Mitochondrial $tRNA^{Ala}$ 5601C>T variant may affect the clinical expression of the LHON-related *ND4* 11778G>A mutation in a family

YU DING[1], YU-FENG YE[2], MEI-YA LI[3], BO-HOU XIA[4] and JIAN-HANG LENG[1]

[1]Central Laboratory; [2]Department of Ophthalmology, School of Medicine, Hangzhou First People's Hospital, Zhejiang University, Hangzhou, Zhejiang 310006; [3]Analytical Testing Center, Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310053; [4]Department of Pharmacy, Hunan Chinese Medical University, Changsha, Hunan 410208, P.R. China

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Abstract. Certain mutations in mitochondrial DNA (mtDNA) are associated with Leber's hereditary optic neuropathy (LHON). In particular, the well-known NADH dehydrogenase 4 (*ND4*) m.11778G>A mutation is one of the most common LHON-associated primary mutations worldwide. However, how specific mtDNA mutations, or variants, affect LHON penetrance is not fully understood. The aim of the current study was to explore the relationship between mtDNA mutations and LHON, and to provide useful information for early detection and prevention of this disease. Following the molecular characterization of a Han Chinese family with maternally inherited LHON, four out of eight matrilineal relatives demonstrated varying degrees of both visual impairment and age of onset. Through PCR amplification of mitochondrial genomes and direct Sanger sequencing analysis, a homoplasmic mitochondrial-encoded *ND4* m.11778G>A mutation, alongside a set of genetic variations belonging to human mtDNA haplogroup B5b1 were identified. Among these sequence variants, alanine transfer RNA $(tRNA)^{Ala}$ m.5601C>T was of particular interest. This variant occurred at position 59 in the TψC loop and altered the base pairing, which led to mitochondrial RNA (mt-RNA) metabolism failure and defects in mitochondrial protein synthesis. Bioinformatics analysis suggested that the m.5601C>T variant altered $tRNA^{Ala}$ structure. Therefore, impaired mitochondrial functions caused by the *ND4* m.11778G>A mutation may be enhanced by the mt-$tRNA^{Ala}$ m.5601C>T variant. These findings suggested that the $tRNA^{Ala}$ m.5601C>T variant might modulate the clinical manifestation of the LHON-associated primary mutation.

Correspondence to: Dr Jian-Hang Leng, Central Laboratory, School of Medicine, Hangzhou First People's Hospital, Zhejiang University, 261 Huansha Road, Hangzhou, Zhejiang 310006, P.R. China
E-mail: lengjh5@163.com

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Introduction

Leber's hereditary optic neuropathy (LHON) is a maternally inherited disease that affects 1 in 31,000-50,000 people and culminates in the bilateral loss of central vision (1-3). In the North East of England, it has been reported that 1:8,500 individuals harbor a primary LHON-causing mutation and 1:31,000 experience visual loss as a result of LHON (4). Patients with LHON may exhibit abnormal symptoms, including movement disorders, dystonia or multiplesclerosislike symptoms, which pose a significant challenge for clinicians (5,6). Few significant improvements in visual acuity are reported following atrophy of the optic discs. LHON demonstrates an incomplete penetrance for both vision loss and gender bias; LHON affects males more frequently than females (7,8). Three primary mutations including the NADH dehydrogenase (ND) 4 m.11778G>A, *ND6* m.14484T>C and *ND1* m.3460G>A have been identified in 90% of patients with LHON (9-11). Yet the molecular mechanisms of these mtDNA mutations in the phenotypic manifestation of LHON have not been elucidated.

To understand the role of mitochondrial dysfunction in LHON, an extended genetic screen for mtDNA variants was performed in a Han Chinese family with a high prevalence of LHON. Sequence analysis of the complete mitochondrial genome identified the occurrence of an *ND4* m.11778G>A mutation and an alanine transfer RNA ($tRNA^{Ala}$) m.5601C>T variant within matrilineal relatives of the proband. In addition, bioinformatics analysis was performed in order to explore whether the m.5601C>T affected the $tRNA^{Ala}$ secondary structure.

