ORIGINAL ARTICLE

Maternally transmitted late-onset non-syndromic deafness is associated with the novel heteroplasmic T12201C mutation in the mitochondrial tRNA^{His} gene

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ABSTRACT

The authors report here the clinical, genetic, molecular and biochemical characterisation of a large five-generation Han Chinese pedigree with maternally transmitted non-syndromic hearing loss. 17 of 35 matrilineal relatives exhibited variable severity and age at onset of sensorineural hearing loss. The average age at onset of hearing loss in matrilineal relatives of this family is 29 years, while matrilineal relatives among families carrying other mitochondrial DNA mutations developed hearing loss with congenital conditions or early age at onset. Molecular analysis of their mitochondrial genome identified the novel heteroplasmic T12201C mutation in the transfer RNA (tRNA)^{His} gene. The levels of T12201C mutation in matrilineal relatives of this family correlated with the severity and age at onset of non-syndromic hearing loss. By contrast, other heteroplasmic mitochondrial DNA mutations often cause syndromic hearing loss. The T12201C mutation destabilises a highly conservative base-pairing (5A-68U) on the acceptor stem of tRNA^{His}. tRNA northern analysis revealed that the T12201C mutation caused an ~75% reduction in the steady-state level of tRNA^{His}. An in vivo protein labeling analysis showed an ~47% reduction in the rate of mitochondrial translation in cells carrying the T12201C mutation. Impaired mitochondrial translation is apparently a primary contributor to the marked reduction in the rate of overall respiratory capacity, malate/glutamate-promoted respiration, succinate/glycerol-3-phosphate-promoted respiration or *N*, *N*,*Ń*,*Ń*-tetramethyl-*p*-phenylenediamine/ascorbate-promoted respiration. These data provide the first direct evidence that mitochondrial dysfunctions caused by the heteroplasmic tRNA^{His} mutation lead to late-onset non-syndromic deafness. Thus, the authors' findings provide new insights into the understanding of pathophysiology and valuable information on the management and treatment of maternally inherited hearing loss.

INTRODUCTION

Deafness is one of the most common human health problems, affecting one in 700–1000 newborns.[1] It can be caused by gene alterations and environmental factors such as aminoglycoside antibiotics.[2] Mutations in mitochondrial DNA (mtDNA) have been associated with syndromic and non-syndromic deafness.[3 4] Non-syndromic-deafness-associated mtDNA mutations such as 12S ribosomal RNA (rRNA) A1555G or transfer RNA (tRNA)$^{Ser(UCN)}$ A7445G often occur in homoplasmy or near homoplasmy,[5–8] while syndromic-deafness-associated mtDNA mutations such as the tRNA$^{Leu(UUR)}$ A3243G mutation and mtDNA large deletions are present in heteroplasmy.[9 10] Furthermore, the A7445G mutation has also been associated with syndromic-deafness-presenting palmoplantar keratoderma.[3] The other non-syndromic-deafness-associated mtDNA mutations are the 12S rRNA C1494T mutation and the 7472insC, T7505C, T7510C and T7511C mutations in the tRNA$^{Ser(UCN)}$ gene.[11–15] The primary defect in tRNA$^{Ser(UCN)}$ mutations appeared to be a failure in tRNA metabolism, thereby altering mitochondrial translation and respiration.[12 16 17] The 12S rRNA mutations impaired mitochondrial translation, leading to deficient respiration.[11 18 19] Mild mitochondrial dysfunctions were observed in cells carrying these mtDNA mutations.[11 16–18] 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.[7 18 19] In respect to syndromic-deafness-associated heteroplasmic mtDNA mutations, there is a threshold level of mutation(s) that is important for phenotypic manifestation and biochemical defects.[20] Cells carrying the threshold level of mtDNA mutations, such as the tRNA$^{Leu(UUR)}$ A3243G mutation or mtDNA large deletions, exhibited profound mitochondrial dysfunctions.[21–23] However, the pathophysiology of deafness-associated mtDNA mutations is far less understood.

As part of a genetic screening program for deafness in the Chinese population,[7 11 14 24] we performed the clinical, genetic, molecular and biochemical characterisation of a large Chinese pedigree with maternally transmitted non-syndromic hearing loss. Seventeen of 34 matrilineal relatives exhibited variable severity and age at onset of hearing loss. Strikingly, the average age at onset of hearing loss was 29 years. Mutational analysis of the entire mtDNA identified the novel heteroplasmic T12201C mutation in the tRNA^{His} gene. The T12201C mutation disrupts a very conservative base-pairing (5A-68U) on the acceptor stem of

tRNA.His [25] Functional significance of the T12201C mutation was evaluated by examining the steady-state levels of mitochondrial tRNAs, including tRNA^{His}, using lymphoblastoid cell lines derived from three affected matrilineal relatives carrying the T12201C mutation and from three control subjects lacking the mtDNA mutation. These cell lines were further assessed for the effects of the T12201C mutation on mitochondrial translation and endogenous and substrate-dependent respiration.

METHODS

Subjects

One Han Chinese family, as shown in figure 1, was ascertained through the otology clinic of the Eye and ENT Hospital of Fudan University. Comprehensive history taking, physical examination and audiological examination were performed to identify any syndromic findings, history of exposure to aminoglycosides and genetic factors related to hearing impairment in all available members of this Chinese pedigree. 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. The 342 control DNAs were obtained from a panel of unaffected Han Chinese subjects from the same region who were clinically tested. Informed consent was obtained from the participants prior to their participation in the study, in accordance with the Cincinnati Children's Hospital Medical Center Institutional Review Board and the ethics committee of Fudan University.

