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ARTICLE

The 12S rRNA A1555G mutation in the mitochondrial haplogroup D5a is responsible for maternally inherited hypertension and hearing loss in two Chinese pedigrees

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We reported here clinical, genetic evaluations and molecular analysis of mitochondrial DNA (mtDNA) in two Han Chinese families carrying the known mitochondrial 12S rRNA A1555G mutation. In contrast with the previous data that hearing loss as a sole phenotype was present in the maternal lineage of other families carrying the A1555G mutation, matrilineal relatives among these two Chinese families exhibited both hearing loss and hypertension. Of 21 matrilineal relatives, 9 subjects exhibited both hearing loss and hypertension, 2 individuals suffered from only hypertension and 1 member had only hearing loss. The average age at onset of hypertension in the affected matrilineal relatives of these families was 60 and 46 years, respectively, whereas those of hearing loss in these two families were 33 and 55 years, respectively. Molecular analysis of their mtDNA identified distinct sets of variants belonging to the Eastern Asian haplogroup D5a. In contrast, the A1555G mutation occurred among other mtDNA haplogroups D, B, R, F, G, Y, M and N, respectively. Our data further support that the A1555G mutation is necessary but by itself insufficient to produce the clinical phenotype. The other modifiers are responsible for the phenotypic variability of matrilineal relatives within and among these families carrying the A1555G mutation. Our investigation provides the first evidence that the 12S rRNA A1555G mutation leads to both of hearing loss and hypertension. Thus, our findings may provide the new insights into the understanding of pathophysiology and valuable information for management and treatment of maternally inherited hearing loss and hypertension.
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INTRODUCTION

Mutations in mitochondrial DNA (mtDNA) have been associated with both syndromic and non-syndromic deafness.[1,2] Those non-syndromic deafness-associated mtDNA mutations, such as 12S rRNA A1555G or tRNA$^{Ser(UCN)}$ A7445G, often occur in the homoplasmy or nearly homoplasmy,[3–6] while the syndromic deafness-associated mtDNA mutations, such as the tRNA$^{Leu(UUR)}$ A3243G mutation and mtDNA large deletions, are present in heteroplasmy.[7,8] Furthermore, the A7445G mutation has also been associated with syndromic deafness presenting palmoplantar keratoderma,[9] while the A1555G mutation has been associated with Leber's hereditary optical neuropathy or with cardiomyopathy or pigmentary disturbances and spinal anomalies.[10–12] The other non-syndromic deafness-associated mtDNA mutations are the 12S rRNA C1494T mutation, the 7472insC, T7505C and T7511C mutations in the *tRNA$^{Ser(UCN)}$* gene ,and the T12201C mutation in the *tRNA^{His}* gene.[13–17] The 12S rRNA mutations impaired mitochondrial translation, leading to deficient respiration.[13,18,19] Mild mitochondrial dysfunctions were observed in cells carrying these mtDNA mutations.[13,18,19] Therefore, these mtDNA mutations are necessary but insufficient to produce a clinical phenotype. Other modifier factors should modulate the phenotypic manifestation of these mtDNA mutations.[5,18,19]

As part of a genetic screening program for deafness in the Chinese population,[5,13,16,20] we ascertained two Chinese pedigrees carrying the known 12S rRNA A1555G mutation through the First Affiliated Hospital, Wenzhou Medical College. In contrast with the previous observation that hearing loss as a sole phenotype was present in the maternal lineage of other families carrying the A1555G mutation,[3–5] only matrilineal relatives among these two Chinese families exhibited both hearing loss and hypertension. In particular, among 21 matrilineal relatives, 9 subjects exhibited both hearing loss and hypertension, 2 individuals suffered from only hypertension and 1 member had only hearing loss. Mutational analysis of their mitochondrial genomes identified distinct sets of variants belonging to the Eastern Asian haplogroup D5a.[21] On other hand, the A1555G mutation in the other Chinese families with aminoglycoside-induced and non-syndromic deafness occurred in the other Eastern Asian mtDNA haplogroups: D, B, R, F, G, Y, M and N, respectively.[5] Out data provide the first

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evidence that the A1555G mutation is associated with hearing loss and hypertension.

