The Mitochondrial tRNA^{Ala} T5655C Mutation May Modulate the Phenotypic Expression of tRNA^{Met} and tRNA^{Gln} A4401G Mutation in a Han Chinese Family With Essential Hypertension

Yunhong XU,¹ MD, Ximing CHEN,¹ MD, Huanliang HUANG,² MD,
and Wanting LIU,³ MD

SUMMARY

Mutations in mitochondrial DNA are associated with the pathogenesis of essential hypertension. We report here the clinical, genetic, and molecular characterization of a three-generation Han Chinese family with essential hypertension. Most strikingly, this family exhibited a high penetrance of essential hypertension. Sequence analysis of the mitochondrial genome showed the presence of a homoplasmic T5655C mutation in tRNA^{Ala}, together with the A4401G mutation in the adjacent region between tRNA^{Met} and tRNA^{Gln}. Notably, the T5655C mutation was localized at the acceptor arm of tRNA^{Ala}, disrupted the high conserved base-pairing (1A-72T), and may impair the tRNA function. Moreover, the A4401G mutation was reported to decrease the steady-state level of tRNA^{Met} and tRNA^{Gln}, and consequently caused the mitochondrial dysfunction responsible for hypertension. Taken together, the combination of T5655C and A4401G mutations in mitochondrial tRNA genes may account for the high penetrance and expressivity of hypertension in this Chinese family. Thus, our findings may provide new insight into the pathogenesis of this disorder. (Int Heart J 2017; 58: 95-99)

Key words: mt-tRNA, Variants, Pedigree, Blood pressure

Essential hypertension (EH) is one of the most important modifiable risk factors for cardiovascular disease and renal disease worldwide. EH is commonly regarded as a multifactorial disease influenced by both genetic and environmental factors. Familial aggregation of high blood pressure, despite different environmental factors, suggests that genetic factors are involved in the etiology of hypertension.¹⁾

EH can be caused by single gene mutations, resulting from interactions between the environment and inherited risk factors. It is now generally believed that human hypertension is a condition associated with endothelial dysfunction and oxidative stress.²⁾ Mitochondrial dysfunction has been potentially implicated in both human and experimental hypertension.^{3,4)} Human mitochondrial DNA (mtDNA) is a double-stranded circular molecule with 16,569 bp encoding 37 genes: 13 for essential subunits of the oxidative phosphorylation (OXPHOS) system, 2 for rRNAs, and 22 for tRNAs required for mitochondrial protein synthesis.⁵⁾ All of these mt-tRNAs form a highly conserved cloverleaf structure, containing the acceptor, anticodon, and T stems, as well as TψC, D, and anticodon loops. Due to the lack of histone protection and a poor DNA repair system, mtDNA has a higher mutation rate than nuclear DNA. Most recently, several mt-tRNA mutations have been reported to be associated with EH; these mutations include the A4435G in the tRNA^{Met} gene,⁶⁾ A12330G in the tRNA^{Leu(CUN)} gene⁷⁾ and

A4295G in the tRNA^{Ile} gene.⁸⁾ These mutations can result in translational defects and consequently mitochondrial respiratory chain dysfunction, and are associated with the pathogenesis of EH.

In order to identify novel mtDNA mutations involved in the pathogenesis of EH in a Chinese population, we initiated a systematic and extended mutational screening of mtDNA in a large cohort of EH subjects from Guangzhou area, Guangdong Province. Here we describe a Han Chinese family with EH carrying the homoplasmic A4401G and T5655C mutations in the mitochondrial genome.

METHODS

Subjects: As part of a genetic screening program for EH, a Chinese Han family with maternally inherited EH was identified at the Third Affiliated Hospital of Guangzhou Medical University. Informed consent, blood samples, and clinical evaluation were obtained from all participating family members under protocols approved by the Ethics Committee of Guangzhou Medical University. Members of this family were interviewed and evaluated to identify both personal and medical histories of EH and other clinical abnormalities. A healthy control population of 200 individuals was obtained from a

From the Departments of ¹ Cardiology, ² Emergency, and ³ Physical Examination Center, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China.