Mutational analysis of mitochondrial genome

Genomic DNA was isolated from whole blood samples of the participants using PAXgene Blood DNA Isolation Kits (Qiagen, Valencia, California, USA). The entire mtDNA of proband (III-11) was PCR amplified in 24 overlapping fragments by using sets of the light-strand (L) and heavy-strand (H) oligonucleotide primers, as described elsewhere.[26] Each fragment was purified and subsequently analysed by direct sequencing in an ABI 3700 automated DNA sequencer using the Big Dye Terminator Cycle sequencing reaction kit (Applied Biosystems, Carlsbad, California, USA). The resultant sequence data were compared with the updated consensus Cambridge sequence (GenBank accession number: NC_012920).[27]

Quantification of the tRNA^{His} T12201C mutation

Quantification of the T12201C mutation was performed using pyrosequencing technology (PSQ96MA; Qiagen, Valencia, California, USA), according to the manufacturer's instructions. For this purpose, 865 bp mtDNA segments at positions 11 929–12 793 derived from proband III-11 carrying the T12201C mutation and a control lacking this mutation were PCR amplified and cloned into a pMD20-T Vector (TaKaRa Biotechnology, Dalian, Liaoning, China). After sequence determination, plasmids carrying the T12201C mutation or wild-type version of the tRNA^{His} gene were isolated for normalisation purpose. The wild-type and mutated DNA amplified from the above-mentioned plasmids were mixed at levels ranging from 0% to 100% of the T12201C mutation. To detect and quantify the levels of the T12201C mutation, we used the following primers:

forward: 5′-CGACATCATTACCGGGTTTTCC-3′ (nt 12112–12133);

reverse: 5′-GGGCCTAAGACCAATGGATAGCT-3′ (nt 12282–12304);

sequencing 5′-AGGCTTACGACCCCT-3′ (nt 12184–12198).

The levels of the T12201C mutation in each subject were quantified using the PSQ SQA software (Biotage AB). Each sample was analysed in triplicate by pyrosequencing assays.

Cell cultures

Lymphoblastoid cell lines were immortalised by transformation with the Epstein–Barr virus, as described elsewhere.[28] Cell lines derived from three hearing-impaired subjects (III-6, III-20 and IV-11) carrying the T12201C mutation and from three Chinese married-in controls (III-5, III-19 and IV-12) lacking the mutation were grown in RPMI 1640 medium (Invitrogen Carlsbad, California, USA) supplemented with 10% fetal bovine serum.

Mitochondrial tRNA analysis

The preparation and electrophoresis of mitochondrial RNA and oligodeoxynucleosides used for digoxigenin (DIG)-labelled probes of tRNA,^{His} tRNA$^{Leu(CUN)}$, tRNA,^{Thr} tRNA,^{Lys} tRNA^{Glu} and tRNA^{Gln} were performed as described elsewhere.[16 17 22 29] DIG-labelled oligodeoxynucleotides were generated by using DIG Oligonucleotide Tailing Kit (Roche Basel, Switzerland). Hybridisation was performed as detailed elsewhere.[15 16] Quantification of density in each band was performed as detailed previously.[15 16 22]

Analysis of mitochondrial protein synthesis

Pulse labelling of the cell lines for 30 min with [^{35}S]methionine–[^{35}S]cysteine in methionine-free Dulbecco's modified Eagle's medium (DMEM) in the presence of emetine, electrophoretic analysis of translation products and quantification of radioactivity in whole electrophoretic patterns or in individual well-resolved bands were carried out as detailed previously.[30]

O_2 consumption measurements

The rates of O_2 consumption in intact cells were determined by using a YSI 5300 oxygraph (Yellow Springs Instruments Yellow Springs, Ohio, USA) on samples of 5×10^6 cells in 1.5 ml of

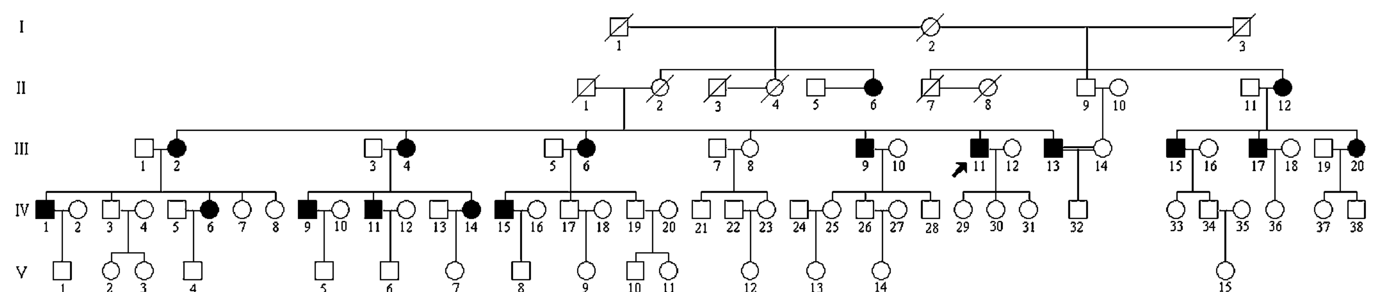

Figure 1 A Han Chinese pedigree with hearing loss. Hearing-impaired individuals are indicated by filled symbols. The arrow denotes proband.