MATERIALS AND METHODS

Subjects

As the part of genetic screening program for hearing impairment, two Han Chinese families, as shown in Figure 1, were ascertained through the Otology Clinic of the First Affiliated Hospital, Wenzhou Medical College. Informed consent was obtained from participants before their participation in the study, in accordance with the Cincinnati Children's Hospital Medical Center Institutional Review Board and Ethnics Committee of Wenzhou Medical College.

Clinical examinations

A comprehensive history and physical examination were performed to identify any syndromic findings, the history of the use of aminoglycosides, genetic factors related to the hearing impairment, laboratory assessment of cardiovascular disease risk factors and routine electrocardiography in members of these pedigrees.

An age-appropriate audiological examination was performed and this examination included pure-tone audiometry and/or auditory brainstem response, immittance testing and distortion product otoacoustic emissions. The pure-tone audiometry was calculated from the sum of the audiometric thresholds at 500, 1000, 2000, 4000 and 8000 Hz. The severity of hearing impairment was classified into five grades: normal <26 dB; mild=26–40 dB; moderate=41–70 dB; severe=71–90 dB; and profound >90 dB.

A physician measured the systolic and diastolic blood pressures of subjects using a mercury column sphygmomanometer and a standard protocol. The first and the fifth Korotkoff sounds were taken as indicators of systolic and diastolic blood pressure, respectively. The average of three such systolic and diastolic blood pressure readings was taken as the examination blood pressure. Hypertension was defined according to the recommendation of the Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure (JNC VI)[22] and the World Health Organization-International Society of Hypertension,[23] as a systolic blood pressure of 140 mm Hg or higher and/or a diastolic blood pressure of 90 mm Hg or greater.

Mutational analysis of mitochondrial genome

Genomic DNA was isolated from whole blood of participants using Paxgene Blood DNA Isolation Kits (QIAGEN, Valencia, CA, USA). Subject's DNA fragments spanning the entire mitochondrial *12S rRNA* gene were amplified by PCR using oligodeoxynucleotides corresponding to positions 618–635 and 1988–2007.[24] For the detection of the A1555G mutation, the amplified segments were digested with a restriction enzyme *BsmA*I.[20] Equal amounts of various digested samples were then analyzed by electrophoresis through 1.5% agarose gel. The proportions of digested and undigested PCR product were determined by laser densitometry after ethidium bromide staining to determine if the A1555G mutation is in homoplasmy in these subjects.

The entire mtDNA of probands (WHP7-II-2 and WHP8-III-3) was PCR amplified in 24 overlapping fragments by using sets of the light-strand (L) and the heavy-strand (H) oligonucleotide primers, as described elsewhere.[24] Each fragment was purified and subsequently analyzed by direct sequencing in an ABI 3700 automated DNA sequencer using the Big Dye Terminator Cycle sequencing reaction kit. The resultant sequence data were compared with the updated consensus Cambridge sequence (GenBank accession number: NC_012920).[25]

Phylogenetic analysis

A total of 17 vertebrate's mtDNA sequences were used in the interspecific analysis. These include: Bos Taurus, Cebus albifrons, Gorilla gorilla, Homo sapiens, Hylobates lar, Lemur catta, *Macaca mulatta*, Macaca sylvanus, Mus musculus, Nycticebus coucang, Pan paniscus, Pan troglodytes, Pongo pygmaeus, Pongo abelii, Papio hamadryas, Tarsius bancanus and Xenopus laevis (Genbank). The conservation index was calculated by comparing the human nucleotide variants with other 16 vertebrates. The conservation index was then defined as the percentage of species from the list of 16 different vertebrates that have the wild-type nucleotide at that position.

