

The mitochondrial tRNA^{Ala} 5587T>C and tRNA^{Leu(CUN)} 12280A>G mutations may be associated with hypertension in a Chinese family

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Abstract. Hypertension is a very common cardiovascular disorder, however, the molecular mechanism underlying this disease remains poorly understood. Recently, an increasing number of studies have demonstrated that mitochondrial (mt)DNA mutations serve important roles in the pathogenesis of hypertension. The current study reported the clinical and molecular characterization of a Chinese family with maternally inherited hypertension (the penetrance of hypertension was 71.4%). In addition, the entire mitochondrial transfer (mt-t)RNA genomes was amplified using a polymerase chain reaction (PCR) and identified through direct Sanger sequencing. Additionally, the mtDNA copy number in matrilineal relatives in this family was evaluated using quantitative PCR. The sequence analysis of the 22 mt-tRNA genes led to the identification of tRNA^{Ala} 5587T>C (thymine to cytosine) and tRNA^{Leu(CUN)} 12280A>G (adenine to guanine) mutations. Notably, the heteroplasmic 5587T>C mutation was located at the 3' end of tRNA^{Ala} (position 73), which is highly conserved from bacteria to human mitochondria. In addition, the 12280A>G mutation was revealed to occurs at the dihydrouridine loop of tRNA^{Leu(CUN)} (position 15) and may decrease the steady-state level of mt-tRNA. As a result, 5587T>C and 12280A>G mutations may lead to the failure of tRNAs metabolism and subsequently cause mitochondrial protein synthesis defects. Molecular analysis revealed that patients carrying the 5587T>C and 12280A>G mutations had a lower copy number of mtDNA compared with a control with

hypertension, but without the mutations, suggesting that these mutations may cause mitochondrial dysfunctions that are responsible for hypertension. Therefore, mt-tRNA^{Ala} 5587T>C and tRNA^{Leu(CUN)} 12280A>G mutations may be involved in the pathogenesis of hypertension in this family.

Introduction

Hypertension is a major public health problem, affecting approximately 1 billion people worldwide (1). Hypertension is also an established risk factor for coronary heart disease, stroke and renal failure (2). Despite significant advances in the understanding of the pathophysiology of hypertension, it remains to be one of the most challenging disorders (3). It is generally believed that hypertension is influenced by genetic and environmental factors. Estimates of genetic variance range from 20-50% (4), and maternal and paternal patterns have been reported (5). In fact, previous studies demonstrated that mitochondrial dysfunction caused by mitochondrial (mt)DNA mutations were important causes for hypertension (6). Several mitochondrial transfer (mt-t)RNA mutations have been reported to be associated with hypertension, these mutations include mt-tRNA^{Ala} 5587T>C (thymine to cytosine) (7); mt-tRNA^{Gln} 4375C>T (8), mt-tRNA^{Met} 4435A>G (9) and mt-tRNA^{Leu(CUN)} 12280A>G (adenine to guanine) (10). The authors of the current study noticed that these mutations may decrease the steady-state level of mt-tRNAs and subsequently cause the mitochondrial dysfunction that is responsible for hypertension. Nevertheless, the association between mtDNA mutations and high blood pressure remains unclear.

To investigate the contribution of mtDNA mutations to hypertension, a mutational analysis for mt-tRNA genes was performed in a large cohort of patients with hypertension. In the current study, the authors described a Han Chinese family with maternally transmitted hypertension. Sequence analysis of the 22 mt-tRNA genes led to the identification of two potential pathogenic mutations: tRNA^{Ala} 5587T>C and tRNA^{Leu(CUN)} 12280A>G. The mtDNA copy number in the patients carrying these mutations was then analysed.