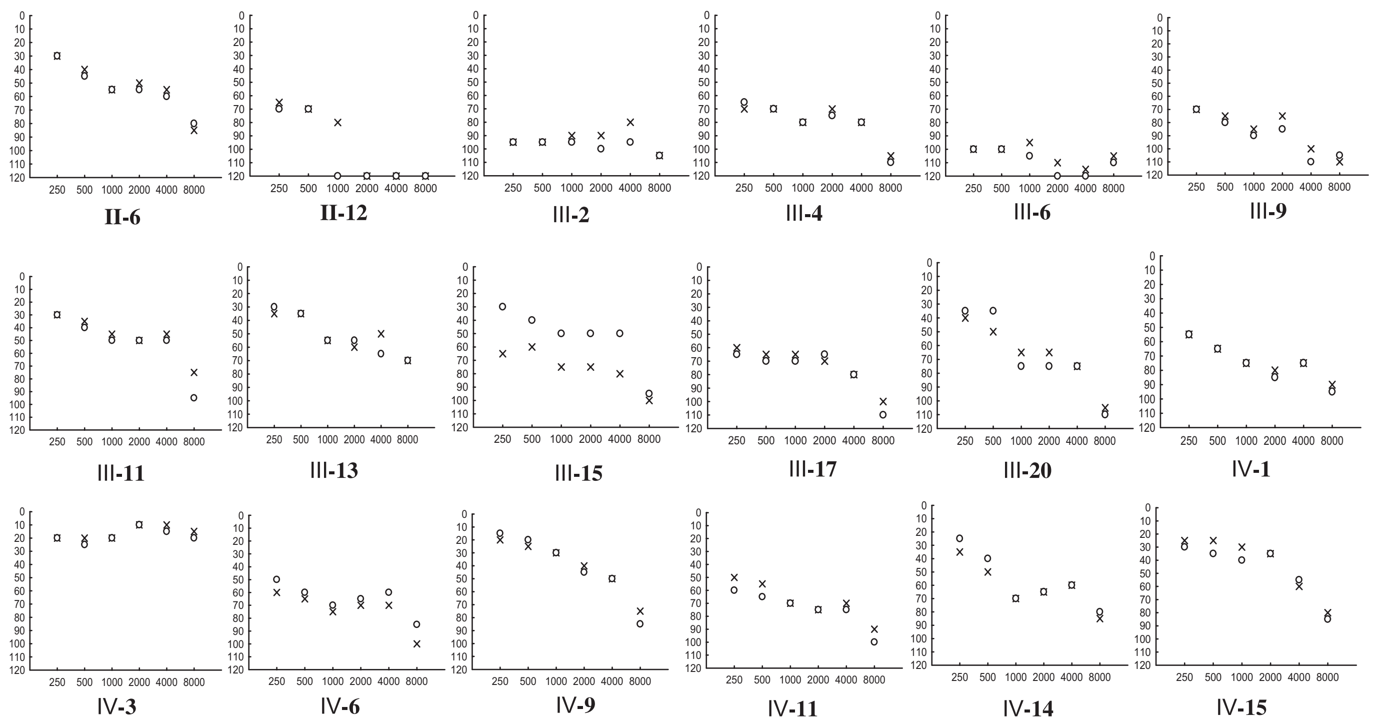

Figure 2 Air conduction audiogram of 18 members of a Chinese family. X, left ear; O, right ear.

a special DMEM lacking glucose, supplemented with 10% dialysed fetal bovine serum.[31] Polarographic analysis of digitonin-permeabilised cells to test the activity of the various respiratory complexes was carried out as detailed previously using different respiratory substrates and inhibitors.[32]

Statistical analysis

Statistical analysis was carried out using Student unpaired two-tailed t test contained in the Microsoft Excel program. Unless indicated otherwise, $p<0.05$ was considered statistically significant.

RESULTS

Clinical and genetic evaluation of a Han Chinese pedigree with hearing loss

The proband (III-11) from Jiangsu province exhibited bilateral hearing impairment at the age of 45 years and was further evaluated at the age of 50 years. He did not have a history

Table 1 Summary of the clinical and molecular data of some maternal members of a Chinese family

Subject	Gender	Age at test (years)	Age at onset (years)	PTA (dB) on right ear	PTA (dB) on left ear	Audiometric configuration	Level of hearing impairment	Level of T12201 mutation (%)	*GJB2* 235delC mutation
II-6	F	81	>65	59	57	Slope	Moderate	28.1	+/+
II-12	F	69	28	100	100	Slope	Profound	90.4	+/+
III-2	F	72	38	98	92	Flat	Profound	88	+/−
III-4	F	63	30	83	81	Slope	Severe	88.3	+/+
III-6	F	57	17	>100	>100	Flat	Profound	83.6	+/+
III-9	M	64	25	94	89	Slope	Profound	94.6	+/+
III-11	M	52	45	57	50	Slope	Moderate	56.7	+/+
III-13	M	44	30	56	54	Slope	Moderate	65.5	+/−
III-15	M	46	18	78	83	Slope	Severe	95.5	+/+
III-17	M	52	10	79	76	Slope	Severe	99.7	+/+
III-20	F	35	21	74	72	Slope	Severe	82.4	+/+
IV-1	M	44	24	79	77	Slope	Severe	94.2	+/−
IV-3	M	37	—	18	15	—	Normal	31.9	+/−
IV-6	F	42	37	68	76	Slope	Severe	96.7	+/−
IV-9	M	41	—	46	44	Slope	Moderate	64.4	+/+
IV-11	M	39	26	77	72	Slope	Severe	99.8	+/+
IV-14	F	35	20	73	76	Slope	Severe	86.7	+/+
IV-15	M	35	—	50	46	Slope	Moderate	60.1	+/−
IV-21	M	27	—	14	21	—	Normal	70.4	+/−
IV-23	F	31	—	11	11	—	Normal	61.8	+/+
IV-38	M	13	—	15	16	—	Normal	7.2	+/+
V-4	M	22	—	15	17	—	Normal	23.4	+/+
V-7	F	15	—	24	22	—	Normal	2.2	+/+
V-12	F	8	—	14	13	—	Normal	1.9	+/+

M, male; F, female; PTA, pure tone audiometry.

of exposure to aminoglycosides. As illustrated in figure 2 with a slope-shaped pattern, he had moderate hearing loss (57 dB on the right ear and 50 dB on the left ear). CT scans of the temporal bones yielded normal results. In addition, he had no other significant medical history.