Address for correspondence: Ximing Chen, MD, Department of Cardiology, the Third Affiliated Hospital of Guangzhou Medical University, No. 63, Duobao Road, Liwan District, Guangzhou, 510150, P.R. China. E-mail: chenxmzg@163.com

Received for publication April 28, 2016. Revised and accepted June 20, 2016.

Released in advance online on J-STAGE January 20, 2017.

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panel of unaffected Han Chinese residents from the same area. Members of this Chinese family underwent a physical examination, laboratory assessment of cardiovascular disease risk factors, and routine electrocardiography. An experienced physician measured the systolic and diastolic blood pressures of subjects with a mercury column sphygmomanometer using a standard protocol. The first and fifth Korotkoff sounds were taken to be indicative of systolic and diastolic blood pressures, respectively. The average of 3 such readings was taken as the examination blood pressure. EH was defined according to the recommendation of the Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure (JNC VI) as a systolic blood pressure of 140 mmHg or higher and/or a diastolic blood pressure of 90 mmHg or greater.⁹⁾

Mutational analysis of mitochondrial tRNA (mt-tRNA) genes: To determine whether mt-tRNA mutations played important roles in EH, we performed a screening for the candidate pathogenic mt-tRNA mutations in the proband (III-4) and matrilineal relatives (I-2, II-4, II-6, III-3). All these experiments were carried out at the Guangzhou Institute of Obstetrics and Gynecology of the Third Affiliated Hospital of Guangzhou Medical University. Briefly, the genomic DNA was isolated from whole blood cells of participants using Puregene DNA Isolation Kits (Gentra Systems, Minneapolis, MN). The entire mt-tRNA genes of the participants were PCR amplified using the primers as previously described.¹⁰⁾ Each fragment was purified and subsequently analyzed by direct sequencing in an ABI 3700 automated DNA sequencer (Applied Biosystems, Inc, Foster City, CA) using a Big Dye Terminator Cycle sequencing reaction kit. The resultant sequence data were compared with the reversed Cambridge sequence (GenBank accession No. NC_012920).¹¹⁾

Phylogenetic conservation analysis: To analyze the phylogeny of mt-tRNA mutations, we used vertebrate mtDNA sequences for inter-specific analysis; these species included *Elephas maximus*, *Macropus robustus*, *Homo sapiens*, *Hylobates lar*, *Macaca mulatta*, *Pan paniscus*, *Pan troglodytes*, *Capra hircus*, *Lama pacos*, and *Orycteropus afer*. The conservation index (CI) was then calculated by comparing the human nucleotide variants with the other species. Notably, the CI $\geq$ 75% was considered as functional potential.¹²⁾

Determination of pathogenicity: We further used the pathogenicity scoring system to predict the potential pathogenic role of mt-tRNA mutations. According to that standard, an mt-tRNA mutation was regarded as “definitely pathogenic” with a total score of more than 11 points, if the score was between 7-10 points, it belonged to “possibly pathogenic”, whereas a score that was less than 6 points should be classified as “neutral polymorphism”.^{13,14)}

RESULTS

Clinical features: The proband (III-4) was a 32-year-old woman who came from Guangzhou area of Guangdong Province. She first experienced hypertension when she was 29 years-old. She went to the hypertension clinic of the Third Affiliated Hospital of Guangzhou Medical University for further clinical evaluations, at which time her blood pressure was 155/90 mmHg. Physical examination, laboratory assessment of cardiovascular risk factors, and routine electrocardiography showed that she did not have any other clinical abnormalities such as diabetes, vision loss, hearing impairment, or renal and neurological disorders. Therefore, she exhibited a typical EH. As shown in Figure 1 and Table I, the average age at onset of EH in the maternal kindred varied from 29 years to 70 years, with an average of 47 years. There was no evidence that any member of this family had any other cause to account for EH. Therefore, the inheritance pattern of this family was consistent with maternal inheritance.