Patients and methods

Patients and genetic screening. To identify mtDNA variations in Chinese patients with LHON, a Han Chinese family was recruited from Hangzhou First People's Hospital (Hangzhou, China) in January 2018, and blood samples (5 ml) were collected from each matrilineal relative of the proband. Blood samples (5 ml) from unrelated control subjects (n=300) from the same geographical region recruited at the Hangzhou First People's Hospital were also used in the present study. These

healthy subjects consisted of 200 males and 100 females, aged 11-48 years, and were enrolled from January 2018 to January 2019. The present study was approved by the Ethics Committee of Hangzhou First People's Hospital. Written informed consent was obtained from all participants, or their parent/guardian, prior to enrollment in the study.

Clinical examinations. The proband (II-12) and other affected matrilineal relatives (II-5, II-7 and III-8; Fig. 1) underwent comprehensive ophthalmic examinations, including visual field tests, examination of visual acuity, fundus photography, visual evoked potentials and determination of the degree of visual impairment, performed as previously described (12,13). The degree of visual impairment was classified based on the following criteria (12,13): healthy, ≥0.300; mild, 0.100-0.299; moderate, 0.050-0.099; severe, 0.020-0.049; and profound, <0.020.

PCR and genetic sequencing to identify mtDNA variants. Genomic DNA from LHON patients and control subjects was extracted using a DNA extraction kit (QIAamp® DNA Blood Mini kit; Qiagen GmbH), according to the manufacturer's protocol. The complete mitochondrial genomes of II-5, II-7, II-12 and III-8 were amplified in 24 overlapping fragments using 200 μM dNTP, 10X buffer, Taq DNA polymerase and 15 mmol/l Mg^2 (cat. no. R004A; Takara Biotechnology, Co., Ltd.). The 24 sequences of light-strand and heavy-strands oligonucleotide primers for amplification of mtDNA genes were used according to a previous report (14). The following thermocycling conditions were used for PCR: 95˚C for 5 min; 30 cycles of 94˚C for 10 sec, 60˚C for 30 sec and 72˚C for 1 min; and a final extension at 72˚C for 5 min. After confirmation of band of interest, the PCR products were purified using the PureLink Gel Extraction kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's recommendations. DNA samples with concentrations >1.0 ng/μl were sequenced using the BigDye™ Terminator Cycle Sequencing reaction kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and an ABI PRISM® 3700 DNA Analyzer. Sequencing data were compared with the updated Cambridge consensus human mitochondrial genome sequence (accession no. NC_012920) using DNASTAR version 5.01 (DNASTAR Inc.) (15).

Phylogenetic conservation analysis. Phylogenetic analysis was performed to determine the potential pathogenic role of the identified mtDNA mutations. Briefly, 17 different species were selected for phylogenetic analysis (Table I). The conservation index (CI) was measured by comparing the human nucleotide alternations with the nucleotide sequences of other species. CI≥70% was implicated to have functional significance (16).

Bioinformatics analysis. To determine whether the m.5601C>T variant affected tRNA^{Ala} secondary structure, the RNAfold web server program was used (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi), as previously described (17).

Determining the pathogenicity of the variant. The role of the tRNA^{Ala} m.5601C>T variant was determined using the pathogenicity scoring system, as described by Yarham *et al* (18). In brief, mutations were classified as: 'neutral polymorphism', ≤6; 'possible pathogenic', 7-10; 'definitely pathogenic', ≥11.

Table I. Mitochondrial DNA sequence accession number of 17 vertebrate species used in the phylogenetic analyses.

Species	GenBank accession no.
Homo sapiens	NC_012920
Cebus albifron	NC_002763
Gorilla gorilla	NC_011120
Hylobates lar	NC_002082
Lemur catta	NC_004025
Macaca mulatta	NC_005943
Macaca sylvanus	NC_002764
Nycticebus coucang	NC_002765
Pan paniscus	NC_001644
Pan troglodytes	NC_001643
Papio hamadryas	NC_001992
Pongo pygmaeus	NC_001646
Pongo pygmaeus abelii	NC_002083
Tarsius bancanus	NC_002811
Mus musculus	NC_006914.1
Bos taurus	HM045018.1
Xenopus laevis	NC_001573.1

Statistical analysis. SPSS 17.0 software (SPSS Inc.) was used for statistical analysis. Fisher's exact test was used to assess the differences between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical presentation of a Han Chinese family with LHON. A pedigree chart from a Han Chinese family with a history of LHON is presented in Fig. 1. There were four LHON patients presented in the pedigree (three males and one female), aged 7-39 years old. Medical history analysis of the proband (II-12) confirmed that no other clinical disorders, such as deafness, diabetes mellitus, cardiovascular diseases, cancer or neurological disorders, were present. Following comprehensive genetic counseling at the Department of Ophthalmology in Hangzhou First People's Hospital, the proband (age, 39), was found to have begun suffering from painless and progressive bilateral loss of vision at the age of 19, manifesting as a dark cloud in the central vision and difficulty differentiating different colors. Ophthalmic examination revealed large centrocecal scotoma in both eyes, a typical clinical feature of LHON (13). A total of three out of seven matrilineal relatives (II-5, II-7 and III-8), in addition to the proband (II-12), suffered from moderate to profound visual impairment (Table II).