As shown in figure 1, this familial history is consistent with a maternal inheritance. Seventeen of 35 matrilineal relatives exhibit sensorineural hearing impairment as the sole clinical symptom. All affected individuals showed their hearing loss at high frequencies, and their hearing impairment was symmetric. Matrilineal relatives of this family displayed late-onset/progressive—but not congenital—hearing impairment. As shown in table 1, audiometric studies showed variable severity of hearing impairment in the maternal kindred, ranging from profound hearing loss (II-12, III-2, III-6 and III-9), to severe hearing impairment (III-4, III-15, III-17, III-20, IV-1, IV-6, IV-11 and IV-14), to moderate hearing impairment (II-6, III-11, III-13, IV-9 and IV-15), to normal hearing. Furthermore, these subjects had variable patterns of audiometric configurations: 15 individuals with sloping pattern and 2 subjects with flat pattern. In addition, there was a wide range in the age at onset of hearing impairment in this family, varying from 10 years to 65 years. Notably, the average age at onset of hearing impairment in this family was 29 years. Moreover, other members of this family showed no other clinical abnormalities, including muscular diseases, visual loss and neurological disorders.

Mutational analysis of mitochondrial genome

The analysis of mtDNA from four matrilineal relatives (proband (III-11), affected male sibling (III-9), unaffected female sibling (IV-23) and affected male sibling (IV-15)) and two unrelated Chinese controls failed to detect the presence of known deafness-associated mtDNA mutations. As shown in table 2, the comparison of the resultant sequence with the Cambridge consensus sequence[27] identified 43 nucleotide changes belonging to the East Asian haplogroup Z3.[33] All of those nucleotide changes were verified in 12 additional matrilineal relatives of this family (six symptomatic and six asymptomatic individuals) by sequence analysis and appeared to be in homoplasmy, except for the novel T12201C mutation in the $tRNA^{His}$ gene (figure 3A). These variants were 14 known variants in the D-loop, 3 known variants in the 12S rRNA gene, 1 known variant in the 16S rRNA gene, and 18 (1 novel (C11782T) and 17 known) silent variants and 6 known missense mutations in the protein-encoding genes.[34] These missense mutations are G8584A (A20T), A8701G (T59A) and A8860G (T112A) in the *ATP6* gene; A10398G (T114A) in the *ND3* gene; and C14766T (T7I) and A15326G (T194A) in the *Cytb* gene. These variants in RNAs and polypeptides were further evaluated by phylogenetic analysis and sequences from 17 other vertebrates, including mouse,[35] bovine[36] and *Xenopus laevis*.[37] The conservation index (CI) of $tRNA^{His}$ T12201C mutation was 89.5%, which was above the threshold level of functional significance in terms of mitochondrial physiology.[38] However, the CIs of other variants were <60% CI, indicating that these variants may not be functionally significant.

As shown in figure 3B, the thymine at position 12 201 is localised at the highly conserved base (corresponding to conventional position 68) on the acceptor stem of $tRNA^{His}$.[25 39] The U-to-C transition at this position is expected to disrupt a very conservative base-pairing (5A-68U) on the acceptor stem of this tRNA, thus causing a failure in tRNA metabolism. Further analysis showed that this mutation was present in all matrilineal relatives, but not in other members of this family and in the 342 Han Chinese controls.

Table 2 MtDNA variants in one Han Chinese subject (III-11) with hearing loss

Gene	Position	Replacement	Conservation (H/B/M/X)*	Previously reported†
D-loop	73	A to G		Yes
	152	T to C		Yes
	249	del A		Yes
	263	A to G		Yes
	309	ins C		Yes
	315	ins C		Yes
	460	T to C		Yes
	489	T to C		Yes
	16 185	C to T		Yes
	16 189	del T		Yes
	16 223	C to T		Yes
	16 260	C to T		Yes
	16 298	T to C		Yes
	16 519	T to C		Yes
12S rRNA	709	G to A	G/A/A/-	Yes
	750	A to G	A/G/G/-	Yes
	1438	A to G	A/A/A/G	Yes
16S rRNA	2706	A to G	A/G/A/A	Yes
ND2	4715	A to G		Yes
	4769	A to G		Yes
COI	6752	A to G		Yes
	7028	C to T		Yes
	7196	C to A		Yes
A6	8584	G to A (Ala to Thr)	A/V/V/I	Yes
	8701	A to G (Thr to Ala)	T/S/L/Q	Yes
	8860	A to G (Thr to Ala)	T/A/A/T	Yes
	9090	T to C		Yes
COIII	9540	T to C		Yes
ND3	10 208	T to C		Yes
	10 398	A to G (Thr to Ala)	T/T/T/A	Yes
	10 400	C to T		Yes
ND4	10 873	T to C		Yes
	11 719	G to A		Yes
	11 782	C to T		No
$tRNA^{His}$	12 201	T to C	T/T/T/T	No
ND5	12 705	C to T		Yes
Cytb	14 766	C to T (Thr to Ile)	T/S/I/S	Yes
	14 783	T to C		Yes
	15 043	G to A		Yes
	15 301	G to A		Yes
	15 326	A to G (Thr to Ala)	T/M/I/I	Yes
	15 487	A to T		Yes
	15 784	T to C		Yes

*Conservation of amino acid for polypeptides or of nucleotide for rRNAs in human (H), mouse (M), bovine (B), and *Xenopus laevis* (X).
†See http//www.mitomap.org and http://www.genpat.uu.se/mtDB/.
A6, subunit 6 of H^{+}-ATPase; COI, COIII, subunits I and III of cytochrome *c* oxidase; ND2, ND3, ND4, ND5, subunits 2, 3, 4 and 5 of the respiratory chain reduced nicotinamide adenine dinucleotide dehydrogenase; rRNA, ribosomal RNA; tRNA, transfer RNA.

To further examine the presence and degree of the T12201C mutation in the members of this Chinese family, we performed a more sensitive experiment involving pyrosequencing technology. Pyrosequencing technology is an accurate method for detecting low-level mtDNA mutations in patient samples.[40] As can be seen in figure 3C and table 1, the quantification of mtDNA from members in this family revealed that wild-type and mutant DNA were present in matrilineal relatives, ranging from 2% to nearly 100% of mutant DNA in cells. As shown in table 1, the levels of heteroplasmic T12201C mutation appeared to be correlated with the severity of hearing loss.