Mutational analysis of the mt-tRNA genes: Because previous studies showed that mt-tRNAs were the hot spots for pathogenic mutations associated with EH,¹⁵⁾ we aimed to evaluate the role of mt-tRNA mutations in EH expression. As shown in Figure 2, the homoplasmic A4401G and T5655C mutations were identified by direct Sanger sequencing. Of note, the well-known A4401G mutation was localized at the adjacent site of tRNA^{Met} and tRNA^{Gln}. In contrast, the T5655C mutation occurred at the 5' end of tRNA^{Ala} and the first nucleotide adenine was replaced with guanine (Figure 4). Moreover, as shown in Figure 3, the T5655C mutation was highly conserved between different species (CI = 100%), which indicated that it may play an important role in EH expression (Figure 3).

Determination of pathogenicity: According to the revised pathogenicity scoring system, we classified the tRNA^{Ala} T5655C mutation as “definitely pathogenic” with a total score of 17 points (Table II).

Figure 1. A Han Chinese family with essential hypertension (EH). Affected individuals are indicated by filled symbols, and the arrow denotes the proband (III-4).

Table I. Summary of Clinical Data for Several Members in This Chinese Family

Subject	Gender	Age at test	Age at onset	Systolic Pressure (mmHg)	Diastolic Pressure (mmHg)	Presence of mt-tRNA mutations
I-2	Female	84	70	145	95	T5655C, A4401G
II-4	Female	59	55	160	80	T5655C, A4401G
II-6	Female	58	54	180	95	T5655C, A4401G
III-3	Female	33	30	145	90	T5655C, A4401G
III-4	Female	32	29	155	90	T5655C, A4401G
III-1	Male	30	/	130	75	None

DISCUSSION

In this study, we have performed the clinical, genetic, and molecular characterization of a Han Chinese family with high penetrance of EH. EH as the sole clinical phenotype only presented in the maternal lineage of this pedigree, the inherited pattern provided a clear indication for the mtDNA mutations being responsible for the phenotype. In particular, the age of onset of this family ranged from 29 to 70 years, and the matrilineal relatives had an earlier age at onset of EH, suggesting that mitochondrial sequence variants may be a risk factor for this disease.

Mitochondria are important vital energy producing organelles in eukaryotic cells and are primarily responsible for generating ATP by OXPHOS.¹⁶⁾ Recently, increasing evidence has shown that mitochondrial dysfunction caused by mtDNA mutations plays an important role in the development of EH, and mitochondrial dysfunction will increase the production of reactive oxygen species (ROS), which will lead to oxidative stress, loss of nitric oxide signaling and endothelial barrier function, and infiltration of leukocytes into the vascular wall, and thus, contribute to high blood pressure.^{17,18)} Today, approximately 200 pathogenic mutations had been mapped to mt-rRNA genes (<http://www.mitomap.org/MITOMAP>),¹⁹⁾ empha-

sizing the importance of mt-tRNAs for mitochondrial function.

In this study, we identified two mutations: A4401G in the junction between the tRNA^{Met} at the heavy strand (H-strand) and tRNA^{Gln} at the light strand (L-strand),²⁰⁾ as well as the T5655C in tRNA^{Ala} (Figure 2 and 4). These mutations were present only in matrilineal relatives of this family in the homoplasmic form but not in 200 healthy controls. Of these, the

Figure 2. Identification of mt-tRNA mutations in the positions: 4401 in tRNA^{Met} and tRNA^{Gln}, 5655 in the tRNA^{Ala}. Partial sequence chromatograms of the fragments spanning the corresponding regions of mt-tRNAs from the proband (III-4) and the healthy control. Arrows indicate the location of the base changes.