Screening for mtDNA mutations. To investigate the molecular basis of LHON, II-5, II-7, II-12 and III-8 were screened for mutations following PCR amplification of the mtDNA genomes. Sequence analysis of the mtDNA PCR products

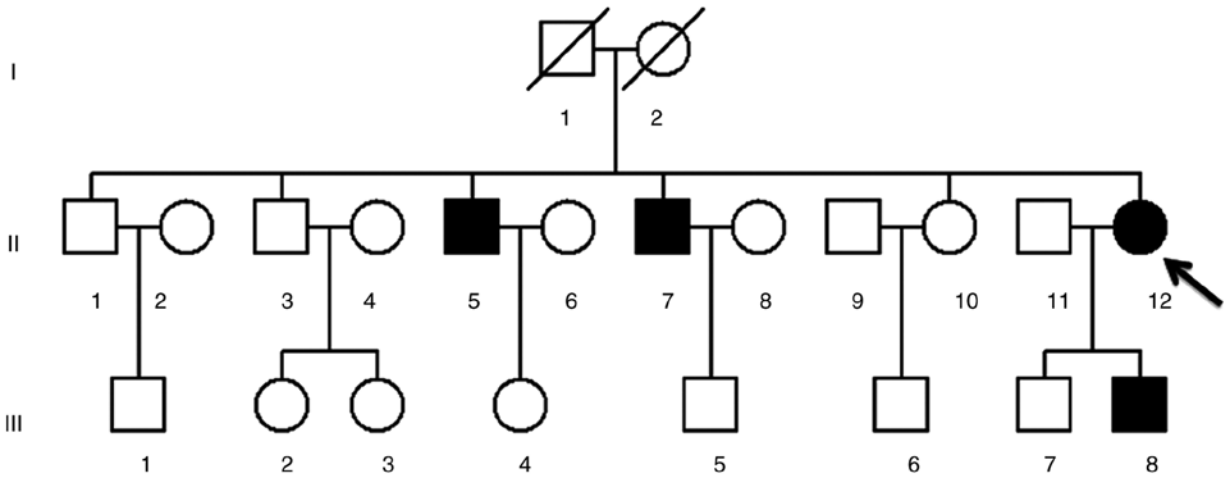

Figure 1. A three-generation Han Chinese family pedigree chart of Leber's hereditary optic neuropathy. The arrow indicates the proband (II-12) and filled symbols represent the visually impaired individuals.

revealed 32 genetic polymorphisms (Table III), all of which belonged to the human mtDNA B5b1 haplogroup (19). Of these, there were nine variants in the D-loop gene, two variants in the 12S rRNA gene, one variant in the 16S rRNA gene and one variant in a tRNA gene (m.5601C>T). The other variants were mainly localized within oxidative phosphorylation encoding genes. Notably, five missense mutations were identified: Mitochondrial encoded NADH dehydrogenase 1 (*ND1*) m.3593T>C (p.V96A), *ND2* m.5442T>C (p.F325L), *ATP 6* m.9103T>C (p.F193L), *ND3* m.10398A>G (p.T114A) and *ND4* m.11778G>A (p.R340H). The CIs of these variants were investigated between different species, including mouse, bovine and *Xenopus laevis* (20-22). Of all identified variants, only $tRNA^{Ala}$ m.5601C>T and *ND4* m.11778G>A were conserved. Notably, the m.5601C>T and m.11778G>A mutations were absent in the 300 control subjects compared with the mtDNA genomes of the matrilineal relatives ($P<0.05$). Taken together, these results indicated that $tRNA^{Ala}$ m.5601C>T and *ND4* m.11778G>A may have active roles in the pathogenesis of LHON.