In addition, the mutational analysis of deafness-associated *GJB2* and *TRMU* genes failed detect homozygous mutations,

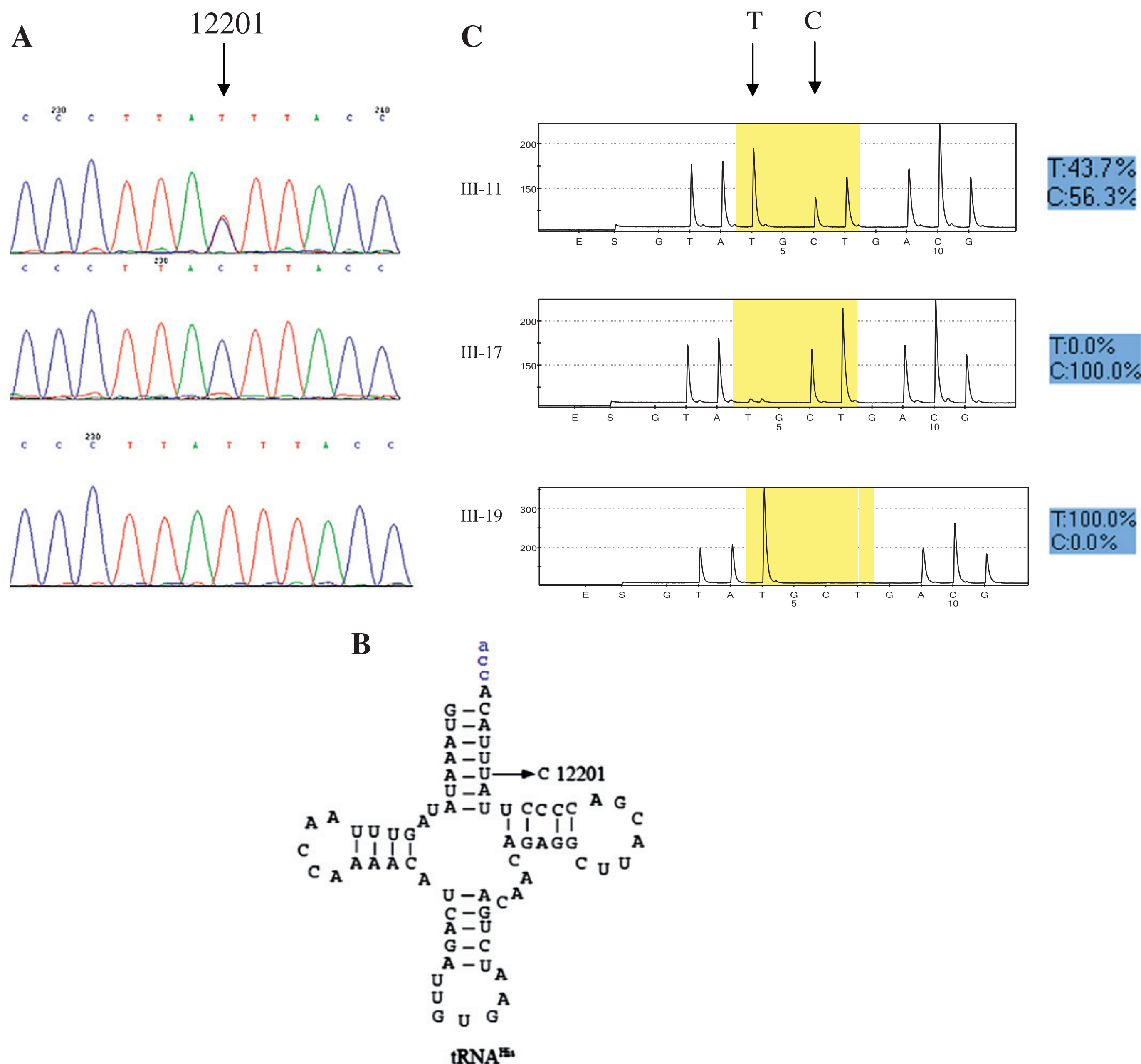

Figure 3 Identification and qualification of the T12201C mutation in the tRNA^{His} gene. (A) Partial sequence chromatograms of the tRNA^{His} gene from two affected individuals (III-11 and III-17) and a married-in control (III-19), respectively. Arrows indicate the locations of the base changes at position T12201C. (B) The locations of the mitochondrial tRNA^{His} T12201C mutation. The cloverleaf structure of tRNA^{His} was derived from Florentz *et al*.[25] (C) Quantification of T12201C mutation by pyrosequencing. The boxes show the AQ values obtained for the allele. The levels of heteroplasmic T12201C mutation in each sample were the average value for the triplicate experiment.

and it suggested that these genes may not play an important role in the phenotypic expression of T12201C mutation (supplemental materials).

Mitochondrial tRNA analysis

To examine if the T12201C mutation alters the tRNA metabolism, we determined the steady-state level of tRNA^{His} by northern analysis using total mitochondrial RNA from cell lines derived from three affected individuals (III-6, III-20 and IV-11) and three Chinese married-in controls (III-5, III-19 and IV-12) in this Chinese family. As shown in figure 4A, the amounts of tRNA^{His} in mutant cells were markedly decreased as compared with those in the controls. For comparison, the average levels of tRNA^{His} in various control or mutant cell lines were normalised to the average levels of tRNA^{Lys}, tRNA$^{Leu(CUN)}$, tRNA^{Thr}, tRNA^{Glu} and tRNA^{Gln}, respectively, in the same cell line. As shown in figure 4B, the levels of tRNA^{His} in the mutant cells were significantly reduced relative to those in the controls. In particular, the average levels of tRNA^{His} in the mutant cell lines derived from the three affected subjects were ~26.1%, 23.4%, 23.4%, 24.3% and 28.6% of the average values of the three control subjects after normalisation to tRNA^{Lys}, tRNA$^{Leu(CUN)}$, tRNA^{Thr}, tRNA^{Glu} and tRNA^{Gln}, respectively.