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Patients and methods

Pedigree information. A Han Chinese family (Fig. 1) with maternally inherited hypertension was recruited to the current study from department of General Medicine, Affiliated Qingdao Hiser Hospital of Qingdao University (Qingdao, China). There were 13 individuals in this family; five matrilineal relatives (I-2, II-4, II-6, III-5 and III-6) and an unrelated member of the family (II-5) suffered from hypertension, although II-5 did not have the investigated mutations (Fig. 1). Notably, members including I-2 (proband's mother), II-4 (proband), II-5 (proband's brother-in-law), II-6 (proband's sister), III-5 (proband's daughter) and III-6 (proband's niece) were involved in the current study. This protocol was approved by the ethics committee of the Affiliated Qingdao Hiser Hospital of Qingdao University. Detailed demographics, anthropometrics, vital parameters and medical history were recorded for each individual during interviews. Additionally, 500 unrelated Han Chinese healthy subjects (200 males and 300 females; age range, 21-55 years; mean age, 40 ± 1.5 years) were collected from the Health Examination Department, Affiliated Qingdao Hiser Hospital of Qingdao University and used as controls; written informed consent was obtained from all subjects involved in the current study. Notably, the control subjects were healthy individuals, without any diseases or any family history of mitochondrial disorders, including deafness, vision loss, neurological disorders or cardiovascular diseases. Control subjects who had a family history of mitochondrial diseases were excluded.

Measurement of the blood pressure (BP). Members of this family, as well as 500 healthy subjects underwent BP assessments; two doctors measured the systolic and diastolic BP via an electronic measuring device, and repeated three times. Hypertension was defined according to the guidelines of the Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure (JNC VI) (11) and the World Health Organization-International Society of Hypertension (12) as a systolic BP of ≥ 140 mmHg and/or a diastolic BP of ≥ 90 mmHg, or a history of hypertension with current antihypertensive drug treatment (13).

Detecting the hypertension-associated mt-tRNA mutations. The blood samples of each individual were collected in sterile ethylenediaminetetraacetic acid test tubes. To analysis the mutations/polymorphisms in mt-tRNA genes, the genomic DNA was extracted from the blood samples using the Puregene DNA Isolation kit (Gentra Systems, Inc., Minneapolis, MN, USA). The 22 mt-tRNA genes were polymerase chain reaction (PCR) amplified using 11 primers as described previously (7). The PCR products were purified and analyzed by direct sequencing in an ABI 3700 automated DNA sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using a Big Dye Terminator Cycle sequencing reaction kit version 3.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.). DNA Star software version 5.01 (DNASTAR Inc., Madison, WI, USA) was used to identify genetic variants in mt-tRNAs by comparing the sequence data with the Cambridge reference sequence (NC_012920) (14) and the protocols of Sanger

sequencing; the Sanger sequencing primers were described in a previous investigation (15).

mt-tRNA structure analysis. The published secondary structures of mt-tRNA^{Leu(CUN)} and tRNA^{Ala} were used to define the stem-and-loop structure (16). Using the cloverleaf structure, the position of the 12280A>G and 5587T>C mutations were localized.

Analysis of the conservation index (CI). To assess the potential pathogenic roles of the tRNA^{Leu(CUN)} 12280A>G and tRNA^{Ala} 5587T>C mutations, the CIs of the mutations were evaluated using phylogenetic conservation analysis (7). The following species were selected for the phylogenetic conservation analysis: *Homo sapiens*, *Gorilla gorilla*, *Macaca mulatta*, *Pan paniscus*, *Papio hamadryas*, *Trachypithecus obscurus*, *Muntiacus reevesi*, *Mus musculus*, *Balaenoptera bonaerensis*, *Cynocephalus variegatus* and *Pongo pygmaeus abelii*. The CI was calculated by comparing the human mtDNA variants with other species; a CI>75% was considered to have functional potential (17).