In addition, the results revealed that the ratio between affected males and females carrying *ND4* m.11778G>A mutations in this case study was 3:1, which was similar to previous studies on families with LHON carrying *ND4* m.11778G>A mutations (Table IV) (23-28). These findings suggested that the m.11778G>A mutation may be the molecular basis for the LHON phenotype.

m.5601C>T variant alters the $tRNA^{Ala}$ *structure*. The m.5601C>T variant is located at a highly conserved position in the TψC loop within the $tRNA^{Ala}$ (29); thus, the point mutation results in a missense mutation that creates a novel base pairing (55T-59C) (Figs. 2 and 3). Subsequent bioinformatics analysis revealed that the m.5601C>T variant caused a structural alteration of $tRNA^{Ala}$ (Fig. 4), which indicated that m.5601C>T may have an impact on $tRNA^{Ala}$ function.

m.5601C>T variant is 'possibly pathogenic' for LHON. The pathogenicity scoring system described by Yarham *et al* (18) was used to determine the role of the $tRNA^{Ala}$ m.5601C>T variant. As presented in Table V, the total pathogenicity score for the m.5601C>T variant was 8, placing it within the 'possibly pathogenic' category for LHON.

Discussion

In the present study, a Han Chinese family with maternally inherited LHON was clinically and molecularly characterized. One of the most common features of LHON is bilateral loss of vision in the matrilineal relatives of the proband (9); this preferential effect on vision has facilitated the positive association between mtDNA mutations and LHON (30). Clinical evaluation of this family revealed that the age of onset for visual impairment between 3 and 19 years. The association between m.11778G>A and LHON was reported as early as 1988 (31). In the present study, patients harboring the m.11778G>A mutation had different mtDNA haplogroups, suggesting that the m.11778G>A mutation occurred sporadically and multiplied through evolution of the mtDNA in China. The varying degree of visual impairment in this Chinese family suggested that modifying factors, such as nuclear genes, environmental factors and mitochondrial genetic polymorphisms, may also contribute to LHON penetrance (32). In particular, secondary LHON-associated variants, such as *ND1* m.4216T>C and *ND5* m.13708G>A mutations in the mtDNA haplogroup J, may increase the penetrance and severity of LHON, in combination with the primary mutations, in European populations (33). mtDNA haplogroups M7b1'2 and M8a have been implicated in the clinical expression of the LHON-associated *ND4* m.11778G>A mutation (33).

In the present study, sequencing of the complete mitochondrial genomes of the matrilineal relatives (II-5, II-7, II-12 and III-8) revealed a set of genetic polymorphisms from the Asian mtDNA haplogroup B5b1 (19). Of these variants, $tRNA^{Ala}$ m.5601C>T was of most interest because this variant is located at a highly conserved nucleotide in the TψC loop of $tRNA^{Ala}$ (position 59), which is thought to be involved in tertiary interactions between the TψC loop and the truncated D-arm (34). Bioinformatics analysis revealed that the m.5601C>T variant created a novel Watson-Crick base-pairing (55T-59C). The $tRNA^{Ala}$ m.5601C>T variant has previously been associated with maternally inherited hypertension and mitochondrial

Table II. Summary of the clinical data for the proband (II-12) and matrilineal relatives (II-5, II-7 and III-8) in the Han Chinese family with maternally inherited Leber's hereditary optic neuropathy.

Subject	Sex	Age at onset (years)	Age at test (years)	Visual impairment score		Degree of visual impairment[a]
				Right eye	Left eye	
II-5	Male	11	35	0.03	0.05	Severe
II-7	Male	16	33	0.1	0.2	Moderate
II-12	Female	19	39	0.01	0.01	Profound
III-8	Male	3	7	0.02	0.01	Profound

[a]The degree of visual impairment was classified based on criteria stated in the clinical examinations section of the Methods.

Table III. Sequence analysis of mitochondrial DNA mutations in a Han Chinese family with maternally inherited Leber's hereditary optic neuropathy.

