Molecular characterization of a pedigree carrying the hypertension-associated mitochondrial tRNA^{Gln} T4363C mutation

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Abstract. Mitochondrial DNA mutations have been reported to be associated with essential hypertension. The present study reported the clinical and molecular features of a Chinese pedigree with maternally inherited hypertension. A total of 6 matrilineal relatives in this pedigree presented with variable degrees of hypertension; the age of onset ranged between 39 and 63 years, and the average age of onset was 53 years. Analysis of the mitochondrial genome in members of this family demonstrated the occurrence of a homoplasmic T4363C mutation in the transfer (t)RNA^{Gln} gene and 25 genetic polymorphisms belonging to mitochondrial haplogroup B4. Notably, the T4363C mutation was localized at the anticodon stem of tRNA^{Gln}, which is highly conserved across various species (conventional position 38). To determine its potential pathogenicity, RNA Fold software was used to predict the secondary structure of tRNA^{Gln} with and without this mutation. The results indicated that the T4363C mutation induced a significant alteration in the secondary structure of tRNA^{Gln}, and may reduce the steady-state levels of tRNA^{Gln}. Furthermore, matrilineal relatives carrying the T4363C mutation exhibited different age of onset and variable degrees of blood pressure, thus indicating that the T4363C mutation itself was insufficient to produce the clinical phenotype. Therefore, other modified factors, including environmental factors, and nuclear gene and epigenetic modifications, may be involved in the pathogenesis of hypertension. In conclusion, the present study provided valuable information regarding the association between tRNA mutations and hypertension.

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Introduction

Cardiovascular disease is a common disease worldwide. Essential hypertension (EH) and coronary heart disease are the most common types of cardiovascular disease. Among them, EH affects ~1 billion people worldwide and ~130 million individuals in China (1). In addition, EH is associated with an increased risk for stroke and renal dysfunction, and it represents one of the greatest public health concerns worldwide. At present, the molecular mechanism underlying EH remains largely unknown. It is generally believed that EH is a complex and multifactorial disorder, which may be caused by single gene defects or environmental conditions. Among these genetic factors, the maternal inheritance of EH has been observed in numerous families, indicating that variation in mitochondrial DNA (mtDNA) is involved in the pathogenesis of EH (2,3). Previous studies have identified some mtDNA pathogenic mutations including the 12S ribosomal (r)RNA A1555 G mutation (4), the transfer (t)RNA^{Met} A4435 G mutation (5), and the tRNA^{Met}/tRNA^{Gln} A4401G and tRNA^{Ile} A4295G mutations (6,7). These mtDNA mutations, mainly located at tRNA genes, may lead to failures in tRNA metabolism, and subsequently result in defects in mitochondrial translation, thus causing mitochondrial dysfunction which in implicated in EH pathophysiology. Therefore, mtDNA mutations may have potential as novel biomarkers for the early detection, prevention and management of maternally inherited EH.

However, the frequency of these mt-tRNA mutations in Han Chinese subjects with EH remains to be elucidated. To understand the contribution of mitochondrial variants to EH, we have initiated an extensive mutational screening program for mtDNA in a large cohort of EH subjects at the Hanchuan People's Hospital (Hanchuan, China). The present study described a Chinese pedigree with EH. Analysis of the entire mitochondrial genome resulted in identification of a homoplasmic tRNA^{Gln} T4363C mutation. In addition, to determine whether mitochondrial genetic background may serve an active role in EH, the present study conducted polymerase chain reaction (PCR)-Sanger sequencing for the fragments spanning the mitochondrial genome, and used RNA Fold Webserver to predict the potential pathogenicity of the tRNA^{Gln} T4363C mutation.

Materials and methods

Subjects. A Han Chinese family (Fig. 1) was recruited at the Department of Cardiology, Hanchuan People's Hospital. The individuals were interviewed, and detailed demographics, anthropometrics, vital parameters and medical history were recorded. Furthermore, 300 DNA samples were collected form age and gender-matched healthy participants from the same area, which were used as controls. The present study was approved by the Ethics Committee of Hanchuan People's Hospital, and written informed consent was obtained from all individuals or relatives prior to enrollment in the present study.