Analysis of mtDNA content. To see whether tRNA^{Leu(CUN)} 12280A>G and tRNA^{Ala} 5587T>C mutations caused mitochondrial dysfunction, the mtDNA content from each individual with hypertension was determined using quantitative PCR and the $2^{-\Delta\Delta C_q}$ method (18). The mtDNA content was normalized to a single copy nuclear gene (β -globin). The primer sequences for amplifying the gene were as follows: mtDNA ND1: Forward, 5'-AACATACCCATGGCCAACCT-3' and reversed, 5'-AGCGAAGGGTTGTAGTAGCCCC-3'; and β -globin: Forward, 5'-GAAGAGCCAAGGACAGGTAC-3' and reverse, 5'-CAACTTCATCCACGTTTACC-3'. The PCR reaction solution (20 μ l) contained 2X Taqman Universal PCR Master Mix (Takara Biotechnology Co., Ltd., Dalian, China), 500 nmol/l of each primer, 200 nmol/l Taqman Probe and 100 ng of total DNA. The PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of denaturation for 15 sec at 95°C and 60 sec annealing/extension at 60°C. Each experiment was repeated three times.

Statistical analysis. The statistical analysis was performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Differences in categorical variables were assessed with Fisher's exact test. $P < 0.05$ indicated that the difference between groups was statistically significant.

Results

Clinical analysis. A maternally transmitted Han Chinese family with hypertension (Fig. 1) was recruited from the Affiliated Qingdao Hiser Hospital of Qingdao University. The proband (II-4) was a 55-year-old female who was admitted to the department of General Medicine, Affiliated Qingdao Hiser Hospital of Qingdao University with a high BP (140/95 mmHg). The proband did not smoke or drink alcohol and did not have a history of coronary heart disease, renal failure or hyperlipidemia. In addition, the authors of the current study observed that the age of onset from the first generation was 66 years, while

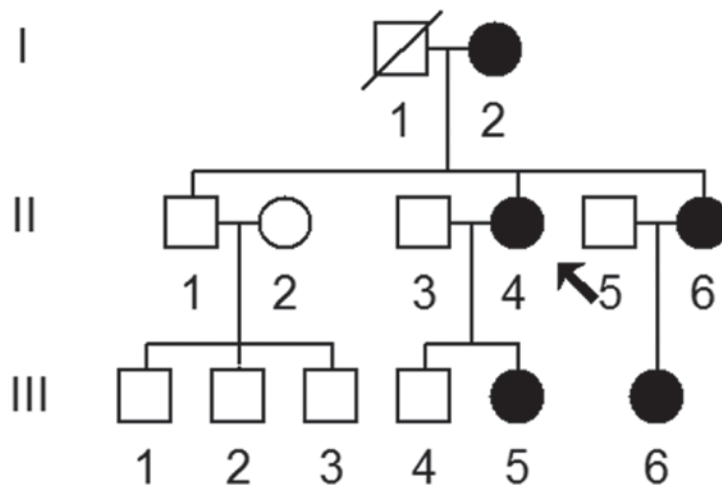


Figure 1. One Han Chinese family with hypertension carrying the mt-tRNA^{Ala} 5587T>C and tRNA^{Leu(CUN)} 12280A>G mutations. The affected individuals are presented as filled symbols, the arrow indicates the proband. mt, mitochondrial; t, transfer.

the mean age of onset were younger for the second generation (43.5 years, ranged between 42 and 45 years) and the third generation (32.0 years, ranged between 30 and 34 years); the mean age of onset of hypertension for the affected members of the family was 43.50 ± 12.52 years. The BP of each individual with hypertension was listed in Table I.