Haplogroup analyses

The entire mtDNA sequences of the Chinese probands carrying the A1555G mutation were assigned to an Asian mitochondrial haplogroup by using the nomenclature of mitochondrial haplogroups.[21]

Genotyping analysis of *TRMU* gene

The genotyping for the nuclear modifier TRMU A10S variant in subjects from two pedigrees was PCR-amplified for exon 1 and was followed by the digestion of a 467-bp segment with the restriction enzyme *Bsp*1286I. The forward and reverse primers for exon 1 are 5′-ACAGCGCAGAAGAAGAGCAGT-3′ and 5′-ACAACGCCACGACGGACG-3′, respectively. The *Bsp*1286I-digested products were analyzed on 1.5% agarose gels.[26] The PCR segments were subsequently analyzed by direct sequencing in an ABI 3700-automated DNA sequencer using the Big Dye Terminator Cycle sequencing reaction kit. The resultant sequence data were compared with the TRMU genomic sequence (GenBank accession number AF448221).[26]

RESULTS

Clinical and genetic evaluation of two Chinese pedigrees carrying the A1555G mutation

As a part of genetic screening, mutational analysis of the *12S rRNA* gene revealed that two Han Chinese subjects, who were diagnosed as both hearing loss and hypertension, harbored the homoplasmic A1555G mutation (Figure 2). A comprehensive history and physical examination as well as audiological examination were performed to identify any syndromic findings, the history of the use of aminoglycosides, genetic factors related to the hearing impairment, laboratory assessment of cardiovascular disease risk factors and routine electrocardiography in all available members of two Han Chinese pedigrees carrying the A1555G mutation. The restriction enzyme digestion and subsequent electrophoresis of available members in two pedigrees indicated that the A1555G mutation was indeed present in

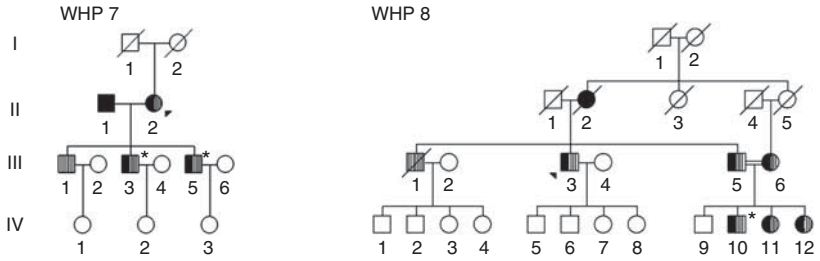

Figure 1 Two Han Chinese pedigrees with hearing loss and hypertension. Hearing impaired and hypertension individuals are indicated by filled symbols and vertical grid, respectively. An arrow denotes probands. Asterisks denote individuals who had a history of exposure to aminoglycosides.

homoplasmy in matrilineal relatives but not other members of these families (data not shown).

Of these, the proband (II-2) of WHP7 family exhibited hearing impairment and hypertension at the age of 33 and 58 years old, respectively. As illustrated in Figure 3, audiological evaluation showed that she had moderate hearing impairment (67 dB at right ear, 62 dB at left ear, with a slope-shaped pattern). As shown in Table 1, her blood pressure was 170/100 mm Hg. Further, comprehensive family history and clinical examination in members of the three-generation family revealed that two male matrilineal relatives (III-3 and III-5) suffered from both hearing loss and hypertension and one male matrilineal relative (III-1) had only hypertension (blood pressure: 170/100 mm Hg). Of those, subjects III-3 and III-5 were administrated with aminoglycosides at the age of 3 and 5 years old, respectively.