Blood pressure (BP) measurement. Members of the Chinese family underwent a complete examination, including physical examination, clinical laboratory evaluation and routine electrocardiography. Using an electronic measuring device, two doctors determined the systolic and diastolic BP of each individual; BP measurements were repeated three times. According to the World Health Organization International Society of Hypertension (8), EH was defined as a systolic BP >140 mmHg or a diastolic BP >90 mmHg.

Analysis of mitochondrial genome mutations. To screen mutations in the mitochondrial genome, genomic DNA was extracted from blood samples using the Puregene DNA Isolation kit (Gentra Systems, Inc., Minneapolis, MN USA). The complete mitochondrial genomes of matrilineal relatives (II-1, II-3, II-5, II-8, II-10, III-5, III-6 and III-7) were amplified by PCR, using a previously described method (9). Following PCR amplification and electrophoresis, the 24 fragments spanning the mitochondrial genome were purified and analyzed using an ABI 3700 automated DNA sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Furthermore, genetic variants were identified in the mitochondrial genome by comparing the sequence data with the Cambridge reference sequence (NC_012920) (10).

Phylogenetic conservation analysis. The entire mitochondrial sequence variants in the matrilineal relatives with EH in the Chinese pedigree were assigned to the Asia mitochondrial haplogroups, as described by Kong *et al* (11). Furthermore, 10 vertebrates' mtDNA sequences were selected to assess evolutionary conservation. The conservation index (CI) was calculated by comparing the human nucleotide variants with 9 other vertebrates. A CI >75% was considered as having functional significance.

Prediction of the secondary structure of tRNA^{Gln} with and without the T4363C mutation. To determine whether the T4363C mutation affected tRNA^{Gln} structure, the RNA Fold Webserver program (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) was used to predict the minimum free energy (MFE) secondary structure of the wild-type tRNA^{Gln} and the mutant tRNA^{Gln} carrying the T4363C mutation (12). The wild-type sequence of tRNA^{Gln} was: 5'-TAGGATGGGGTGTGATAGGTGGCACGGAGAATTTTGGATTCTCAGGGATGGGTTCGATTCTCATAGTCCTAG-3', whereas the sequence of tRNA^{Gln} carrying the T4363C mutation was: 5'-TAGGATGGGGTGTGATAGGTGGCACGGAGAATTTTGGGTTCTCAGGGATGGGTTCGATTCTCATAGTCCTAG-3'. The structure was predicted using the loop based energy model and dynamic programming algorithm, as described by Zuker and Stiegler (13).

Statistical analysis. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Differences in categorical variables were assessed with Fisher's exact test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical characterization of the Chinese pedigree carrying EH. The proband (III-7) was a 45-year-old woman born in Wenzhou, who now lived in Hanchuang. The patient had suffered from EH for ~5 years, and her BP was 150/95 mmHg. Recently, she visited the Department of Cardiology, Hanchuan People's Hospital for treatment of EH. A comprehensive examination, including physical examination, clinical laboratory assessment of risk factors for EH and electrocardiography, indicated that she did not carry other abnormalities, such as diabetes mellitus, myopia, deafness, cancer, and renal and neurological disorders. Therefore, she suffered from only one syndrome: EH. According to the family history of the patient, it was determined that 7 individuals from her family suffered from a variable degree of hypertension. The grandmother (I-2) of the proband had succumbed several years ago, due to high BP (180/95 mmHg). As presented in Fig. 1, the pattern of transmission in this family was maternal inheritance. As presented in Table I, the age of onset of EH in the pedigree ranged between 39 and 63 years, with an average of 53 years.

Mutational analysis of the mitochondrial genome. As shown in Fig. 1, the pattern of transmission of EH in this family was consistent with maternal inheritance, indicating that mitochondrial genome mutations may be the molecular basis for this disease. To determine the contribution of mtDNA mutations to EH, PCR amplification of the mitochondrial genome was conducted on samples from matrilineal relatives (II-1, II-3, II-5, II-8, II-10, III-5, III-6 and III-7) and the PCR fragments were subsequently sequenced from each affected individual. As presented in Table II, after comparing with the Cambridge reference sequence by phylogenetic analysis, 25 genetic polymorphisms were identified, belonging to human mitochondrial haplogroup B4 (11). Of these, there were 7 variants in the D-loop gene, 2 known variants in the 12S rRNA gene and 1 variant in the 16S rRNA gene, as well as a 9-bp common deletion in the conjunction between the tRNA^{Lys} and cytochrome *c* oxidase subunit 2 genes. The missense mutations included NADH dehydrogenase subunit 2 C5263T mutation (A265V), ATPase subunit 6 A8701G (T59A) and A8860G (T112A) mutations, NADH dehydrogenase subunit 3 A10398G (T114A) mutation and cytochrome B C14766T (I7T) mutation. All of these genetic variants can be found by searching Google and specific databases, and therefore should not be regarded as novel (14). Furthermore, evolutionary conservation was assessed for these identified variants in 9 organisms, including mice (15), cattle (16) and *Xenopus laevis* (17). We found that other variants were not conserved, with the exception of the T4363C mutation