Gene	Position	Base change	Conservation (H/B/M/X)[a]	CI (%)	Previously reported[b]
D-loop	73	A>G			Yes
	152	T>C			Yes
	189	A>C			Yes
	263	A>G			Yes
	489	T>C			Yes
	16117	T>C			Yes
	16172	T>C			Yes
	16223	T>C			Yes
	16519	T>C			Yes
12S rRNA	709	G>A	G/A/A/-		Yes
	1438	A>G	A/A/A/G		Yes
16S rRNA	2706	A>G	A/G/A/A		Yes
ND1	3593	T>C (p.V96A)	V/I/I/A	25	Yes
	4102				Yes
ND2	4769	A>G			Yes
	4833	A>G			Yes
	5108	T>C			Yes
	5442	T>C (p. F325L)	F/F/M/L	23	Yes
tRNA^{Ala}	**5601**	**C>T**	**C/C/C/C**	100	Yes
CO1	7028	C>T			Yes
	7600	G>A			Yes
CO2	8167	C>T			Yes
ATP6	8547	C>T			Yes
	8748	C>T			Yes
	9103	T>C (p. F193L)	F/F/F/S	52	Yes
ND3	10398	A>G (p. T114A)	T/T/T/A	36	Yes
ND4	11719	G>A			Yes
	11778	**G>A (p. R340H)**	**R/R/R/R**	100	Yes
ND5	12705	C>T			Yes
ND6	14668	C>T			Yes
Cyt b	15043	G>A			Yes
	15301	G>A			Yes

[a]Conservation of amino acid for polypeptide. [b]From Mitomap database (www.mitomap.org). Conserved nucleotide residues are shown in bold font. B, bovine; *CO*, cytochrome *c* oxidase; *cyt b,* cytochrome b; H, human; M, mouse; *ND*, mitochondrial encoded NADH dehydrogenase; rRNA, ribosomal RNA; tRNA, transfer RNA; X, *Xenopus laevis*.

Table IV. Clinical and molecular data for eight Han Chinese pedigrees carrying the *ND4* 11778G>A primary mutation in LHON.

Author, year	Pedigree number	Affected ratio (male:female)	Penetrance of LHON (%)	Secondary variants	MtDNA haplogroup	(Refs.)
Ding *et al*, 2019	1	3:1	40	$tRNA^{Ala}$ m.5601C>T	B5b1	-
Qu *et al*, 2006	2	3:1	61.5	$tRNA^{Met}$ m.4435A>G	D5	(23)
Li *et al*, 2006	3	2:1	60	$tRNA^{Thr}$ m.15951A>G	D4	(24)
Zhang *et al*, 2010	4	3:0	37.5	*ND1* m.3394T>C	M9a	(25)
Qu *et al*, 2007	5	3.5:1	33	*ND4* m.11696G>A	D4	(26)
Zhang *et al*, 2010	6	1:1	57.1	*ND6* m.14502T>C	M10a	(27)
Qu *et al*, 2009	7	1:0	14.2	None	M8a2	(28)
Qu *et al*, 2009	8	2:0	8	None	D4g2	(28)

LHON, Leber's hereditary optic neuropathy; ND, mitochondrial encoded NADH dehydrogenase; tRNA, transfer RNA.

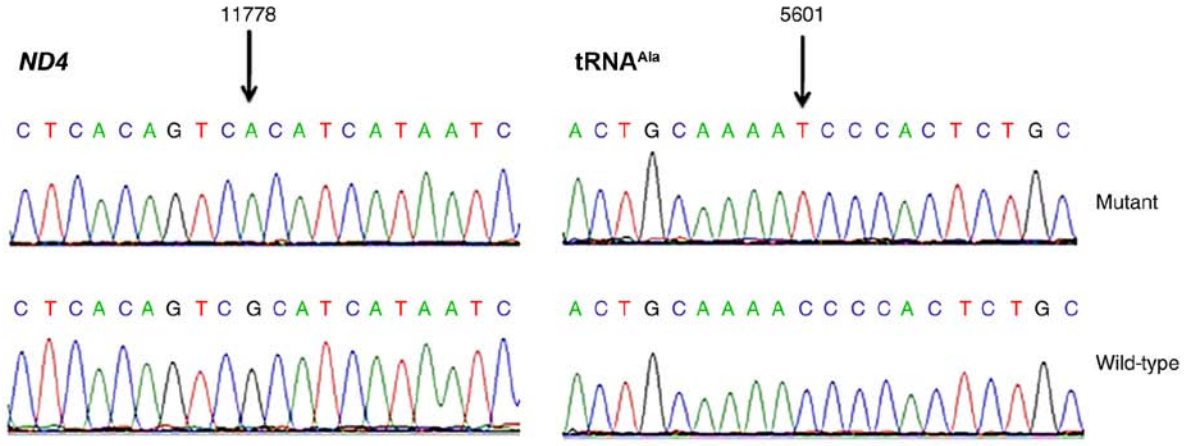

Figure 2. Identification of the (A) *ND4* m.11778G>A mutation and (B) $tRNA^{Ala}$ m.5601C>T variant in the mitochondrial genome. Partial sequence chromatograms of *ND4* and $tRNA^{Ala}$ genes from the proband (II-12; mutant) and a control subject (wild-type). Arrows indicate the location of the base change. *ND4*, mitochondrial encoded NADH dehydrogenase 4; $tRNA^{Ala}$, alanine transfer RNA.