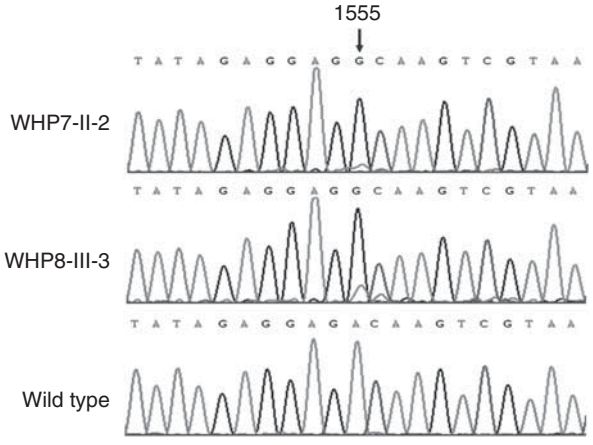

Figure 2 Identification of the A1555G mutation in the *12S rRNA* gene. Partial sequences chromatograms of *12S rRNA* gene from two affected individuals (WHP7-II-3, WHP8-III-3) and a control, respectively. Arrows indicate the locations of the base changes at positions 1555.

In the family WHP8, the proband III-3 suffered hearing impairment and hypertension at the age of 65 and 69 years old, respectively. He had moderate hearing impairment (36 dB at right ear, 46 dB at left ear, with a flat-shaped pattern) and his blood pressure was 140/90 mm Hg. Familiar history and clinical evaluation revealed that six matrilineal relatives (III-3, III-5, III-6, IV-10, IV-11, IV-12) suffer from both hearing loss and hypertension, one subject (II-2) exhibited hearing loss as the sole clinical symptom and one individual (III-1) had only hypertension. Of these, subject IV-10 was administrated with aminoglycosides at the age of 5 years. As shown in Table 1, the age-at-onset of hypertension in the maternal kindred varied from 45 to 71 years, with an average of 60 years, while the average age-at-onset of hearing loss in this family varied from 45 to 65 years, with an average of 55 years. It is worthwhile to note that the proband III-3 had two boys (IV-5 and IV-6) with hypertension. However, molecular analysis showed that the subjects IV-5 and IV-6 and their mother III-4 did not carry the A1555 mutation. The cause of their hypertension is unclear. Furthermore, there was no evidence that any member of this family had any other cause to account for hypertension. However, none of other clinical abnormalities were observed in the maternal kindred.

Mutational analysis of mitochondrial genomes

To assess the role of mtDNA variants in the phenotypic expression of the A1555G mutation, we performed a PCR-amplification of fragments spanning entire mitochondrial genome and subsequent DNA sequence analysis in two probands. In addition to the identical A1555G mutation, as shown in Table 2, these subjects exhibited distinct sets of mtDNA polymorphisms (43 variants in WHP7-II-2

