGGACAACCAGCTATCACCA	58	802 bp
$tRNA^{Leu(UUR)}$	MT-4F MT-4R	AAATCTTACCCCGCCTGTTT AGGAATGCCATTGCGATTAG	60	887 bp
$tRNA^{Ile}$ $tRNA^{Gln}$ $tRNA^{Met}$	MT-6F MT-6R	TGG CTC CTT TAA CCT CTC CA AAG GAT TAT GGA TGC GGT TG	60	898 bp
$tRNA^{Ala}$ $tRNA^{Asn}$ $tRNA^{Cys}$	MT-8F MT-8R	CTAACCGGCTTTTTGCCC ACCTAGAAGGTTGCCTGGCT	60	814 bp
$tRNA^{Ser(UCN)}$ $tRNA^{Asp}$	MT-11F MT-11R	ACGCCAAAATCCATTTCACT CGGGAATTGCATCTGTTTTT	58	987 bp
$tRNA^{Lys}$	MT-12F MT-12R	ACG AGT ACA CCG ACT ACG GC TGG GTG GTT GGT GTA AAT GA	60	900 bp
$tRNA^{Gly}$ $tRNA^{Arg}$	MT-15F MT-15R	TCTCCATCTATTGATGAGGGTCT AATTAGGCTGTGGGTGGTTG	60	891 bp
$tRNA^{His}$ $tRNA^{Ser(AGY)}$ $tRNA^{Leu(CUN)}$	MT-18F MT-18R	TATCACTCTCCTACTTACAG AGAAGGTTATAATTCCTACG	55	866 bp
$tRNA^{Glu}$	MT-21F MT-21R	GCATAATTAAACTTTACTTC AGAATATTGAGGCGCCATTG	55	938 bp
$tRNA^{Thr}$ $tRNA^{Pro}$	MT-22F MT-22R	TGAAACTTCGGCTCACTCCT GAGTGGTTAATAGGGTGATAG	60	1162 bp

[a]Tm: Annealing Temperature

Orycteropus afer, *Zaglossus bruijni*, *Ornithorhynchus anatinus*, *Dromiciops gliroides*, and *Microtus kikuchii*.

Assessment of the pathogenicity

To further assess the pathogenicity of mt-tRNA variants, we used the following criteria: first, the variant itself occurred < 1% in control subjects; second, the CI ≥ 75%, as proposed by Ruiz-Pesini and Wallace [19]; third, the variant may have functional impact on mt-tRNA genes; finally, the pathogenicity scoring system which was originally proposed by Yarham et al. [20], the variants were classified as "definitely pathogenic" with a score of ≥ 11 points, "possibly pathogenic" with a score of 7–10 points and a "neutral polymorphism" with a score of ≤ 6 points. Patients carrying potential pathogenic mt-tRNA variants that met these criteria were selected for further molecular and biochemical analysis.

Mutational screening for the complete mitochondrial genomes in probands carrying putative pathogenic mt-tRNA variants

To see the contributions of mitochondrial genetic background to LHON expression, we screened the entire mtDNA genes in 23 probands carrying one of the putative pathogenic LHON-associated tRNA variants. Briefly, 24 overlapping primers were used to amplify the whole mtDNA genes, as described previously [16]. The sequence results were compared with the rCRS (GenBank Accession No. NC_012920.1) [17], as described above.

Determining the mitochondrial haplogroups

The mtDNA sequences of the 23 subjects carrying the putative pathogenic mt-tRNA variants were assigned to the Asian mitochondrial haplogroups proposed by Kong et al. [21].

Cell lines

Lymphoblastoid cell lines were immortalized by transformation with the EB virus, as described previously [22]. Cell lines derived from the LHON patients carrying $tRNA^{Phe}$ 593 T > C, $tRNA^{Leu(UUR)}$ 3275C > T, $tRNA^{Gln}$ 4363 T > C, $tRNA^{Met}$ 4435A > G, $tRNA^{Ala}$ 5587 T > C, $tRNA^{Glu}$ 14693A > G, $tRNA^{Thr}$ 15927G > A, and 15951A > G variants, as well as 10 healthy subjects without these variants were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS).

Analysis of ATP production

The mitochondrial ATP levels of LHON patients with pathogenic mt-tRNA variants and control subjects were analyzed by using the Cell Titer-Glo® Luminescent Cell Viability Assay kit (Promega), according to the protocol provided by the manufacturer [23]. Briefly, the assay buffer and substrate were equilibrated to room temperature, and the buffer was transferred to and gently mixed with the substrate to obtain a homogeneous solution. After a 30-min equilibration of the cell plate to room temperature, 100 µl of the assay reagent was added into each well with 20,000 cells and the content was mixed for 2 min on an orbital shaker to induce cell lysis. After 10-min incubation in room temperature, the luminescence was read on a microplate reader (Syneregy H1, Bio-Tek).