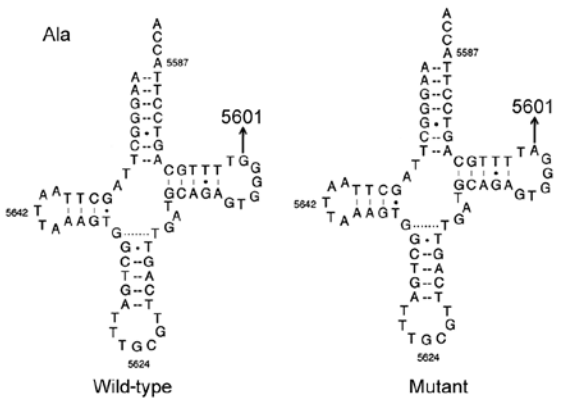

Figure 3. Location of the $tRNA^{Ala}$ m.5601C>T variant. The secondary structure of $tRNA^{Ala}$ protein (wild-type) was derived from the Mitomap database (www.mitomap.org). Arrow indicates the m.5601C>T variant. $tRNA^{Ala}$, alanine-transfer RNA.

myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (35,36). Therefore, the m.5601C>T may alter the secondary structure of $tRNA^{Ala}$ and impair the mt-tRNA metabolism and protein translation, and contribute to the LHON phenotype. A previous study has demonstrated that the m.12192G>A mutation, occurring at a similar position on $tRNA^{His}$, modulates the clinical expression of deafness in a Chinese pedigree (37), whereas the m.5601C>T variant may increase the penetrance of the hypertension-associated $tRNA^{Met}$ m.4435A>G mutation (35). Zhou *et al* have previously described the association between the $tRNA^{Ala}$ m.5601C>T variant and LHON in seven Han Chinese families (38). However, these families only carried the m.5601C>T variant, and did not harbor the three LHON-associated primary mutations (*ND4* m.11778G>A, *ND6* m.14484T>C and *ND1* m.3460G>A), thus exhibiting very low penetrance and severity of visual impairment (4.5-25.0%) (38). In the present study, the penetrance of LHON-induced visual impairment was 40%, which suggested that the combination of the *ND4* m.11778G>A mutation and the $tRNA^{Ala}$ m.5601C>T variant may be responsible for the higher prevalence of LHON in this family.

Results from the present study suggested that the $tRNA^{Ala}$ m.5601C>T variant could increase both the prevalence and

Table V. Pathogenicity scoring system for the m.5601C>T mutation.

Scoring criteria	m.5601C>T mutation	Score	Classification
More than one independent report	Yes	2	
Evolutionary conservation of the base pair	No changes	2	
Variant heteroplasmy	No	0	
Segregation of the mutation with disease	Yes	2	
Histochemical evidence of mitochondrial disease	Strong evidence	2	
Biochemical defect in complex I, III or IV	No	0	
Evidence of mutation segregation with biochemical defect from single-fiber studies	No	0	
Mutant mt-tRNA steady-state level or evidence of pathogenicity in trans-mitochondrial cybrid studies	No	0	
Total score		8	Possibly pathogenic