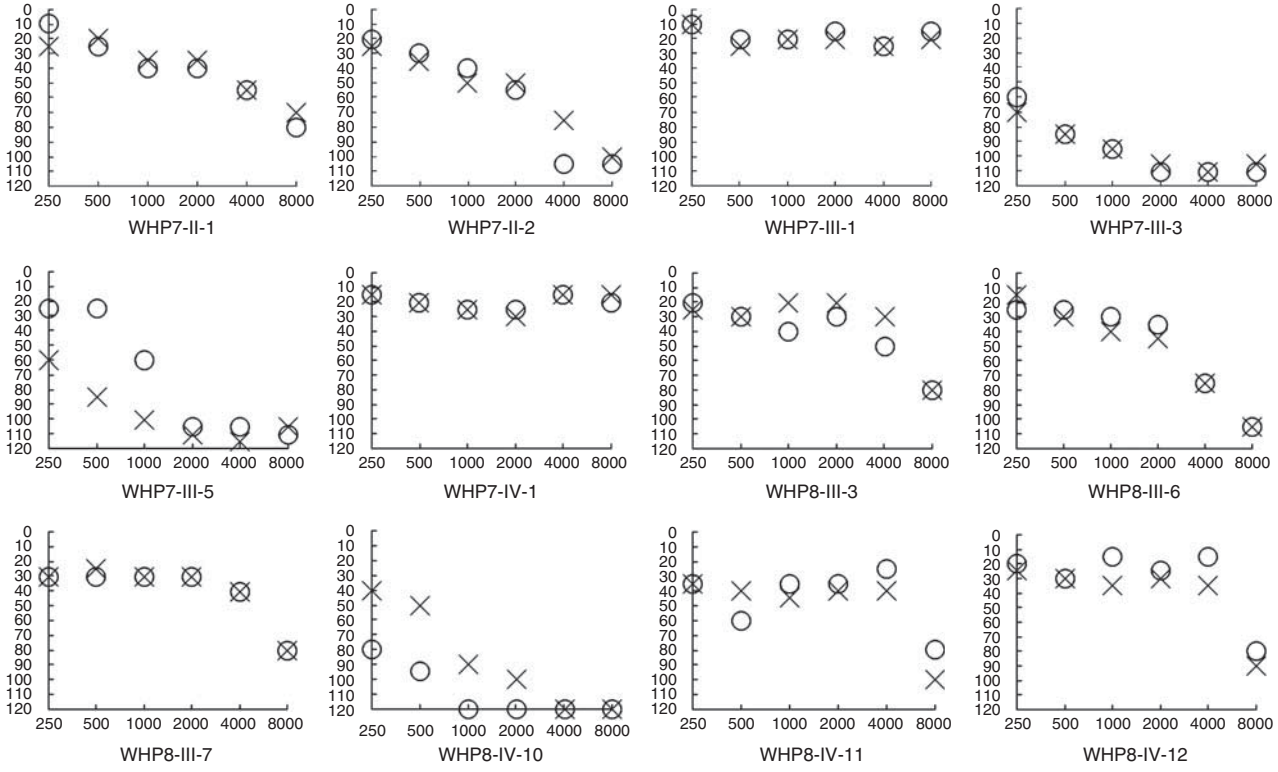

Figure 3 Air conduction audiogram of some members in two Chinese families. Symbols: X-left, O-right ear.

Table 1 Summary of clinical data for some members in two Chinese pedigrees

				Hearing loss					Hypertension	
Subject	*Gender*	*Age at test (year)*	*Age-at-onset (year)*	*PTA(dB) right ear*	*PTA(dB) left ear*	*Use of aminoglycosides*	*Level of hearing impairment*	*Audiometric configuration*	*Age-at-onset (year)*	*Blood pressure (mm Hg) (SBP/DBP)*
WHP7-II-1	M	71	65	48	43	No	Moderate	Slop	—	130/75
WHP7-II-2	F	68	33	67	62	No	Moderate	Slop	58	170/100
WHP7-III-1	M	46	—	19	22	No	Normal	Flat	43	160/100
WHP7-III-3	M	42	3	100	102	Gentamycin	Profound	Slop	42	135/110
WHP7-III-5	M	37	5	81	103	Gentamycin	Profound	Slop	37	170/80
WHP7-IV-1	F	25	—	21	21	No	Normal	Flat	—	110/70
WHP7-IV-2	F	22	—	23	20	No	Normal	Flat	—	115/65
WHP-IV-3	F	20	—	24	21	No	Normal	Flat	—	120/75
WHP8-III-3	M	80	65	36	46	No	Moderate	Flat	69	140/90
WHP8-III-5	M	77	55	54	59	No	Moderate	Slop	71	190/110
WHP8-III-6	F	72	58	42	41	No	Moderate	Flat	69	170/108
WHP8-IV-10	M	53	5	115	96	Gentamycin	Profound	Slop	53	140/90
WHP8-IV-11	F	51	50	47	53	No	Moderate	Flat	53	140/90
WHP8-IV-12	F	47	45	33	44	No	Mild	Flat	47	150/100

Abbreviations: PTA, pure-tone audiometry; SBP, systolic blood pressure; DBP, diastolic blood pressure.