ROS analysis

The ROS level of the LHON patients carrying these mt-tRNA variants and controls were analyzed by fluorometry, as described in a previous study [24]. Cells were incubated with the fluorescent probe (5×10^{-6} mol/L) 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min. The ROS production was assessed by fluorimetry using a Synergy Mx plate reader (BioTek Instruments, Winooski, VT). Subsequently, it was assessed using a fluorescence microscope (IX81, Olympus, Hamburg, Germany) coupled with the static cytometry software "ScanR" version 2.03.2 (Olympus).

Determining the MMP

Loss of mitochondrial membrane potential (MMP) was implicated to be involved in apoptosis [25]. For evaluating MMP, cells were plated onto 96-well cell culture plate overnight in growth medium. JC-10 dye-loading solution was added for 30 min at 37 °C, 5% CO_2. Alternatively, plated cells were preincubated with 10 µM of the protonophore uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 30 min at 37 °C, 5% CO_2 prior to staining with JC-10 dye. The fluorescent intensities for both J-aggregates and monomeric forms of JC-10 were measured at $E_x/E_m = 490/530$ and 490/590 nm with a microplate reader, as described in a previous study [23].

Statistical analysis

The SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis. Student *t* test was used to calculate the *P* values; moreover, the Fisher's exact test was carried out to evaluate the difference in mt-tRNA variants between LHON and controls; the $P < 0.05$ was regarded as having statistical significance.

Results

Clinical information for the LHON patients and controls

The study samples lacking the known LHON-related three primary mutations (*ND1* 3460G > A, *ND4* 11778G > A, and *ND6* 14484 T > C) consisted of 88 males and 50 females. All participants were Han Chinese subjects from separate families who came to the Department of Ophthalmology, the First Affiliated Hospital of Nanjing Medical University. Clinical examination revealed that 55 subjects exhibited profound visual loss, 32 patients had severe visual impairment, and 51 patients suffered from moderate visual impairment, whereas the 266 controls had normal vision. The age at onset of LHON ranged from 3 to 39 years, with an average of 17 years. However, these LHON patients did not harbor other clinical abnormalities, such as hearing loss, diabetes, cancer, or other mitochondrial diseases.

Mutational screening for LHON-associated mt-tRNA variants

To analyze the frequencies of LHON-related mt-tRNA variants, the DNA fragments spanning 22 mt-tRNA genes from 138 LHON patients and 266 control subjects were PCR amplified and analyzed by Sanger sequencing. Compared with the rCRS [17], we identified 27 mt-tRNA variants, as shown in Table 2. All of these genetic variants were well-known mutational hot spots, and none of these variants could be classified as "novel" [26]. These variants were as follows: $tRNA^{Phe}$ 593 T > C and 628C > T, $tRNA^{Val}$ 1647 T > C and 1655A > G, $tRNA^{Leu(UUR)}$ 3275C > T and 3290 T > C, $tRNA^{Gln}$ 4363 T > C, 4386 T > C and 4395 T > C, $tRNA^{Met}$ 4435A > G and 4454 T > C, $tRNA^{Cys}$ 5802 T > C and 5821G > A, $tRNA^{Ser(UCN)}$ 7492C > T and 7498C > T, $tRNA^{Lys}$ 8343A > G, $tRNA^{Arg}$ 10454 T > C, $tRNA^{Glu}$ 14693A > G, $tRNA^{Thr}$ 15889 T > C, 15896A > G, 15927G > A, 15930G > A, 15941 T > C and 15951A > G, $tRNA^{Pro}$ 16000C > T. Moreover, we noticed that there were 7 variants only presented in controls but absent in LHON patients: $tRNA^{Val}$ 1647 T > C, $tRNA^{Gln}$ 4386 T > C, $tRNA^{Cys}$ 5821G > A, $tRNA^{Ser(UCN)}$ 7498C > T, $tRNA^{Thr}$ 15889 T > C, 15896A > G, and 15930G > A, suggesting that they were neutral polymorphisms.