Mutational screening for mt-tRNA genes and structural analysis. The hypertension was maternally transmitted, which suggested that mitochondrial dysfunction may be the molecular basis for this disease; in addition, recent experimental studies suggested a positive association between mt-tRNA mutations and hypertension (19,20). Therefore, the mt-tRNA mutations were analyzed from the matrilineal relatives (I-2, II-1, II-4, II-6, III-4, III-5 and III-6) from this pedigree. The PCR was performed to amplify the entire mt-tRNAs, the PCR products were then purified and analyzed by direct sequencing. As a result, two mutations were identified: A homoplasmic tRNA^{Leu(CUN)} 12280A>G mutation and a heteroplasmic tRNA^{Ala} 5587T>C mutation in the matrilineal relatives (I-2, II-1, II-4, II-6, III-4, III-5 and III-6) with hypertension (Fig. 2), and no other mt-tRNA mutations were identified in the family. The 12280A>G mutation was localized at position 15 in the dihydrouridine (DHU)-loop of tRNA^{Leu(CUN)} (Fig. 3), which was highly conserved from various species (Table II). Notably, the 12280A>G created a novel base-pairing (15C-19G) and may cause the failure in tRNA metabolism. While the 5587T>C mutation occurred at position 73 near the end of tRNA^{Ala}; notably, the T to C transition at position 73 was extremely conserved, suggesting that the 5587T>C mutation may alter the secondary structure of tRNA^{Ala} (21). The 5587T>C and 12280A>G mutations was not identified in 500 healthy subjects; the Fisher's exact test was performed and it was revealed that the 5587T>C and 12280A>G had statistical significance (both $P < 0.05$; Table III).

Evolutionary conservation assessment. To evaluate potential pathogenic mutations, evolutionary conservation was assessed. The CIs of 12280A>G and 5587T>C mutations were analyzed, demonstrating them as 100% (Table II); a recent report by

Ji *et al* (21) also revealed that the CI of the 5587T>C mutation was 100%.

mtDNA copy number analysis. As shown in Fig. 4, patients carrying mt-tRNA^{Leu(CUN)} 12280A>G and tRNA^{Ala} 5587T>C mutations have markedly a lower copy number of mtDNA compared with a control with hypertension, but without the mutations, suggesting that the 12280A>G and/or 5587T>C mutation may cause mitochondrial dysfunction by altering the mtDNA content.

Discussion

The present study reported that the clinical and molecular characterization of a Chinese pedigree with maternally inherited hypertension. Although many studies revealed the genetics of mitochondrial disorders, the molecular mechanism underlying hypertension remains unclear (22,23). Experimental studies identified a positive association between mtDNA mutations and hypertension (24,25). In particular, Watson *et al* (26) reported a double ND3 10398A>G DdeI CO1 HaeIII 6620T>C or 6260G>A mutation in hypertensive African-Americans with end-stage renal disease. In addition, in a recent case-control study, Liu *et al* (27) identified several mt-tRNA mutations that were associated with hypertension, including tRNA^{Phe} 586G>A, tRNA^{Lys} 8313G>A and tRNA^{His} 12147G>A, suggesting that mt-tRNA genes were likely to contain pathogenic mutations associated with hypertension.

For this purpose, the mutations/variants in 22 mt-tRNA genes from the matrilineal relatives in the pedigree were screened and two potential pathogenic mutations identified were: mt-tRNA^{Ala} 5587T>C and tRNA^{Leu(CUN)} 12280A>G. It was interesting to note that the heteroplasmic 5587T>C mutation occurred at the 3' end of tRNA^{Ala}, which is very important for tRNA identity (28). Additionally, this mutation has been reported to be associated with Leber's hereditary optic neuropathy (21), hearing loss, progressive unstable gait, dysarthria, muscle cramps and myalgias (29). Functional analysis indicated that the 5587T>C mutation affected the aminoacylation

Table I. Clinical information for family members with hypertension.

Subjects	Sex	Age of onset (years)	Systolic pressure (mmHg)	Diastolic pressure (mmHg)
I-2	F	66	155	90
II-4	F	45	140	95
II-6	F	42	145	85
III-5	F	34	145	90
III-6	F	30	150	75
II-5	M	n/a	130	75

F, female; M, male; n/a, not applicable.