Mitochondrial protein synthesis defect

Cells from the cell lines derived from the three affected matrilineal relatives carrying the T12201C mutation and from the three controls were labelled for 30 min with [^{35}S]methionine–

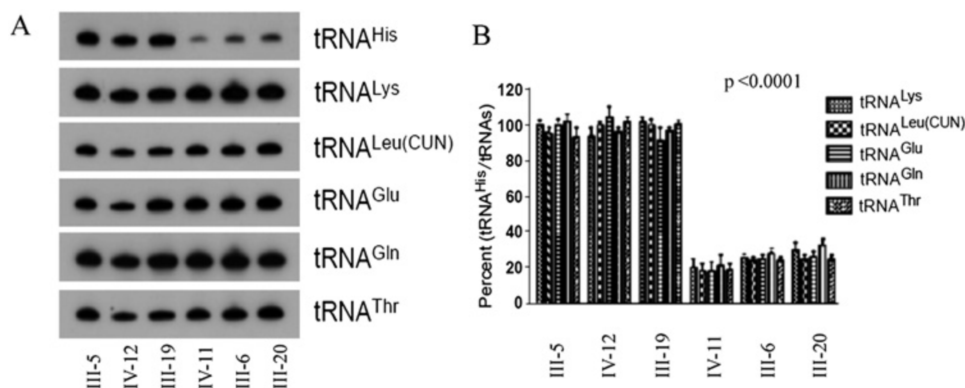

Figure 4 Northern blot analysis of mitochondrial tRNA. (A) Equal amounts (2 μg) of total mitochondrial RNA from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridised with DIG-labelled oligonucleotide probes specific for $tRNA^{His}$ and five other tRNAs, respectively. (B) Quantification of mitochondrial tRNA levels. Average relative $tRNA^{His}$ content per cell, normalised to the average content per cell of five other tRNAs in cells derived from the three affected subjects (III-6, III-20 and IV-11) carrying the T12201C mutation and from the three Chinese married-in controls (III-5, III-19 and IV-12) lacking the mutation. The values for the latter are expressed as percentages of the average values for the control cell lines. The calculations were based on three independent determinations of each tRNA content in each cell line and three determinations of the content of reference tRNA marker in each cell line.

[^{35}S]cysteine in methionine-free regular DMEM in the presence of 100 μg/ml emetine, which was used to inhibit cytosolic protein synthesis.[30] Figure 5A shows the typical electrophoretic patterns of the mitochondrial translation products of the mutant and control cell lines. Patterns of the mtDNA-encoded polypeptides of cells carrying the T12201C mutation were qualitatively identical to those of the control cells in terms of the electrophoretic mobility of the various polypeptides. However, cell lines carrying the T12201C mutation trended to a decrease in the total rate of labelling of the mitochondrial translation products relative to those of the control cell line. Figure 5B shows a quantification of the results of a large number of labelling experiments and electrophoretic runs, which were carried out using an Image-Quant program (GE Healthcare, Piscataway) first analysis of appropriate exposures of fluorograms and normalisation of data obtained for the $143B.TK^{-}$ sample. In fact, the overall rates of labelling of the mitochondrial translation products in the cell lines derived from the three affected individuals (III-6, III-20 and IV-11) carrying the T12201C mutation were 39%, 49% and 72%, with an average of 53% ($p=0.0013$) relative to the mean value measured in the control cell lines.

Respiration deficiency

The endogenous respiration rates of the cell lines derived from the three affected individuals (III-6, III-20 and IV-11) and the three controls were measured by determining the O_2 consumption rate in intact cells, as described previously.[31] As shown in figure 6A, the rate of total O_2 consumption in the lymphoblastoid cell lines derived from the three affected individuals were 60%, 63% and 72%, with an average of ∼65% ($p=0.0012$) relative to the mean value measured in the control cell lines.

O_2 consumption measurements were carried out on digitonin-permeabilised cells, using different substrates and inhibitors, in order to investigate which enzyme complexes of the respiratory chain were affected in the mutant cell lines.[32] As shown in figure 6B, in the cell lines derived from the three affected individuals, the rate of malate/glutamate-driven respiration, which depends on the activities of NADH:ubiquinone oxidoreductase (complex I), ubiquinol−cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV) but usually reflects the rate-limiting activity of complex I,[32] was very significantly decreased, relative to the average rate in the control cell lines, by 52%−64% (58% on average; $p=0.003$). Similarly, the rate of succinate/glycerol-3-phosphate-driven respiration, which depends on the activities of complexes III and IV but usually reflects the rate-limiting activity of complex III, was significantly affected in the mutant cell lines, relative to the average rate in the control cell lines, by 62%−69% (65% on average; $p=0.0002$). Furthermore, the rate of *N*,*N*,Ń,Ń-tetramethyl-*p*-phenylenediamine/ascorbate-driven respiration, which reflects the activity of complex IV, exhibited a 55%−67% reduction in complex IV activity (62% on average) in the mutant cell lines relative to the average rate in the control cell lines.