To evaluate the potential pathogenicity of mt-tRNA variants, we used the following criteria: (a) the variant presented in < 1% of controls, (b) the CI ≥ 75%, (c) potential structural

Table 2 Summary of mt-tRNA variants identified in this study

Gene	Alternations	CI (%)	W–C base pair[a]	No. of nucleotide in tRNAs	Location	No. of 138 LHON patients (%)	No. of 266 controls (%)	*P* value	Previously reported[b]	Disease association
$tRNA^{Phe}$	593 T > C	42.3		17	D-arm	3 (2.17)	0 (0)	0.015	Yes	LHON, deafness
	628C > T	51.9		52	TψC loop	1 (0.72)	1 (0.37)	0.64	Yes	Neutral polymorphism
$tRNA^{Val}$	1647 T > C	26.9		46	TψC loop	0 (0)	3 (1.13)	0.21	Yes	Neutral polymorphism
	1655A > G	28.8		54	TψC loop	1 (0.72)	1 (0.37)	0.64	Yes	Neutral polymorphism
$tRNA^{Leu(UUR)}$	3275C > T	100		44	Variable region	2 (1.44)	0 (0)	0.049	Yes	LHON, PCOS, MetS
	3290 T > C	23.1		60	TψC loop	1 (0.72)	2 (0.74)	0.98	Yes	Hypertension
$tRNA^{Gln}$	4363 T > C	75.0	T-A↓	38	Anticodon stem	3 (2.17)	0 (0)	0.015	Yes	PCOS, MetS; Hypertension
	4386 T > C	59.6		15	D-arm	0 (0)	4 (1.50)	0.15	Yes	Neutral polymorphism
	4395 T > C	82.7	C-G↑	6	Acceptor arm	2 (1.44)	1 (0.37)	0.23	Yes	Hypertension
$tRNA^{Met}$	4435A > G	100		37	Anticodon stem	3 (2.17)	0 (0)	0.015	Yes	LHON; Hypertension; POI
	4454 T > C	59.6		56	TψC loop	1 (0.72)	2 (0.74)	0.98	Yes	Neutral polymorphism
$tRNA^{Ala}$	5587 T > C	94.0		73	Acceptor arm	2 (1.44)	0 (0)	0.049	Yes	LHON; Deafness
	5601C > T	47.0		59	TψC loop	2 (1.44)	4 (1.50)	0.96	Yes	LHON
$tRNA^{Cys}$	5802 T > C	86.5	A-T↓	30	Anticodon stem	1 (0.72)	0 (0)	0.16	Yes	Deafness
	5821G > A	53.8	C-G↓	6	Acceptor arm	0 (0)	4 (1.50)	0.15	Yes	Deafness
$tRNA^{Ser(UCN)}$	7492C > T	75.0		26	Anticodon stem	1 (0.72)	1 (0.37)	0.63	Yes	Deafness, PCOS
	7498C > T	32.7		17	D-arm	0 (0)	3 (1.13)	0.21	Yes	Neutral polymorphism
$tRNA^{Lys}$	8343A > G	37.5		54	TψC loop	1 (0.72)	4 (1.5)	0.50	Yes	PD, PCOS, MetS
$tRNA^{Arg}$	10454 T > C	69.2		55	TψC loop	1 (0.72)	3 (1.13)	0.69	Yes	Deafness
$tRNA^{Glu}$	14693A > G	96.1		54	TψC loop	5 (3.62)	1 (0.37)	0.01	Yes	Deafness, LHON
$tRNA^{Thr}$	15889 T > C	66.6	T-A↓	2	Acceptor arm	0 (0)	2 (0.74)	0.31	Yes	Neutral polymorphism
	15896A > G	100		9	D-arm	0 (0)	1 (0.37)	0.47	Yes	Neutral polymorphism
	15927G > A	26.7	C-G↓	42	Anticodon stem	2 (1.44)	0 (0)	0.049	Yes	Deafness; LHON; CHD
	15930G > A	13.3		45	Variable region	0 (0)	4 (1.5)	0.15	Yes	Neutral polymorphism
	15941 T > C	46.6		61	TψC loop	1 (0.72)	3 (1.13)	0.69	Yes	Neutral polymorphism
	15951A > G	66.6	T-A↓	71	Acceptor arm	3 (2.17)	0 (0)	0.015	Yes	LHON; POI
$tRNA^{Pro}$	16000C > T	11.5		24	D-arm	1 (0.72)	1 (0.37)	0.64	Yes	Neutral polymorphism

LHON Leber's hereditary optic neuropathy, PCOS polycysticovary syndrome, PD Parkinson's disease: MetS metabolic syndrome: POI prematureovary insufficiency, CHD coronary heart disease

[a]Classic Watson–Crick (W–C) base pair: created (↑) or abolished (↓)

[b]Please visit Mitomap database (www.mitomap.org)