and 44 variants in WHP8-III-3) belonging to the Eastern Asian haplogroup D5a on their maternal lineage.[21] In fact, there were 40 identical mtDNA variants between two probands. These variants were 17 known variants in the D-loop, 4 known variants in the *12S rRNA* gene, 1 known variant in the *16S rRNA* gene, 17 (3 novel (A6359G, A8479G, C11944T) and 14 known) silent variants and 8 known missense mutations in the protein encoding genes.[27] These missense mutations are the C5178A (L237M) and A5301G (I278V) in the *ND2* gene, A8701G (T59A) and A8860G (T112A) in the *ATP6* gene, the A10398G (T114A) in the *ND3* gene, the A12026G (I423V) in the *ND4* gene, the C14766T (T7I) and A15326G (T194A) in the *Cytb* gene. These variants in RNAs and polypeptides were further evaluated by phylogenetic analysis of these variants and sequences from other 17 vertebrates including mouse,[28] bovine[29] and *Xenopus laevis*.[30] Conservation indexes of all variants were $<60\%$, which was below the threshold level to be functionally significant in terms of mitochondrial physiology, proposed by Wallace.[31]

Mutational analysis of *TRMU* gene

Our previous study showed that the *TRMU* A10S mutation modulated the phenotypic manifestation of the A1555G mutation in the Israeli/European pedigrees.[26] To assess if the *TRMU* A10S variant also has a role in phenotypic expression of the A1555G mutation in these Chinese families, we carried out a mutational screening of exon 1 in *TRMU* gene in eight affected matrilineal relatives of these pedigrees. We failed to detect any variant in *TRMU* exon 1 in these matrilineal relatives of these Chinese pedigrees.

DISCUSSION

In the present study, we have performed the clinical, genetic and molecular characterization of two Han Chinese families carrying the known 12S rRNA A1555G mutation. In contrast to the previous data that the Chinese, Spanish and Arab-Israeli families harboring the A1555G mutation exhibited only hearing loss,[3–5,32] the variable severity and age at onset in both hypertension and hearing loss were observed in the matrilineal relatives of these two Chinese pedigrees carrying the A1555G mutation. In particular, the average age at onset of hypertension in the affected matrilineal relatives of these families was 60 and 46 years, respectively, while those of other Chinese families carrying the $tRNA^{Met}$ A4435G, $tRNA^{Ile}$ A4263G, and $tRNA^{Gln}$ and $tRNA^{Met}$ A4401G mutations were 50, 52, and 44 years, respectively.[33–36] On the other hand, the penetrances of hypertension (affected matrilineal relatives/total matrilineal relatives) in these Chinese pedigrees (80% in the family WHP7 and 58.3% in the pedigree WHP8) were higher than other Chinese families carrying the A4435G, A4263G or A4401G mutations.[33–36] The striking feature is that the average age-of-onset for hearing loss in these two families were 33 and 55 years old, respectively, when aminoglycoside-induced deafness was excluded. By contrast, the average age-at-onset for hearing loss without aminoglycoside exposure was 15 and 20 years among 69 Chinese families and 19 Spanish families carrying the A1555G mutation, respectively,[4,5] and some of matrilineal relatives in a large Arab-Israeli family exhibited congenital profound hearing loss.[32] Unlike the fact that there were low penetrances of hearing loss among a Scottish family carrying the A7445G mutation[6] and some Chinese families harboring the A1555G mutation,[5] the penetrances of hearing impairment in these two Chinese family carrying the A1555G mutation were relatively high (60 and 50%, when the effect of aminoglycosides was excluded). Furthermore, the hearing impairment in the both Chinese families appeared to be less severe than other families carrying the homoplasmic $tRNA^{Ser(UCN)}$A7445G[9] and T7505C mutations[16] and some Chinese families carrying the A1555G mutation.[5]

Our previous investigation showed that there were mild biochemical defects in the cells carrying the A1555G mutation.[18,37] These suggest that the A1555G mutation is necessary but by itself insufficient to produce a clinical phenotype. The genetic and environmental modifier factors are apparently responsible for the phenotypic variability of matrilineal relatives within and among these families carrying the A1555G mutation. Of these, three matrilineal relatives of these families suffered from profound hearing loss after administration of aminoglycosides, as in the case of other families carrying the A1555G mutation.[3–5] Here, the lack of functionally significant variant in their mtDNA indicates that mitochondrial haplogroups may not play an important role in the phenotypic expression of the A1555G mutation. The absence of the TRMU A10S mutation suggests the contribution of