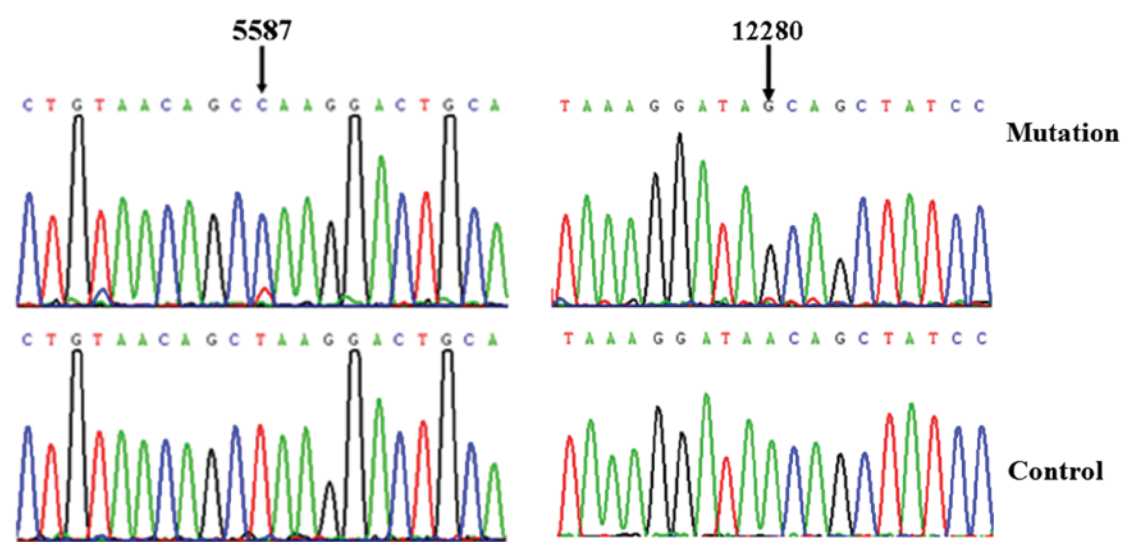


Figure 2. Identification of 5587T>C and 12280A>G mutations using direct sequencing.

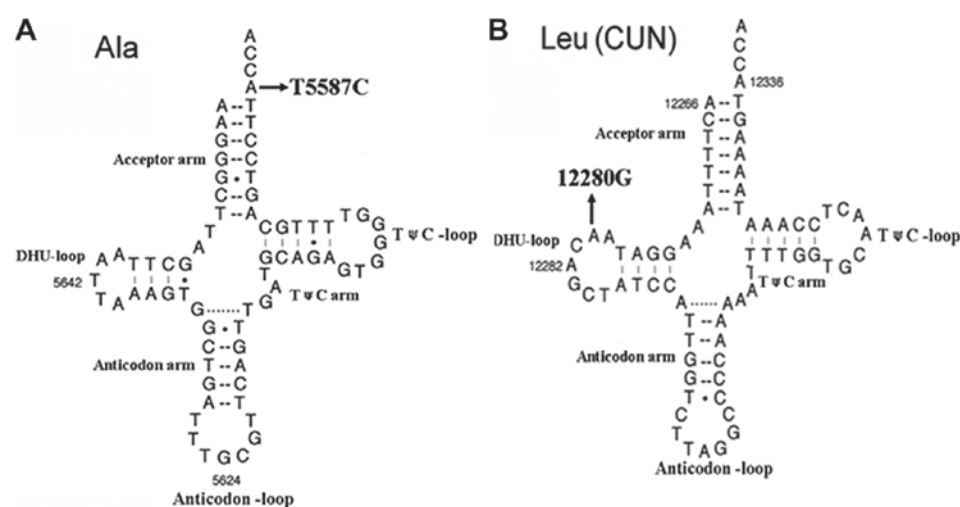


Figure 3. Structure of tRNA and location of the mutations. (A) The 5587T>C mutation is localized at the 3' end of tRNA^{Ala}. (B) The 12280A>G mutation occurs at DHU loop of tRNA^{Leu(CUN)}. DHU, dihydrouridine.

of tRNA^{Ala} and efficiency of mitochondrial translation (21). Thus, the 5587T>C mutation may have the same impact for the pathogenesis of hypertension in this family.