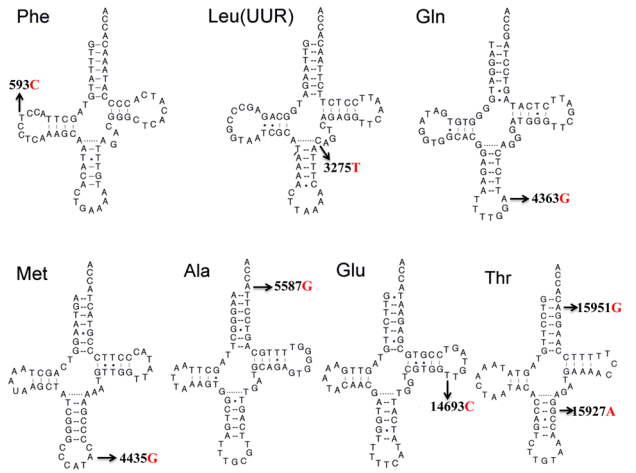

Fig. 1 Mt-tRNA variants in Chinese subjects with LHON. Cloverleaf structures of 7 mt-tRNAs are shown. Arrows indicate the position of tRNA variants

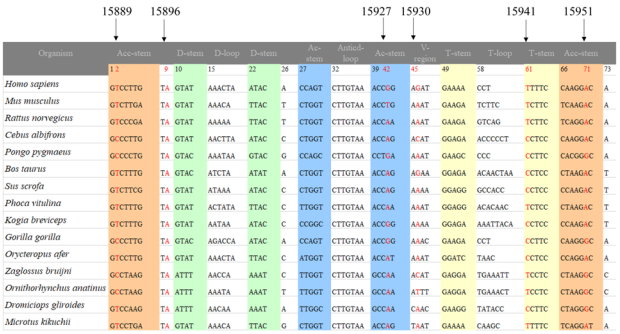

Fig. 2 Sequence alignment of $tRNA^{Thr}$ gene from 15 different species; arrows indicate the positions of 2, 9, 42, 45, 61, and 71, corresponding to the 15889 T > C, 15896A > G, 15927G > A, 15930G > A, 15941 T > C, and 15951A > G variants

and functional alterations, and (d) the pathogenicity scoring system [20]. Since most mt-tRNA from all domains of life had a highly conserved cloverleaf structure, consisting of acceptor arm, D-arm, anticodon stem, variable region, and TψC loop [27]. As shown in Fig. 1 and Table 2, 5 variants were localized at acceptor arm, 5 variants occurred at D-arm, 5 variants were located at anticodon stem, 1 variant occurred at variable region, and 10 variants occurred at TψC loop. Moreover, the 4363 T > C variant disrupted the T-A base-pairing, by contrast, the 4395 T > C variant created a novel C-G base-pairing in $tRNA^{Gln}$, while the 5802 T > C and 5821G > C variants abolished the A-T and C-G base-pairings in $tRNA^{Cys}$, respectively. In addition, the 15889 T > C, 15927G > A, and 15951A > G variants disrupted the T-A, C-G, and T-A base-pairings in $tRNA^{Thr}$, respectively. Furthermore, phylogenetic conservation analysis was performed by comparing the human tRNA nucleotide variants with those in 14 other vertebrates. As shown in Fig. 2 and Table 2, the CIs among these variants ranged from 11.5% ($tRNA^{Pro}$ 16000C > T) to 100% ($tRNA^{Leu(UUR)}$ 3275C > T, $tRNA^{Met}$ 4435A > G, and $tRNA^{Thr}$ 15896A > G). Furthermore, the Fisher's exact test was used to evaluate the difference in mt-tRNA variants between LHON patients and controls, the variants with $P < 0.05$ were $tRNA^{Phe}$ 593 T > C, $tRNA^{Leu(UUR)}$ 3275C > T, $tRNA^{Met}$ 4435A > G, $tRNA^{Ala}$ 5587 T > C, $tRNA^{Glu}$ 14693A > G, $tRNA^{Thr}$ 15927G > A, and 15951A > G, while the P values of other variants > 0.05.

Clinical features of 23 probands carrying one of the putative pathogenic mt-tRNA variants

As shown in Table 3, 23 probands with LHON carried the pathogenic/likely pathogenic tRNA variants, accounting for 16.67% of cases in our cohort. The age at onset of LHON in these subjects ranged from 7 to 36 years. Moreover, comprehensive medical histories showed that none of these subjects' relatives suffered from LHON. Notably, these subjects carrying the putative pathogenic mt-tRNA variants did not manifestate diabetes, deafness, or cardiovascular diseases. There were variable degrees of vision loss among these probands, 3 patients suffered from profound vision loss, 2 subjects had severe vision loss, 7 individuals had moderate vision loss, and 11 patients had mild vision loss.