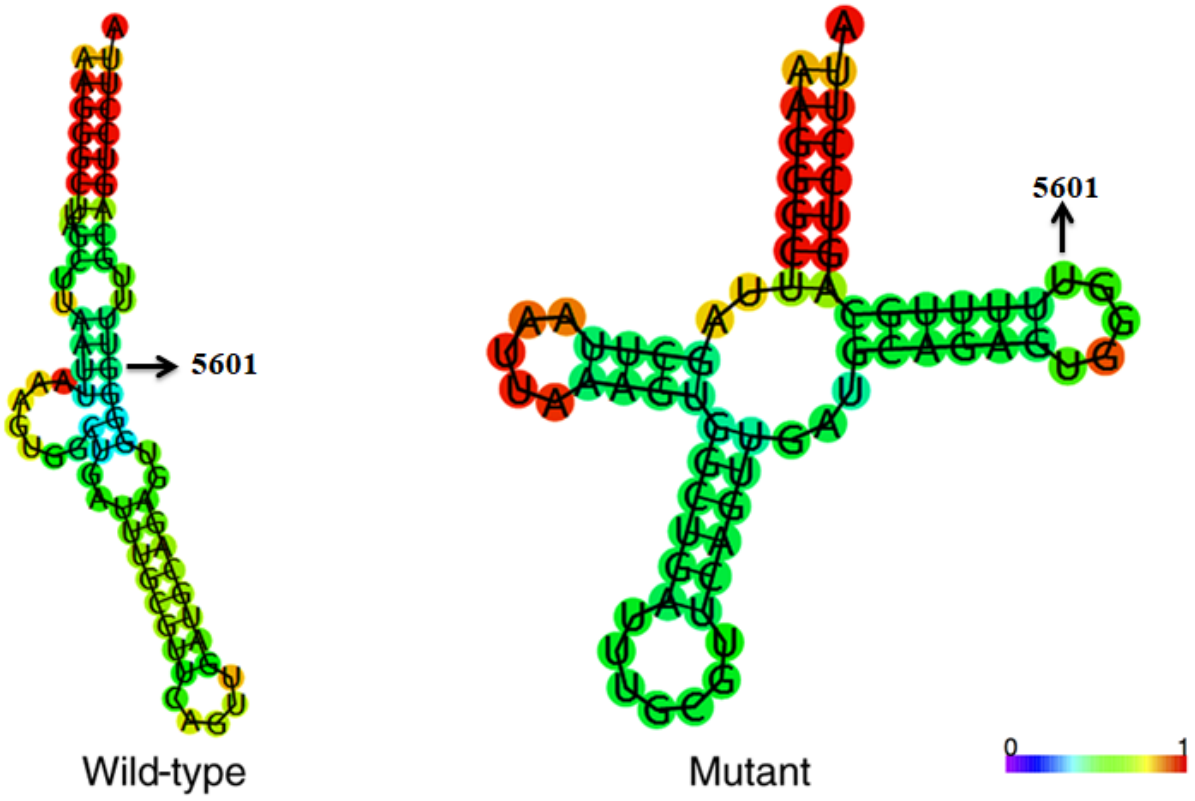

Figure 4. Predicted secondary protein structure of tRNA^{Ala} with (mutant) and without (wild-type) the m.5601C>T variant (indicated by arrow). The RNA Fold Webserver program (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) was used to predict the structure. The structure is colored according to the base-pairing probability (0-1, as denoted in the color scale bar).

the expression of the LHON-associated *ND4* m.11778G>A mutation. Evidence to support this includes the fact that the mutation occurs at a highly conserved nucleotide of tRNA^{Ala}, which is critical for basal tRNA activity and normal function (29). The present data demonstrated that the m.5601C>T variant alters the secondary structure of the tRNA^{Ala} gene. Finally, the pathogenicity scoring system generated indicated that the m.5601C>T variant was 'possibly pathogenic' (18). Therefore, the mitochondrial dysfunction, caused by the *ND4* m. 11778G>A mutation, may be worsened by the m. 5601C>T variant. In conclusion, the m. 5601C>T variant may have a modified role in clinical expression of LHON-associated m. 11778G>A mutation in this family.

Nevertheless, the incomplete penetrance of visual impairment in this family (as evidenced by family members harboring these mutations but exhibiting normal vision) indicated that the *ND4* m.11778G>A and tRNA^{Ala} m.5601C>T variants are insufficient alone to produce the observed clinical phenotypes. Therefore, it is likely that other risk factors, including environmental factors, nuclear genes and epigenetic

modifications, may contribute to the clinical manifestation of LHON in this pedigree. The main limitation of this study is the lack of functional analysis of the $tRNA^{Ala}$ m.5601C>T variant. Further studies, such as the use of cytoplasmic hybrid cells carrying the $tRNA^{Ala}$ m.5601C>T variant are required to confirm our conclusions and to identify additional contributing risk factors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YD and YFY designed the study. MYL and BHX performed the molecular analysis. YFY collected the samples and performed the clinical examinations. JHL analyzed the datasets and carried out the phylogenetic analysis. JHL and YD wrote the paper. All authors discussed the results and implications and commented on the manuscript at all stages. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of Hangzhou First People's Hospital (Hangzhou, China). Written informed consent was obtained from all participants, or their parent/guardian, prior to enrollment in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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