DISCUSSION

In the present study, we identified a novel heteroplasmic $tRNA^{His}$ T12201C mutation that causes hearing loss. This mutation is only present in matrilineal relatives of this family in heteroplasmy and not in the 342 Chinese controls. The T12201C mutation is localised at a highly conserved base-pairing (5A-68U) on the acceptor stem of $tRNA^{His}$, where the position is important for the stability and identity of tRNA.[26 41] The U-to-C transition at this position is expected to destabilise the highly conservative base-pairing (5A-68U) of this tRNA and to alter the secondary structure, thereby causing a failure in tRNA metabolism. Here, an ∼75% reduction in the steady-state level of $tRNA^{His}$ was observed in three mutant cell lines, relative to that in the three wild-type cell lines. This reduced level of $tRNA^{His}$ is below the proposed threshold to support a normal protein synthesis rate in lymphoblastoid cells.[16−18] The shortage of $tRNA^{His}$ causes an ∼47% reduction in the rate of mitochondrial translation and subsequent respiration defects, as in the cases of cell lines carrying the $tRNA^{Ser(UCN)}$ mutations.[12 14−16] Indeed, an ∼50% decrease in mitochondrial translation capacity in lymphoblastoid cells carrying the A1555G mutation was the proposed threshold level to support a normal respiratory phenotype.[18 19] As a consequence, this respiratory deficiency results in a decline in ATP production and an increased production of oxygen reactive species.[3 4] In particular, in cell types with high oxidative phosphorylation demands, such as cochlear cells (hair cells/or stria vascularis), the ∼50% decrease in mitochondrial translation rate has disastrous consequences.[18]

There was a wide range of phenotypic variability among the maternal lineage of this Chinese family carrying the T12201C mutation. In contrast to the low penetrance of hearing loss among Scottish families carrying the A7445G mutation[8] and in some Chinese families harbouring the A1555G mutation,[7] the penetrance of hearing impairment in this Chinese family was relatively high (49%). The striking feature is that matrilineal relatives developed hearing loss at the average age of 29 years, but it was not congenital. By contrast, the average age at onset

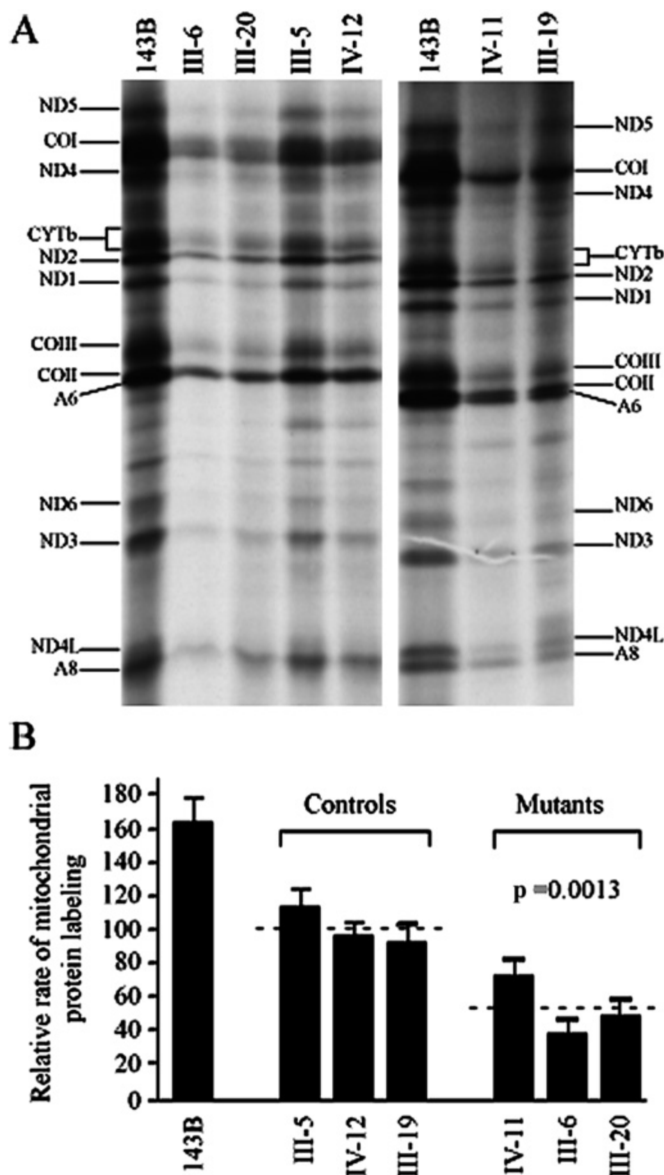

Figure 5 Mitochondrial translation assay. (A) Electrophoretic patterns of the mitochondrial translation products of lymphoblastoid cell lines and 143B.TK^{-} cells. COI, COII, and COIII indicate subunits I, II, and III of cytochrome *c* oxidase. ND1, ND2, ND3, ND4, ND4L, ND5 and ND6 indicate subunits 1, 2, 3, 4, 4L, 5 and 6 of the respiratory chain reduced nicotinamide adenine dinucleotide dehydrogenase. A6 and A8 indicate subunits 6 and 8 of H^{+}-ATPase. CYTb, apocytochrome *b*. (B) Quantification of the rates of mitochondrial translation labelling. The rates of mitochondrial protein labelling, as detailed elsewhere,[30] were expressed as percentages of the value for 143B.TK^{-} in each gel, with error bars representing two SEMs. A total of three independent labelling experiments and three electrophoretic analyses of each labelled preparation were performed on cell lines.

for hearing loss without aminoglycoside exposure was 15 and 20 years among 69 Chinese families and 19 Spanish families carrying the A1555G mutation, respectively,[6 7] and some matrilineal relatives in a large Arab–Israeli family exhibited congenital profound hearing loss.[42] Furthermore, hearing impairment in the maternal kindred appeared to be more severe than in other families carrying the homoplasmic tRNA$^{Ser(UCN)}$ mutations[8 14] and in some Chinese families carrying the A1555G mutation.[7] In addition, the sloping patterns of the audiometric