Analysis of entire mitochondrial genomes of 23 LHON patients carrying one of pathogenic/likely pathogenic variants

The affected subjects carrying one of pathogenic/likely pathogenic tRNA variants were further examined to ascertain if there were any other functional mtDNA variants. As shown in Table 3, 6 variants were identified to be coexisted with these potential pathogenic tRNA variants, but we failed to detect any LHON patients carrying more than one mt-tRNA variant. Among them, the *ND1* 4216 T > C (p.Y304H) variant coexisted with $tRNA^{Phe}$ 593 T > C variant. In fact, the 4216 T > C variant changed the conserved thymine to cytosine at position 304, occurred at *ND1* gene which encoded a key member of Complex I of electron transport chain (ETC), which was regarded as a risk factor for maternally inherited diabetes according to a recent study [28]. In addition, *ND1* 3394 T > C and 3308 T > C coexisted with $tRNA^{Leu(UUR)}$ 3275C > T and $tRNA^{Glu}$ 14693A > G variants, respectively. Interestingly, the 3394 T > C (p.Y30H) was localized at extremely conserved nucleotide of *ND1* gene and was involved in the pathogenesis and progression of LHON [29]. While the well-known 3308 T > C (p.M1T) variant resulted in the replacement of the first amino acid, translation-initiating methionine with a threonine in *ND1* [30]. Furthermore, the 3308 T > C variant located in two nucleotides adjacent to the 3′ end of

Table 3 Mitochondrial genetic background and clinical features of 23 probands carrying one of the putative pathogenic LHON-associated tRNA variants

Genes	Mutations	Probands	Sex	Age at onset (year)	Visual acuity (right/left)	Level of vision loss	Family history	Other functional mtDNA variants	Haplogroup
$tRNA^{Phe}$	593 T > C	LHON-005	M	15	0.1/0.2	Mild	No	*ND1* 4216 T > C	D4j
		LHON-013	M	21	0.05/0.12	Moderate	No	/	F2
		LHON-029	M	8	0.01/0.01	Profound	No	/	D4h
$tRNA^{Leu(UUR)}$	3275C > T	LHON-035	F	9	0.1/0.12	Mild	No	*ND1* 3394 T > C	F3
		LHON-055	M	8	0.05/0.05	Moderate	No	/	G2a
$tRNA^{Gln}$	4363 T > C	LHON-027	M	20	0.25/0.25	Mild	No	*ND4* 11696G > A	D4j
		LHON-080	F	11	0.1/0.08	Moderate	No	/	B4c1b
$tRNA^{Met}$	4435A > G	LHON-012	M	30	0.1/0.1	Mild	No	/	M12
		LHON-028	M	9	0.25/0.25	Mild	No	/	F1a
		LHON-094	F	7	0.02/0.01	Profound	No	/	B5
$tRNA^{Ala}$	5587 T > C	LHON-019	M	10	0.15/0.12	Mild	No	*ND6* 14502 T > C	M7b
		LHON-121	M	18	0.06/0.06	Moderate	No	/	M10
$tRNA^{Glu}$	14693A > G	LHON-009	F	33	0.1/0.1	Mild	No	*ND1* 3308 T > C	D4
		LHON-024	M	36	0.1/0.1	Mild	No	/	D4c1
		LHON-037	M	17	0.08/0.08	Moderate	No	/	G1a1
		LHON-079	F	14	0.04/0.04	Severe	No	*ND5* 12338 T > C	F2a
		LHON-088	M	26	0.03/0.04	Severe	No	/	D4b2b
$tRNA^{Thr}$	15927G > A	LHON-099	F	15	0.02/0.01	Profound	No	/	B5b1
		LHON-130	M	18	0.05/0.05	Moderate	No	/	F3b
	15951A > G	LHON-022	M	14	0.15/0.15	Mild	No	/	Z4a
		LHON-031	M	8	0.08/0.08	Moderate	No	/	G3a2
		LHON-058	F	9	0.1/0.1	Mild	No	/	M71
		LHON-111	M	17	0.1/0.12	Mild	No	/	D4b2b2