Table I. Summary of clinical data for the matrilineal relatives in a family with essential hypertension.

Subject	Sex	Age at test	Age of onset	Diastolic blood pressure (mmHg)	Systolic blood pressure (mmHg)	Occurrence of the T4363C mutation
II-1	Male	66	61	95	145	Yes
II-3	Male	63	/	80	120	Yes
II-5	Male	68	63	80	150	Yes
II-8	Female	65	60	100	160	Yes
II-10	Female	61	59	95	175	Yes
III-6	Female	41	39	90	145	Yes
III-7	Female	45	40	95	150	Yes
III-5	Male	46	/	75	130	Yes
III-3	Female	40	/	80	135	No
III-1	Male	36	/	75	135	No

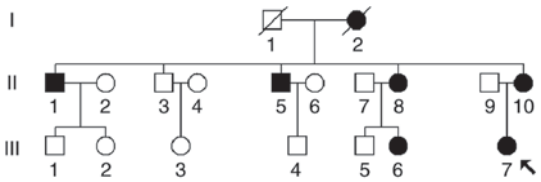

Figure 1. A Han Chinese family with EH; patients with EH are indicated by filled symbols. Arrow indicates the proband; circles indicate females and squares indicate males; dashes indicate deceased patients.

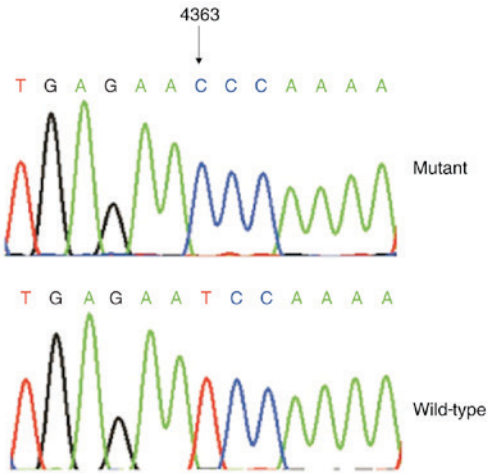

Figure 2. Sequence analysis of the transfer RNA^{Gln} T4363C mutation. Particle chromatogram of mitochondrial DNA sequence from a patient with EH and a healthy subject. Arrows indicate the T4363C mutation.

(Figs. 2 and 3). Notably, some matrilineal relatives (II-3 and III-5) carried the T4363C mutation, but did not have high BP. Fisher's exact frequency difference test demonstrated that the T4363C mutation was significant when compared with the frequency in control samples ($P<0.05$).

T4363C mutation induces structural alterations to tRNA^{Gln}. To determine whether the T4363C mutation induced secondary structure alterations to tRNA^{Gln}, the RNA Fold program was used to predict the MFE structure of tRNA^{Gln} with and without the T4363C mutation (12). As presented in Fig. 4, this mutation appeared to alter the secondary structure of tRNA^{Gln}, thus suggesting that the T4363C mutation may serve an important role in the development of EH.

Discussion

The present study investigated the contribution of mitochondrial mutations in the clinical manifestation of EH in a Han Chinese family. Notably, members of this pedigree presented with hypertension as the sole phenotype. Clinical and genetic assessment revealed a variable degree of EH, with differing severities and age of onset. Notably, the age of onset of EH in matrilineal relatives (II-1, II-3, II-5, II-8, II-10, III-5, III-6 and III-7) ranged between 39 to 63 years, with an average age of 53 years. Furthermore, it was observed that compared with the first and second generation, the members in the third generation in this family had an earlier age of onset of EH; indicating that screening for the presence of pathogenic mtDNA mutations may be useful for the early diagnosis and prevention of EH.