Furthermore, the homoplasmic 12280A>G mutation was found at position 15 in the DHU loop of the tRNA^{Leu(CUN)} gene, which was extremely conserved in various species. Notably, the

Table II. Alignment of the mt-tRNA^{Leu(CUN)} gene from different species.

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
<i>Homo sapiens</i>	ACTTTT AAA	GGAT	AACA GCT	ATCCA	TTGGT	CTTA GGC	CCCAA	AAAT	TTTGG	TGCA ACT	CCAAA	TAAAA GTA
<i>Gorilla gorilla</i>	ACTTTT AAA	GGAT	AACA GCT	ATCCA	TTGGT	CTTA GGA	CCCAA	AAAT	TTTGG	TGCA ACT	CCAAA	TAAAA GTA
<i>Macaca mulatta</i>	ACTTTT AAA	GGAT	AACA GCT	ATCCA	TTGAC	CTTA GGA	GTCAA	AAAC	ATTGG	TGCA ACT	CCAAA	TAAAA GTA
<i>Pan paniscus</i>	ACTTTT AAA	GGAT	AACA GCC	ATCCG	TTGGT	CTTA GGC	CCCAA	AAAT	TTTGG	TGCA ACT	CCAAA	TAAAA GTA
<i>Papio hamadryas</i>	ACTTTT AAA	GGAT	AACA GCT	ATCCA	TTGGT	CTTA GGA	ACCAA	AAAC	ATTGG	TGCA ACT	CCAAA	TAAAA GTA
<i>Trachypithecus obscurus</i>	ACTTTT AAA	GGAT	AACA GAT	ATCCG	TTGGT	CTTA GGA	ACCAA	AAAT	ATTGG	TGCA ACT	CCAAA	TAAAA GTA
<i>Muntiacus reevesi</i>	ACTTTT AGA	GGAT	GACA GAT	ATCCG	TTGGT	CTTA GGA	ATCAA	AAA	ATTGG	TGCA ACT	CCAAA	TAAAA GTA
<i>Mus musculus</i>	ACTTTT ATA	GGAT	AATA GTA	ATCCA	TTGGT	CTTA GGA	ACCAA	AAAC	CTTGG	TGCA AAT	CCAAA	TAAAA GTA
<i>Balaenoptera bonaerensis</i>	ACTTTT ACA	GGAT	AACA GTT	ATCCA	TTGGT	CTTA GGA	ACCAA	AAA	ATTGG	TGCA ACT	CCAAA	TAAAA GTA
<i>Cynocephalus variegatus</i>	ACTTTC AAA	GGAT	AAAA GCA	ATCCA	TTGGT	CTTA GGA	ACCAA	AAAA	TTTGG	TGCA ACT	CCAAA	TGAAA GTA
<i>Pongo pygmaeus abelii</i>	ACTTTT AAA	GGAT	AACA GCT	ATCCC	TTGGT	CTTA GGA	CCCAA	AAAT	TTTGG	TGCA ACT	CCAAA	TAAAA GTA

The red letter indicates position 15, which corresponds to the 12280A>G mutation.

Table III. 5587T>C and 12280A>G mutations identified in affected individuals, but not controls.

Genes	Position	Replacement	Number of individuals with the two mutations		P-value
			Patients (%)	Controls (%)	
tRNA ^{Ala}	5,587	T to C	5 (100)	0	<0.05
tRNA ^{Leu(CUN)}	12,280	A to G	5 (100)	0	<0.05

A total of 500 healthy tissues were used as controls.