$tRNA^{Leu(UUR)}$. Thus, this variant caused an alteration on the processing of the H-strand polycistronic RNA precursors or the destabilization of *ND1* mRNA [31]. Similarly, the 12338 T > C (p.M1T) variant resulted in the replacement of the first amino acid, translation-initiating methionine with a threonine in *ND5* polypeptide; thus, the truncated *ND5* mRNA was expected to be shortened by two amino acids [32]. Moreover, 12338 T > C variant located in two nucleotides adjacent to the 3′ end of $tRNA^{Leu(CUN)}$ [32]. Therefore, the 12338 T > C variant altered the respiratory function, as well as the processing of RNA precursors, thereby leading to a reduction in $tRNA^{Leu(CUN)}$ level [33]. Furthermore, the 11696G > A (p.V313I) variant was identified in patient harboring the $tRNA^{Gln}$ 4363 T > C variant. The G-to-A transition at position 11,696 (11696G > A) in *ND4*, caused by the substitution of an isoleucine for valine at amino acid position 313 [34]. In fact, the 11696G > A variant had been associated with LHON in a large Dutch family [35], and acted as a risk factor for increasing the penetrance and expressivity of deafness-associated 12S rRNA 1555A > G mutation in a Chinese family [36], while the 14502 T > C (p.I58V) caused the substitution of a highly conserved isoleucine for valine at position 58 in *ND6* [37]. Previous studies had suggested that the 14502 T > C variant may play a synergistic role with the primary mutations (14484 T > C and 11778G > A) [38]. According to their distinct sets of polymorphisms, the mtDNA of these probands belonged to Eastern Asian haplogroups D4j, F2, D4h, F3, G2a, B4c1b, M12, F1a, B5, M7b, M10, D4, D4c1, G1a1, F2a, D4b2b, B5b1, F3b, Z4a, G3a2, M71, and D4b2b2, respectively (Table 3) [21].

The pathogenicity scoring system

We next utilized the pathogenicity scoring system [20] to evaluate the status of $tRNA^{Phe}$ 593 T > C, $tRNA^{Leu(UUR)}$ 3275C > T, $tRNA^{Gln}$ 4363 T > C, $tRNA^{Met}$ 4435A > G, $tRNA^{Ala}$ 5587 T > C, $tRNA^{Glu}$ 14693A > G, $tRNA^{Thr}$ 15927G > A, and 15951A > G variants, which showed the statistical significance between LHON group and controls. As shown in Table 4, we noticed that the total scores of 15927G > A and 15951A > G were 12 and 9 points, and belonging to "definitely pathogenic" and "possibly pathogenic" at this stage. Similarly, the total scores of 593 T > C, 3275C > T, 4435A > G, 5587 T > C, and 14693A > G variants were 11, 11, 12, 13, and 10 points, respectively.

Table 4 The predicted pathogenicity of $tRNA^{Thr}$ 15927G > A and 15951A > G mutations

Scoring criteria	15927G > A mutation	Score/20	15951A > G mutation	Score/20	Classification
More than one independent report	Yes	2	Yes	2	≤ 6 points: neutral polymorphisms; 7 ~ 10 points: possibly pathogenic; 11–13 points (not including evidence from single fiber, steady-state level or trans-mitochondrial cybrid studies): probably pathogenic ≥ 11 points (including evidence from single fiber, steady-state level or trans-mitochondrial cybrid studies): definitely pathogenic
Evolutionary conservation of the base pair	Multiple changes	0	Multiple changes	0	
Variant heteroplasmy	No	0	No	0	
Segregation of the mutation with disease	No	0	Yes	2	
Histochemical evidence of mitochondrial disease	No evidence	0	No evidence	0	
Biochemical defect in complex I, III, or IV	Yes	5	No	0	
Evidence of mutation segregation with biochemical defect from single-fiber studies	No	0	No	0	
Mutant mt-tRNA steady-state level or evidence of pathogenicity in trans-mitochondrial cybrid studies	Strong evidence	5	Strong evidence	5	
Maximum score	Definitely pathogenic	12	Possibly pathogenic	9	

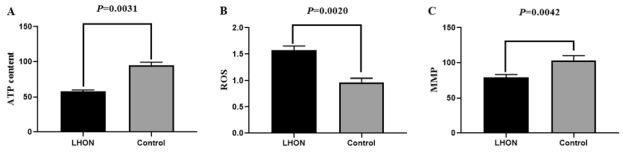

Fig. 3 Analysis of mitochondrial functions: **A** ATP analysis. **B** ROS analysis. **C** MMP analysis

Reduced in mitochondrial ATP production

Because mitochondria generated ATP via OXPHOS, defects in ATP synthesis were found to be an important cause for mitochondrial dysfunction [39]. For this purpose, we constructed the cybrid cells containing the LHON-associated putative pathogenic variants ($tRNA^{Phe}$ 593 T > C, $tRNA^{Leu(UUR)}$ 3275C > T, $tRNA^{Gln}$ 4363 T > C, $tRNA^{Met}$ 4435A > G, $tRNA^{Ala}$ 5587 T > C, $tRNA^{Glu}$ 14693A > G, $tRNA^{Thr}$ 15927G > A, and 15951A > G), as well as 10 healthy subjects lacking these variants, according to the study as described in elsewhere [22]. As shown in Fig. 3A, we found that LHON patients carrying these variants had a lower level of ATP when compared with the controls ($P = 0.0031$).