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	8	10		22		27	32	39		44	49				61		66		73				
<i>Elephas maximus</i>	AAGGGCT	TA	GTTT	AGTTA	AAGC	G	TCTGA	TTTGCAT	TCAGT	TGAT	GTAAG	GTGATAT	CTTGC	AGTCCTT	A									
<i>Macropus robustus</i>	AAGAATT	TA	GCTT	AATGA	AAGT	G	TTCGA	TTTGGGT	TCGGA	TGAT	GCAAG	ATAAAGG	CTTGT	AATTCTT	A									
<i>Homo sapiens</i>	AAGGGCT	TA	GCTT	AATTA	AAGT	G	GCTGA	TTTGGGT	TCAGT	TGAT	GCAGA	GTGGGGT	TTTGC	AGTCCTT	A									
<i>Hylobates lar</i>	AAGGGCT	TA	GCTT	AATTA	AAGT	G	ACTGA	TTTGGGT	TCGGT	TGAT	GCAAA	GTGGGC	TTTGC	AGTCCTT	G									
<i>Macaca mulatta</i>	AAGGGCT	TA	GCTT	AATTA	AAGT	G	GTTGA	TTTGGGT	TCAAT	TGAT	GCAGA	GTAGGTG	TTTGC	AGTCCTT	A									
<i>Pan paniscus</i>	AAGGGCT	TA	GCTT	AATTA	AAGT	G	GCTGA	TTTGGGT	TCAGT	TGAT	GCAGA	GTGGGGT	TTTGC	AGTCCTT	A									
<i>Pan troglodytes</i>	AAGGGCT	TA	GCTT	AATTA	AAGT	G	GCTGA	TTTGGGT	TCAGT	TGAT	GCAGA	GTGGGGT	TTTGC	AGTCCTT	A									
<i>Capra hircus</i>	AAGGATT	TA	GCTT	AATTA	AAGT	G	GTTGA	TTTGCAT	TCAAT	TGAT	GTAAG	ATATGGT	CTTGC	AGTCCTT	A									
<i>Lama pacos</i>	AAGGGCT	TA	GCTT	AATTA	AAGT	A	GTTGA	TTTGCAT	TCAAT	TGAT	GTAGG	ATAGAGT	CTTGC	AGTCCTT	A									
<i>Orycteropus afer</i>	AAGGGCT	TA	GCTT	AATTA	AAGT	A	ACTGA	TTTGGGT	TCAGT	GGAT	GTAGG	ATAGAGT	CTTGC	AGTCCTT	A									

Figure 3. Alignment of tRNA^{Ala} gene from different species. Arrow indicates the position 1, corresponding to the T5655C mutation.

Figure 4. Location of the A4401G mutation in the junction of tRNA^{Met} and tRNA^{Gln}, and the T5655C mutation in the tRNA^{Ala}. Cloverleaf structures of human mt-tRNAs are derived from the Mitomap database (<http://www.mitomap.org/MITOMAP>). The arrows indicate the positions of these mtDNA mutations.

Table II. Pathogenicity Scoring System for T5655C Mutation

Scoring criteria	T5655C mutation	Score	Classification
More than one independent report	Yes	2	≤ 6 points: neutral polymorphisms;
Evolutionary conservation of the base pair	No changes	2	
Variant heteroplasmy	No	0	7-10 points: possibly pathogenic;
Segregation of the mutation with disease	Yes	2	
Histochemical evidence of mitochondrial disease	Strong evidence	2	11-13 points (not including evidence from single
Biochemical defect in complex I, III or IV		2	fiber, steady-state level): probably pathogenic;
Evidence of mutation segregation with biochemical defect from single-fiber studies	Yes	2	
Mutant mt-tRNA steady-state level or evidence of pathogenicity in trans-mitochondrial cybrid studies	Strong evidence	5	≥ 11 points (including trans-mitochondrial cybrid studies): definitely pathogenic.
Maximum score		17	Definitely pathogenic