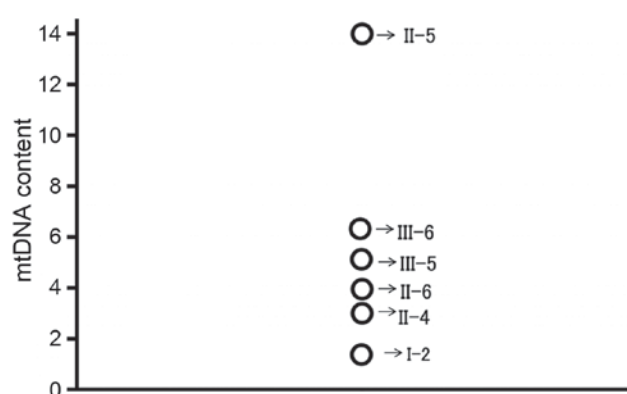


Figure 4. Quantification of mtDNA copy number. mt, mitochondrial.

12280A>G mutation created a novel base-pairing (15G-19C), which may alter its tertiary structure. Importantly, nucleotides at the same positions in mt-tRNA^{Ile} gene (4277T>C mutation) has been reported to be associated with hypertrophic cardiomyopathy (30). Therefore, the authors of the current study propose that the 12280A>G mutation may have the same impact on hypertension.

To see whether 5587T>C and 12280A>G mutations caused the mitochondrial dysfunction, the mtDNA copy number in patients carrying these mutations were evaluated using quantitative PCR. Consequently, it was determined that patients with these mutations have a lower copy number of mtDNA compared with a control with hypertension, but without the mutations, which is consistent with a previous study (31). In fact, the alteration of the mtDNA copy number, which reflects oxidant-induced cell damage, had been observed in a wide range of human mitochondrial diseases (32). Additionally, a decreased mtDNA copy number has been demonstrated to lead to increased ROS levels; ROS induced by mitochondrial dysfunction can increase mitochondrial Ca²⁺ accumulation and may act as potential pathophysiological mechanism in hypertension (33,34).

In conclusion, the authors of the current study hypothesise that mt-tRNA^{Ala} 5587T>C and tRNA^{Leu(CUN)} 12280A>G mutations possibly lead to molecular mechanisms that underlie the progression of maternally inherited essential hypertension. The molecular mechanisms may be as follows: The mutations altered the secondary structure of tRNA^{Ala} and tRNA^{Leu(CUN)}, subsequently, the structural alternations led to a decrease

in the steady-state levels of tRNA^{Ala} and tRNA^{Leu(CUN)}, and caused the impairments of mt-tRNAs metabolism. As a result, mitochondrial protein synthesis, the respiration chain and ATP levels declined significantly; additionally, decreased tRNA steady-state levels may also affect tRNA aminoacylation, mtDNA copy number and ROS generation (35,36). Therefore, the mitochondrial dysfunction, caused by 5587T>C and 12280A>G mutations, may contribute to the progression of hypertension in this family. In fact, mutations that caused the mt-tRNA metabolism failure suggest a possible metabolic pathway, as indicated in several studies (37-39). However, the incomplete penetrance of hypertension and variable degree of blood pressure indicated that the 5587T>C and 12280A>G mutations were insufficient to produce the clinical phenotypes; thus, other modifiable factors, including environmental factors, nuclear genes and epigenetic modification may account for hypertension expression. The tRNA^{Ala} 5587T>C and tRNA^{Leu(CUN)} 12280A>G mutations should be added as risk factors for familial hypertension. The main limitation of the current study was the lack of functional experiments. Thus further investigations using trans-mitochondrial cybrid cells should be employed to determine the mitochondrial dysfunctions caused by 5587T>C and 12280A>G mutations, including assessing ROS production, ATP production and mitochondrial membrane potential.

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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

LL and HZ designed the current study. LL, PC, ZQ, MW, YY, JW and QS performed clinical and molecular analyses. HZ wrote the manuscript. All authors approved the final manuscript.

Ethics approval and consent to participate

The protocol of the current study was approved by the ethics committee of the Affiliated Qingdao Hiser Hospital of Qingdao University (Qingdao, China). Written informed consent was obtained from all subjects involved in the current study.

Patient consent for publication

All patients agreed for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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