ROS increased

The levels of ROS from LHON patients carrying the pathogenic mt-tRNA variants and controls were determined by using the fluorometry. As shown in Fig. 3B, approximately ~ 50% in the increasing ROS production when compared with the controls ($P = 0.0020$).

MMP decreased

The MMP generated by proton pumps was an essential component in the process of energy storage during OXPHOS [40]. To see whether mt-tRNA pathogenic variants affected mitochondrial functions, we analyzed MMP in cybrids cells with and without mt-tRNA pathogenic variants. As shown in Fig. 3C, we noticed that MMP decreased significantly in mutant cell lines when compared with the controls ($P = 0.0042$).

Discussion

Dysfunction of mitochondrial activities was frequently associated with LHON [41]. Mt-tRNA point mutations typically caused a loss of mt-tRNA stability leading to defective mitochondrial translation and a combined respiratory chain deficiency. Today, approximately 200 pathogenic variants had been mapped to mt-tRNA genes (https://www.mitomap.org/MITOMAP) [42], emphasizing the importance of mt-tRNAs for mitochondrial function.

In the current study, we analyzed the frequencies of mt-tRNA variants in 138 genetically unrelated LHON patients lacking three primary mtDNA mutations and 266 control subjects by using PCR-Sanger sequencing. Mutational screening for the entire mt-tRNA genes identified 27 nucleotide alternations in 13 mt-tRNA genes. Among these, 8 mt-tRNA variants exhibited the statistical significance between LHON and controls. In fact, the 593 T > C variant occurred at the D-arm of $tRNA^{Phe}$ (conventional position 17), variation at that position was important for the structure and function of tRNA. Functional analysis showed that approximately ~ 46% decreases in the steady-state level of $tRNA^{Phe}$ in the cell lines harboring this variant [43]. In addition, the 593 T > C variant was implicated to play a synergistic effect on LHON-associated *ND4* 11778G > A mutation [44], while the homoplasmic 3275C > T variant was localized at the variable region of $tRNA^{Leu(UUR)}$, which had been reported to be associated with LHON and polycystic ovary syndrome (PCOS) [45, 46]. Moreover, the 4363 T > C variant occurred at the anticodon stem of $tRNA^{Gln}$, which was highly conserved across various species (conventional position 38). Nucleotide at that position was often chemically modified during $tRNA^{Gln}$ processing and function [27]. Thus, the 4363 T > C variant may reduce the steady-state level of $tRNA^{Gln}$ and cause mitochondrial dysfunction [47]. In addition, the well-known 4435A > G variant affected a highly conserved adenosine at position 37, 3′ adjacent to the $tRNA^{Met}$ anticodon, which was important for the fidelity of codon recognition and stabilization. Functional analysis revealed that the 4435A > G variant introduced an m^1G37 modification of $tRNA^{Met}$, altering its structure and function [48–50].

While the T to C transition at position 5587 was localized at the end of $tRNA^{Ala}$ and may alter the tertiary structure of this tRNA, the 5587 T > C variant had been reported to be associated with deafness and LHON [51, 52]. Furthermore, the homoplasmic 14693A > G variant occurred at the first base (conventional position 54) of the TψC loop of $tRNA^{Glu}$, nucleotide at position 54 was often modified, and contributed to the structural and stabilization of functional tRNAs [27]; thus, the 14693A > G variant may lead to mitochondrial dysfunction which was responsible for LHON [53]. Recent experimental studies revealed that the 14693A > G variant was associated with hearing loss and may increase the penetrance and expressivity of deafness-associated 12S rRNA mutations [54, 55]. Moreover, 2 variants in $tRNA^{Thr}$ were found to be associated with LHON: 15927G > A and 15951A > G. The 15927G > A variant abolished the C-G base-pairing in the anticodon stem of $tRNA^{Thr}$ and caused approximately 60% reductions in the steady-state level of $tRNA^{Thr}$ [14]. Furthermore, this variant decreased the activities of mitochondrial complex I and III, marked diminished mitochondrial ATP level [14]. Interestingly, the 15927G > A had been regarded as pathogenic variant associate with hearing loss [56] and coronary heart disease [57], while the 15951A > G variant was located at the acceptor arm of $tRNA^{Thr}$, which was important for tRNA identity and pre-tRNA processing [58]. In fact, the significant reduction of the steady-state levels in $tRNA^{Thr}$ was observed in cells carrying the 15951A > G variant [59]. Therefore, these mt-tRNA variants may lead to the failure in tRNA metabolism and impair the mitochondrial translation and respiration.