Table 2 mtDNA variants in two Han Chinese probands (WHP7 II-2 and WHP8 III-3) with hearing loss and hypertension

Gene	*Position*	*Replacement*	*Conservation (H/B/M/X)*[a]	*CRS*[b]	*WHP7*	*WHP8*
D-loop	73	A to G		A	G	G
	150	C to T		C	T	T
	263	A to G		A	G	G
	310	T to CTC/CTCC		T	CTCC	CTC
	489	T to C		T	C	C
	514	C to Del		C	Del C	Del C
	515	A to Del		A	Del A	Del A
	16092	T to C		T		C
	16164	A to G		A	G	
	16172	T to C		T	C	C
	16182	A to C		A	C	C
	16183	A to C		A	C	C
	16189	T to C		T	C	C
	16223	C to T		C	T	T
	16259	C to T		C	T	
	16266	C to T		C		T
	16362	T to C		T	C	C
12S rRNA	750	A to G	A/A/A/–	A	G	G
	752	C to T	C/C/A/–	C	T	T
	1107	T to C	T/C/T/T	T	C	C
	1555	A to G	A/A/A/A	A	G	G
16S rRNA	2706	A to G	A/G/A/A	A	G	G
ND2	4769	A to G		A	G	G
	4883	C to T		C	T	T
	4973	T to C		T	C	
	5178	C to A (Leu to Met)	L/T/T/T	C	A	A
	5301	A to G (Ile to Val)	I/I/M/L	A	G	G
CO1	6359	A to G		A		G
	7028	C to T		C	T	T
ATP8	8479	A to G		A		G
ATP6	8701	A to G (Thr to Ala)	T/S/L/Q	A	G	G
	8860	A to G (Thr to Ala)	T/A/A/T	A	G	G
	9180	A to G		A	G	G
CO3	9540	T to C		T	C	C
ND3	10397	A to G		A	G	G
	10398	A to G (Thr to Ala)	T/T/T/A	A	G	G
	10400	C to T		C	T	T
	10873	T to C		T	C	C
ND4	11719	G to A		G	A	A
	11944	T to C		T	C	C
	12026	A to G (Ile to Val)	I/I/M/I	A	G	G
ND5	12705	C to T		C	T	T
Cytb	14766	C to T (Thr to Ile)	T/S/T/S	C	T	T
	14783	T to C		T	C	C
	15043	G to A		G	A	A
	15301	G to A		G	A	A
	15326	A to G (Thr to Ala)	T/M/I/I	A	G	G

Abbreviations: B, bovine; CRS, Cambridge reference sequence; H, human; M, mouse; mtDNA, mitochondrial DNA; X, *Xenopus laevis*.
[a]Conservation of amino acid for polypepides or nucleotide for RNAs in H, B, M and X.
[b]CRS.[25]

other nuclear modifier genes to the phenotypic variability and tissue-specific effect of both hypertension and hearing loss in these Chinese families. Furthermore, environmental and epigenetic factors, and personal lifestyles may also contribute to the development of hypertension in these subjects carrying the A1555G mutation.[38,39] Indeed, ~50% decrease of mitochondrial translation capacity in lymphoblastoid cells carrying the A1555G mutation was the proposed threshold level to support a normal respiratory phenotype.[18,37] Abnormal mitochondrial respiration causes oxidative stress, uncoupling of the oxidative pathways for ATP synthesis and subsequent failure of cellular energetic processes.[40] An inefficient metabolism caused by mitochondrial dysfunctions in skeletal and vascular smooth muscles may lead to the elevation of systolic blood pressure and therefore may be involved in the development of hypertension.[41–43]

In summary, our investigation provides the first direct evidence that the known 12S rRNA A1555G mutation leads to both of hearing loss and hypertension. The A1555G mutation should be added to the list of inherited factors for future molecular diagnosis for hypertension. Thus, our findings may provide the new insights into the understanding of pathophysiology and valuable information for management and treatment of maternally inherited hearing loss and hypertension.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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