A4401G mutation was identified in several Chinese families with EH and was implicated to cause a failure in tRNA metabolism.^{21,22)} The 5' end of the flanking sequence is 4401A/AG-TAAG in the tRNA^{Met} gene, whereas the 5' end of the flanking sequence is 4401T/TGAGAT in the tRNA^{Gln} gene.²³⁾ In fact, the processing of mt-tRNAs requires the precise endonucleolytic cleavage at both the 3' and 5' ends catalyzed by RNase P and 3' endonuclease.^{24,25)} Thus, the A4401G mutation may affect the reaction efficiency of the RNase P involved in tRNA^{Met} and tRNA^{Gln} 5' end metabolism. Functional characterization of cell lines carrying this mutation showed a significant reduction of the steady-state level of tRNA^{Met} and tRNA^{Gln}. In addition, the A4401G mutation caused the defects in mitochondrial protein synthesis and respiratory chain function. On the other hand, the homoplasmic T5655C mutation, combined with the *ND1* T3308C mutation, was first described in an African family with maternally inherited aminoglycoside-induced and non-syndromic hearing impairment carrying the tRNA^{Ser(UCN)} T7511C mutation.²⁶⁾ As shown in Figure 3, the T5655C mutation occurred at the position which was highly conserved between different species. Interestingly, the T5655C mutation produced an approximate 50% reduction in the tRNA^{Ala} level, whereas the *ND1* T3308C mutation caused significant decreases both in the amount of *ND1* mRNA and co-transcribed tRNA^{Leu(UUR)} in mutant cells. Therefore, the co-existence of the T5655C mutation and T3308C mutation may contribute to the high penetrance and expressivity of deafness-associated T7511C mutation.²⁷⁾ Moreover, a previous study indicated that the T5655C mutation played an important role in the recognition of aminoacyl-tRNA synthetase.²³⁾ The significant decrease in tRNA^{Ala} steady-state levels in the mutant cybrid carrying the T5655C mutation may result from a failure to aminoacylate properly and of post-transcriptional modification of this tRNA.²⁷⁾

Based on these findings, we propose that the molecular mechanism underlying the T5655C and A4401G mutations in the pathogenesis of EH may be as follows: first, these mutations caused the alternation of mt-tRNA secondary structure which will subsequently result in the failure in mt-tRNA metabolism, including decreases in the tRNA steady state level and aminoacylation, which will eventually lead to decreased mitochondrial protein synthesis. Defects in mitochondrial translation consequently lead to a respiratory phenotype and a decline in ATP production below the threshold level required for endothelial cell function,²⁸⁾ thereby contributing to the high blood pressure. This is the first time that a study has provided direct evidence for the hypertension associated mitochondrial

T5655C and A4401G mutations.

DISCLOSURE

The authors have no conflicts of interest to disclose.

REFERENCES

1. Nakanishi T. Pulmonary arterial hypertension associated with congenital heart disease. Personal perspectives. *Int Heart J* 2015; 56: S1-3. (Review)
2. Redón J, Oliva MR, Tormos C, *et al.* Antioxidant activities and oxidative stress byproducts in human hypertension. *Hypertension* 2003; 41: 1096-101.
3. Arrell DK, Elliott ST, Kane LA, *et al.* Proteomic analysis of pharmacological preconditioning: novel protein targets converge to mitochondrial metabolism pathways. *Circ Res* 2006; 99: 706-14.
4. Yagi H, Sumino H, Yoshida K, *et al.* Biological antioxidant potential negatively correlates with carotid artery intima-media thickness. *Int Heart J* 2016; 57: 220-5.
5. Chomyn A, Attardi G. MtDNA mutations in aging and apoptosis. *Biochem Biophys Res Commun* 2003; 304: 519-29. (Review)
6. Liu Y, Li R, Li Z, *et al.* Mitochondrial transfer RNAMet 4435A>G mutation is associated with maternally inherited hypertension in a Chinese pedigree. *Hypertension* 2009; 53: 1083-90.
7. Teng L, Zheng J, Leng J, Ding Y. Clinical and molecular characterization of a Han Chinese family with high penetrance of essential hypertension. *Mitochondrial DNA* 2012; 23: 461-5.
8. Li Z, Liu Y, Yang L, Wang S, Guan MX. Maternally inherited hypertension is associated with the mitochondrial tRNA(Ile) A4295G mutation in a Chinese family. *Biochem Biophys Res Commun* 2008; 367: 906-11.
9. The sixth report of the Joint National Committee on prevention, detection, evaluation and treatment of high blood pressure. *Arch Intern Med* 1997; 157: 2413-46.
10. Qin Y, Xue L, Jiang P, *et al.* Mitochondrial tRNA variants in Chinese subjects with coronary heart disease. *J Am Heart Assoc* 2014; 3: e000437.
11. Anderson S, Bankier AT, Barrell BG, *et al.* Sequence and organization of the human mitochondrial genome. *Nature* 1981; 290: 457-65.
12. Wang L, Chen ZJ, Zhang YK, *et al.* The role of mitochondrial tRNA mutations in lung cancer. *Int J Clin Exp Med* 2015; 8: 13341-6.
13. González-Vioque E, Bornstein B, Gallardo ME, Fernández-Moreno MÁ, Garesse R. The pathogenicity scoring system for mitochondrial tRNA mutations revisited. *Mol Genet Genomic Med* 2014; 2: 107-14.
14. Yarham JW, Al-Dosary M, Blakely EL, *et al.* A comparative analysis approach to determining the pathogenicity of mitochondrial