It has been suggested that mitochondrial genetic background (haplogroups) may contribute to the phenotypic expression of LHON. For instance, the haplogroup J specific *ND1* 4216 T > C and *ND5* 13708G > A variants may increase the penetrance and expressivity of LHON-related primary mutations in European countries [60, 61]. Furthermore, mtDNA haplogroups M7b1′2 and M8a had been implicated in the clinical expression of the LHON-associated *ND4* 11778G > A mutation in Han Chinese population [62]. More recently, mitochondrial haplogroup D4j specific variant 11696G > A was found to increase the penetrance and expressivity of the LHON-associated 11778G > A mutation in several Han Chinese pedigrees [63]. To see the contributions of rare mutations to the clinical expression of LHON-related mt-tRNA variants, we sequenced the whole mitochondrial genomes of 23 probands carrying one of the pathogenic/likely pathogenic mt-tRNA variants. Consequently, 6 probands carrying functional mtDNA variants were identified. These mtDNA variants included the following: *ND1* 3308 T > C, 3394 T > C and 4216 T > C, *ND4* 11696G > A, *ND5* 12338 T > C and *ND6* 14502 T > C. We noticed that these variants occurred at extremely conserved nucleotides of mtDNA and may affect the respiratory chain function, which aggravated mitochondrial dysfunction caused by putative pathogenic mt-tRNA variants (Table 3).

To whether mt-tRNA variants caused mitochondrial dysfunction, we generated the cybrid cell lines containing these pathogenic/likely pathogenic variants. As shown in Fig. 3, we found that LHON patients harboring these variants had lower levels of ATP and MMP when compared with the controls; by contrast, the ROS level increased significantly ($P < 0.05$ for all). In fact, the shortage in ATP generation in LHON patients was most probably a result of the decreased in the proton electrochemical potential gradient of impaired mitochondria [64]. Moreover, reduction of MMP can contribute to abnormal mitochondrial function and was an important parameter for indicating the early cell death [65]. The impairment of MMP will in turn increase the ROS generation; on the other hand, overproduction of ROS may lead to serious consequence such as increasing the oxidative stress in cells, damaging DNA, RNA, and lipids and contributing to programmed cell death [66]. Therefore, these mt-tRNA variants were involved in the pathogenesis of LHON.

Based on these observations, we proposed that the possible molecular mechanism underlying the roles of mt-tRNA mutations in the pathogenesis of LHON may be as follows: first, the mutation itself disrupted the secondary structure of mt-tRNA, thus causing a failure in tRNA metabolism, such as CCA addition, posttranscriptional modification, or aminoacylation [67, 68]. Failures in mt-tRNA metabolism caused by these mutations would impair mitochondrial protein synthesis and respiration. As a result, abnormal mitochondrial respiration caused oxidative stress and uncoupling of oxidative pathways for ATP synthesis [69], which caused the RGC dysfunction and apoptosis [5], thus contributing to the LHON progression.

Several limitations existed in the present study; first, this study only included Chinese individuals and the results needed to be validated in other ethnic groups. Second, the statistical power may be limited due to the small sample size. Third, further investigations such as examining tRNA steady-state level, mitochondrial protein expression were required to verify the conclusions.

In summary, our data provided the evidence that mt-tRNA variants may be associated with LHON. Mt-tRNA^{Phe} 593 T > C, tRNA$^{Leu(UUR)}$ 3275C > T, tRNA^{Gln} 4363 T > C, tRNA^{Met} 4435A > G, tRNA^{Ala} 5587 T > C, tRNA^{Glu} 14693A > G, tRNA^{Thr} 15927G > A, and 15951A > G variants should be added as risk factors for LHON. Thus, our findings may provide novel insights into the understanding of the pathophysiology and valuable information for the management and prevention of LHON.

Author contribution Zhilan Yuan and Jie Shuai designed the study. Jie Shuai and Jian Shi performed the mutational screening of mt-tRNA genes in all subjects involved in this study. Ya Liang and Fangfang Ji performed the mitochondrial functional analysis. Luo Gu analyzed the data. Zhilan Yuan wrote the paper. All authors have read and approved final draft.

Declarations

Ethical approval This work is approved by the Ethics Committee of First Affiliated Hospital of Nanjing Medical University.

Informed consent Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare no competing interests.

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