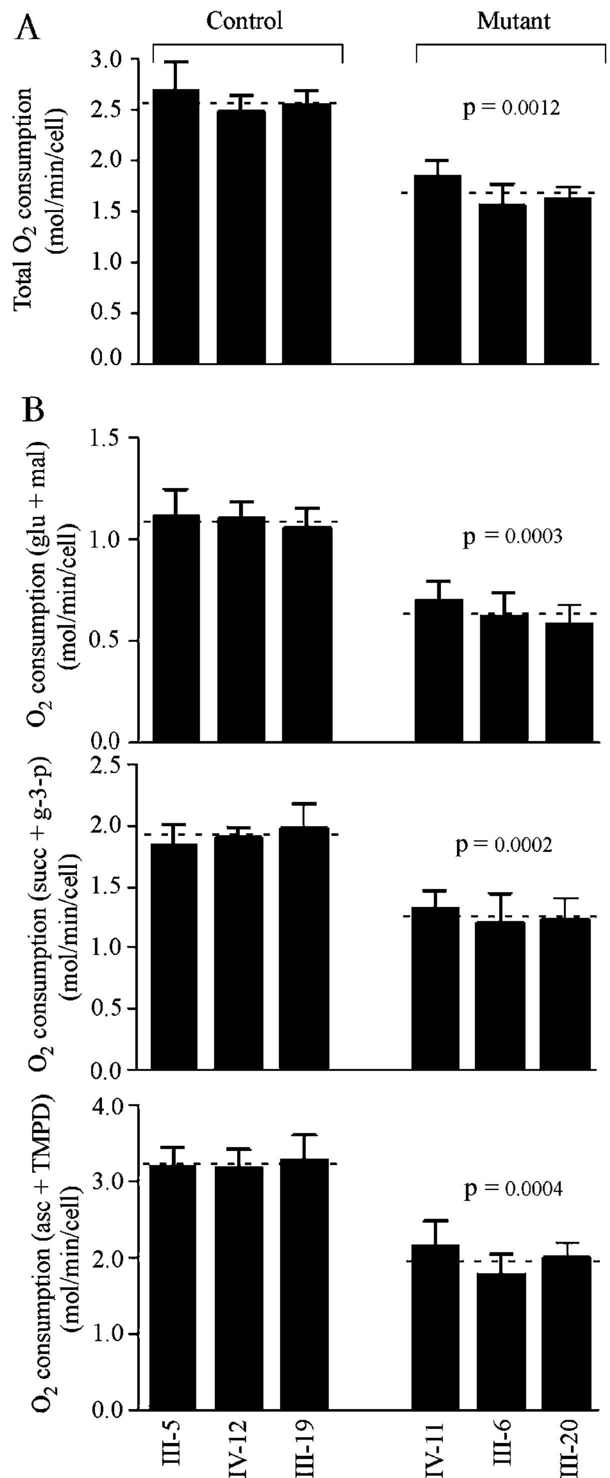

Figure 6 Respiration assays. (A) Average rates of endogenous O_2 consumption per cell measured in different cell lines, with error bars representing two SEs. A total of four determinations were performed on each of the lymphoblastoid cell lines. (B) Polarographic analysis of O_2 consumption in digitonin-permeabilised cells of the various cell lines using different substrates and inhibitors. The activities of the various components of the respiratory chain were investigated by measuring on 1×10^7 digitonin-permeabilised cells the respiration dependent on malate/glutamate, succinate/G3P and TMPD/ascorbate. A total of four determinations were performed on each of the lymphoblastoid cell lines. Graph details and symbols are explained in the legend to figure 4. mal/glu, malate/glutamate-dependent respiration; succ/G-3-P, succinate/G3P-dependent respiration; TMPD/asc, TMPD/ascorbate-dependent respiration.

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configuration are common in these hearing-impaired subjects carrying the T12201C mutation, as in the cases of subjects carrying the A1555G mutation.[24]

The late onset and variable severity of hearing loss in matrilineal relatives in this family suggest the involvement of other modifier factors in the phenotypic manifestation of T12201C mutation in this Chinese family. The levels of heteroplasmic T12201C mutation in cells correlated with the severity of hearing loss. In particular, the cells of matrilineal relatives with profound or severe hearing loss had >80% mutated mtDNA molecules. However, matrilineal relatives with moderate hearing loss carried 28.1–65.5% mutated mtDNA molecules. Furthermore, the level of the T12201C mutation among the six matrilineal relatives with normal hearing varied from ~2% to 70.4%. This suggests that ~80% of T12201C mutation in the cells is the threshold level for the development of severe hearing loss. It is likely that the late onset and severity of hearing loss in matrilineal relatives are due to the accumulation of the T12201C mutation in cochlear cells during the ageing process, as in the case of $tRNA^{Leu(UUR)}$ A3243G mutation[43] and mtDNA large deletions.[44] The accumulation of mutated mtDNA molecules may result from the replication advantage of mutated mtDNA molecules such as MELAS-associated $tRNA^{Leu(UUR)}$ A3243G mutation[45] and large deletions.[46] The A3243G mutation or mtDNA large deletions caused syndromic hearing loss,[9 10 45 46] while the T12201C mutation is associated with non-syndromic hearing loss. In addition, the lack of functionally significant variants in their mtDNA indicated that mitochondrial haplogroups may not play an important role in phenotypic expression. The 235delC mutation in the *GJB2* gene is the most important cause of hereditary deafness in the Chinese population.[47] The absence of a nuclear modifier *TRMU* A10S variant in matrilineal relatives and the presence of the heterozygous *GJB2* 235delC mutation[47] in seven matrilineal relatives (five with hearing loss and two with normal hearing) indicated that other nuclear modifier genes may contribute to phenotypic variability and tissue-specific effect. Alternatively, tissue-specific differences in tRNA metabolism may contribute to the development of the deafness phenotype.[48 49]

In summary, this is the first study to investigate the molecular pathogenesis underlying the novel heteroplasmic $tRNA^{His}$ T12201C mutation leading to late-onset hearing loss. The T12201C mutation should be added to the list of inherited factors for future molecular diagnosis of hearing loss. Thus, our findings may provide new insights into the understanding of pathophysiology and valuable information on the management and treatment of maternally inherited hearing loss.

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Competing interests None.

Patient consent Obtained.

Ethics approval IRB from Cincinnati Children's Hospital Medical Center.

Contributors XY, ZW, SS, GC, YH, QL, MS, WL, YB and JZ collected clinical data and clinical evaluation; XW, JQM, RL and QY performed biochemical assays; YZ performed the statistical analysis; HL and MXG designed the experiment; and MXG wrote the manuscript.

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