- tRNA mutations. *Hum Mutat* 2011; 32: 1319-25.
15. Ding Y, Xia B, Yu J, Leng J, Huang J. Mitochondrial DNA mutations and essential hypertension. *Int J Mol Med* 2013; 32: 768-74. (Review)
16. Wallace DC. Bioenergetics and the epigenome: interface between the environment and genes in common diseases. *Dev Disabil Res Rev* 2010; 16: 114-9.
17. Marian AJ. Mitochondrial genetics and human systemic hypertension. *Circ Res* 2011; 108: 784-6.
18. Kinugawa S, Takada S, Matsushima S, Okita K, Tsutsui H. Skeletal Muscle Abnormalities in Heart Failure. *Int Heart J* 2015; 56: 475-84.
19. Ruiz-Pesini E, Lott MT, Procaccio V, *et al.* An enhanced MITO-MAP with a global mtDNA mutational phylogeny. *Nucleic Acids Res* 2007; 35: D823-8.
20. Ojala D, Montoya J, Attardi G. tRNA punctuation model of RNA processing in human mitochondria. *Nature* 1981; 290: 470-4.
21. Zhu HY, Wang SW, Liu L, *et al.* A mitochondrial mutation A4401G is involved in the pathogenesis of left ventricular hypertrophy in Chinese hypertensives. *Eur J Hum Genet* 2009; 17: 172-8.
22. Li R, Liu Y, Li Z, Yang L, Wang S, Guan MX. Failures in mitochondrial tRNAMet and tRNAGln metabolism caused by the novel 4401A>G mutation are involved in essential hypertension in a Han Chinese Family. *Hypertension* 2009; 54: 329-37.
23. Florentz C, Sohm B, Tryoen-Tóth P, Pütz J, Sissler M. Human mitochondrial tRNAs in health and disease. *Cell Mol Life Sci* 2003; 60: 1356-75. (Review)
24. Holzmänn J, Frank P, Löffler E, Bennett KL, Gerner C, Rossmannith W. RNase P without RNA: identification and functional reconstitution of the human mitochondrial tRNA processing enzyme. *Cell* 2008; 135: 462-74.
25. Levinger L, Jacobs O, James M. In vitro 3'-end endonucleolytic processing defect in a human mitochondrial tRNA(Ser(UCN)) precursor with the U7445C substitution, which causes nonsyndromic deafness. *Nucleic Acids Res* 2001; 29: 4334-40.
26. Sue CM, Tanji K, Hadjigeorgiou G, *et al.* Maternally inherited hearing loss in a large kindred with a novel T7511C mutation in the mitochondrial DNA tRNA(Ser(UCN)) gene. *Neurology* 1999; 52: 1905-8.
27. Li X, Fischel-Ghodsian N, Schwartz F, Yan Q, Friedman RA, Guan MX. Biochemical characterization of the mitochondrial tRNASer(UCN) T7511C mutation associated with nonsyndromic deafness. *Nucleic Acids Res* 2004; 32: 867-77.
28. Yang Q, Kim SK, Sun F, *et al.* Maternal influence on blood pressure suggests involvement of mitochondrial DNA in the pathogenesis of hypertension: the Framingham Heart Study. *J Hypertens* 2007; 25: 2067-73.