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Maternally inherited nonsyndromic hearing loss is associated with the T7511C mutation in the mitochondrial tRNA$^{Ser(UCN)}$ gene in a Japanese family

Ronghua Li[a], Kotaro Ishikawa[b], Jian-Hong Deng[c], Selena Heman-Ackah[a], Yuya Tamagawa[b], Li Yang[a], Yidong Bai[c], Keiichi Ichimura[b], Min-Xin Guan[a,d,e,*]

[a] *Division and Program in Human Genetics, and Center for Hearing and Deafness Research, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA*
[b] *Department of Otolaryngology, Jichi Medical School, Minamikawachi, Tochigi, Japan*
[c] *Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA*
[d] *School of Life Sciences, Wenzhou Medical College, Wenzhou, Zhejiang 325003, China*
[e] *Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA*

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Abstract

We report here the characterization of a Japanese family with maternally transmitted nonsyndromic hearing loss. Fourteen of 21 matrilineal relatives in this family exhibited early or late-onset/progressive but noncongenital hearing impairment with a wide range of severity, ranging from severe to normal hearing. The age-of-onset varies from 3 to 30 years. Sequence analysis of the complete mitochondrial genome in one matrilineal relative of this family revealed the presence of T7511C mutation and other variants. However, the levels of heteroplasmy of T7511C mutation did not correlate with the severity and age-of-onset of hearing loss in this family. Furthermore, none of other mtDNA variants are evolutionarily conserved and implicated to have significantly functional consequence. The absence of the ND1 T3308C and tRNA^{Ala} T5655C mutations in this Japanese family but the presence of these mtDNA mutations in an African family with a high penetrance seems to account for different penetrance between two pedigrees. Incomplete penetrance in this family indicates the involvement of modulatory factors in the phenotypic expression of hearing impairment associated with the T7511C mutation. Here, two known variants G79A and G109A in the *GJB2* gene were identified in the hearing-impaired and normal hearing matrilineal relatives of this Japanese family. However, the lack of correlation in the severity and age-of-onset in hearing impairment with homozygous or heterozygous G79A or G109A or combination of both variants in the *GJB2* gene in those subjects with hearing impairment and normal hearing indicates that those variants of *GJB2* gene may not be a modifier of the phenotypic effects of the T7511C mutation in those subjects. Thus, the phenotypic variability in this family is due to the involvement of other modifier factor(s).

Keywords: Hearing loss; Mitochondria; tRNA; Mutation; Haplotype; Penetrance; Inherited; Japanese; GJB2

Mutations in mitochondrial DNA (mtDNA) have been found to be associated with both syndromic and nonsyndromic forms of sensorineural hearing loss (SNHL) [1,2]. Of these, the homoplasmic A1555G mutation in the highly conserved decoding site of the mitochondrial 12S rRNA has been found to be associated with both aminoglycoside-induced and nonsyndromic SNHL in many families of various ethnic backgrounds [3–7]. Similarly, the homoplasmic C1494T mutation in the same gene has also been found to be associated with aminoglycoside-induced and nonsyndromic SNHL in a

* Corresponding author. Fax: +1 513 636 3486.
E-mail address: min-xin.guan@cchmc.org (M.-X. Guan).

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large Chinese family [8]. The tRNA$^{Ser(UCN)}$ appears to be another hot spot for mutations associated with nonsyndromic hearing loss, as four mutations: A7445G [9,10], 7472insC [11], T7510C [12], and T7511C mutations [13–15], have been identified in this gene. Unlike other pathogenic mtDNA mutations such as the MELAS A3243G mutation in the tRNA$^{Leu(UUR)}$ gene [16], the nonsyndromic hearing-loss-associated mutations in the tRNA$^{Ser(UCN)}$ gene often occur in homoplasmy or in high levels of heteroplasmy, indicating a high threshold for pathogenicity. Those mutations such as the A7445G mutation [9,10] are often incomplete penetrance since some individuals carrying the mutation have normal hearing. Thus, other factors including other mtDNA mutations/polymorphisms and/or nuclear backgrounds modulate the phenotypic variability and penetrance of hearing-loss-associated with those mtDNA mutations.

The T7511C mutation has been identified to be associated with nonsyndromic hearing loss in several families from different ethnic groups, including African [13], French [15], and Japanese [14]. The T7511C transition disrupts a very conservative base-pairing, converting an A–U to a G–U base pairing on the 5′ side of the acceptor stem of the tRNA$^{Ser(UCN)}$ [17]. The T7511C mutation often exists in homoplasmy in most matrilineal relatives of these pedigrees and in a high level of heteroplasmy in some matrilineal relatives of those families [13,15]. Despite sharing some common features, including bilateral, symmetric, and sensorineural hearing impairment, matrilineal relatives of intra-families or inter-families carrying the T7511C mutation exhibited the variable severity, age-of-onset, and progression in hearing impairment [13–15]. Strikingly, these pedigrees differ considerably in the penetrance of the T7511C mutation [13–15]. In particular, 36 of 43 matrilineal relatives in a large African pedigree exhibited hearing impairment [13,18], whereas only a small portion of matrilineal relatives in two French and one Japanese pedigrees developed hearing impairment [14,15].