Analysis of the mutations in the mitochondrial genome identified 25 genetic polymorphisms belonging to human mitochondrial haplogroup B4d. Of them, the tRNA^{Gln}T4363C mutation is of particular interest. This mutation was present in 6 matrilineal relatives with EH, but was also present in 2 matrilineal relatives without EH. Notably, the T4363C mutation was localized at the immediate 3' end of the anticodon, corresponding to position 38 of tRNA^{Gln} (18). Notably, the nucleotide at this position is highly conserved among9 other vertebrates, and is often modified during tRNA^{Gln} processing and function. Thus, the T4363C mutation may reduce the steady-state level of tRNA^{Gln} (19). Previous studies have reported that the T4363C mutation is associated with deafness, developmental delay and pseudoexfoliation glaucoma (20,21). Furthermore, the results of an RNA Fold analysis indicated that the T4363C mutation altered the structure of tRNA^{Gln}, strongly suggesting that this mutation will result in the failure of tRNA^{Gln} metabolism, consequently impairing mitochondrial translation and finally leading to mitochondrial dysfunction associated with EH.

Table II. Mitochondrial DNA sequence variants in a family with essential hypertension.

Gene	Position	Replacement	Conservation (H/B/M/X)	Members carrying these mutations
D-loop	73	A to G		II-1, II-3, II-5, II-8, II-10, III-5, III-6 , III-7
	152	T to C		II-1, II-3, II-5, II-8, II-10, III-5, III-6 , III-7
	263	A to G		II-1, II-3, II-5, II-8, II-10, III-5, III-6 , III-7
	310	InsC		II-1, II-3, II-5, II-8, II-10, III-5, III-6 , III-7
	16,136	T to C		II-1, II-3, II-5, II-8, II-10, III-5, III-6 , III-7
	16,189	T to C		II-1, II-3, II-5, II-8, II-10, III-5, III-6 , III-7
	16,519	T to C		II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
12S rRNA	750	A to G	A/A/A/-	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
	827	A to G	A/A/A/A	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
16S rRNA	3,107	delC		II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
ND1	3,970	C to T		II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
tRNA^{Gln}	**4,363**	**T to C**	**Y/Y/Y/Y**	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
ND2	4,715	A to G	G/G/G/G	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
	5,263	C to T (Ala to Val)	A/A/I/F	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
CO1	7,028	C to T	A/A/A/A	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
NC_7	8,281-8,289	9-bp del		II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
A6	8,701	A to G (Thr to Ala)	T/S/L/Q	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
	8,860	A to G (Thr to Ala)	T/A/A/T	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
CO3	9,540	T to C		II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
ND3	10,398	A to G (Thr to Ala)	T/T/T/A	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
	10,400	C to T	T/T/T/A	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
ND5	12,705	C to T	I/L/L/T	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
Cytb	14,766	C to T (Thr to Ile)	T/S/T/S	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
	14,783	T to C	I/I/I/I	II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7
	15,301	G to A		II-1, II-3, II-5, II-8, II-10, III-5, III-6, III-7

Bold indicates the only gene conserved in the other species. H, human; B, bovine; M, mouse; X, *Xenopus laevis*; Ins, insertion; del, deletion; A, adenine; Y, Tyrosine; G, Guanine; I, Isoleucine; F, Phenylalanine; T, Threonine; S, Serine; L, Leucine; Q, Glutamine.

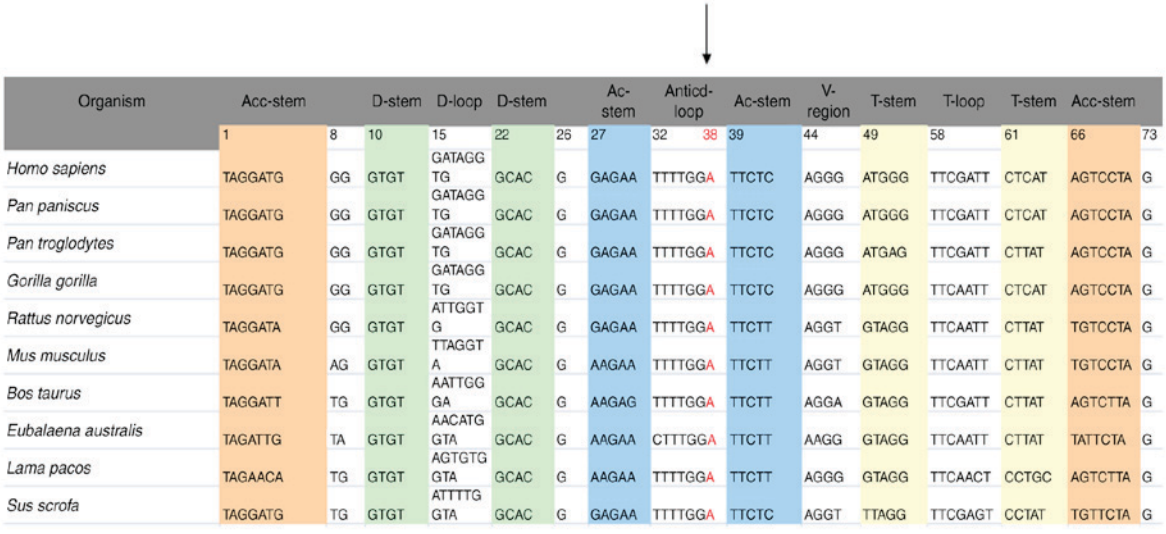

Figure 3. Alignment of transfer RNA^{Gln} gene sequences from 10 vertebrates, arrow indicates position 38, which corresponds to the T4363C mutation.

In conclusion, the identification of a homoplasmic tRNA^{Gln} T4363C mutation in members of this Chinese pedigree suggested that this mutation may serve an active role in the pathogenesis of EH. However, the family members (II-3 and III-5) that carried

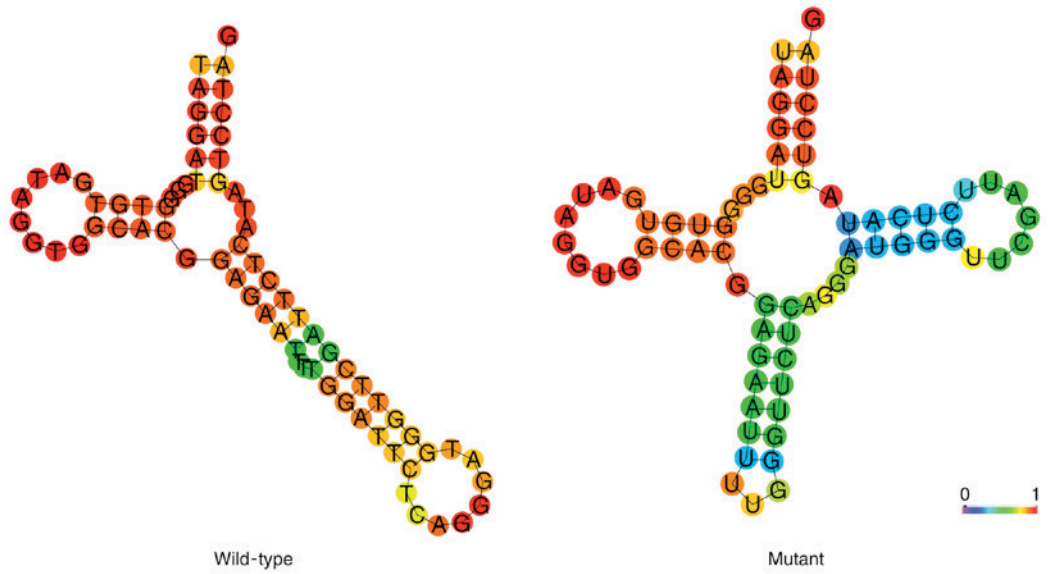

Figure 4. Prediction of the secondary structure of the wild-type version of $tRNA^{Gln}$ and the mutant $tRNA^{Gln}$ carrying the T4363C mutation. tRNA, transfer RNA.

the T4363C mutation but did not suffer from EH suggested that environmental factors, nuclear gene and epigenetic modifications may also serve important roles in the pathogenesis of EH. It is recommended that the T4363C mutation in $tRNA^{Gln}$ may be considered a risk factor for the early diagnosis of EH. Therefore, the present study provided a novel insight into the molecular mechanism, prevention and potential treatment of EH, particularly for those with a family history of EH.

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