Recently, a Japanese family with maternally inherited hearing loss was ascertained in the ENT Clinic at the Jichi Medical School. PCR-amplification and subsequent nucleotide analysis of fragments spanning the tRNA$^{Ser(UCN)}$ gene revealed the presence of the T7511C mutation in this family [14]. In the present study, clinical characterization of matrilineal relatives of this family was carried out in further detail. To elucidate the molecular basis of maternally inherited hearing loss and the role of mitochondrial haplotype in the phenotypic expression of the T7511C mutation, we performed PCR-amplification of fragments spanning entire mitochondrial genome and subsequent DNA sequence analysis in the matrilineal relatives of this family. To examine the role of *GJB2* gene in the phenotypic manifestation of the T7511C mutation, we also conducted the mutational screening of *GJB2* gene in the members of this family.

Materials and methods

A Japanese family. The four generation family with 27 members is shown in Fig. 1 as described elsewhere [14]. A comprehensive history and physical examination was performed to identify any syndromic findings or genetic factors related to the hearing loss. An age-appropriate audiological examination was performed and this included pure-tone audiometry (PTA) and/or auditory brainstem response (ABR), immittance testing and transiently evoked otoacoustic emissions (TEOAEs). The PTA was calculated from the sum of the audiometric thresholds at 500, 1000, 2000, and 4000 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). Informed consent was obtained from all participants prior to their participation in the study, in accordance with the Cincinnati Children's Hospital Medical Center and Jichi Medical School Institutional Review Board. The 141 control DNA used for screening for the presence of mtDNA and *GJB2* mutations was obtained from a panel of unaffected individuals from comparable ethnic background.

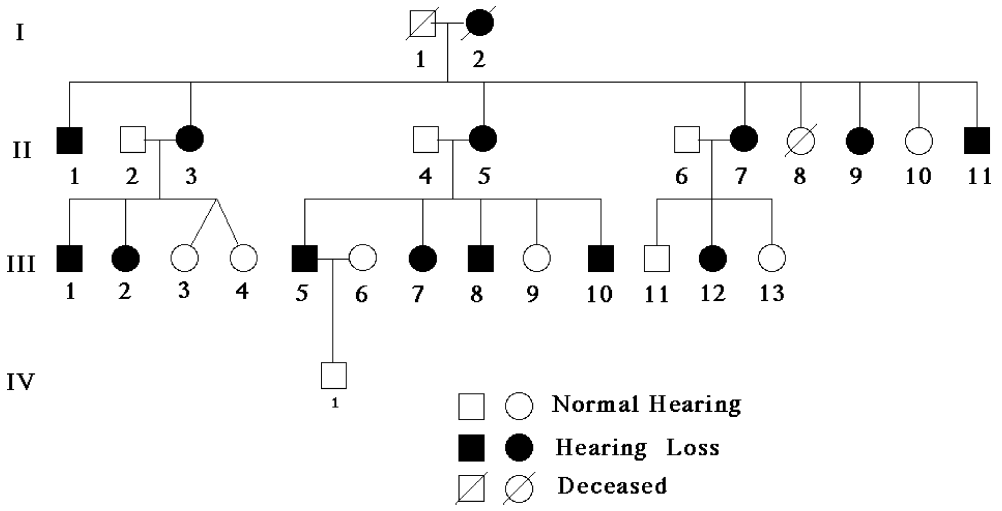

Fig. 1. The Japanese pedigree with nonsyndromic hearing impairment. Hearing impaired individuals are indicated by filled symbols.

Mutational screening of the mitochondrial genome. Genomic DNA was isolated from whole blood of participants using the Puregene DNA Isolation Kits (Gentra Systems). The entire mitochondrial genomes of one affected matrilineal relative III-1 carrying the T7511C mutation were PCR-amplified in 24 overlapping fragments by use of sets of the light-strand and the heavy-strand oligonucleotide primers, as described elsewhere [19]. 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. mtDNA sequence alignments were carried out using seqweb program GAP (GCG). The resultant sequence data were compared with the updated consensus Cambridge sequence (GenBank Accession No.: NC_001807) [20].

Quantification of the T7511C mutation. Affected and control subject's DNA fragments spanning the entire mitochondrial $tRNA^{Ser(UCN)}$ gene were amplified by PCR using oligodeoxynucleotides corresponding to positions 7396–7417 and 7657–7676 [21]. For the detection of the T7511C mutation, the amplified segments were digested with a restriction enzyme *Mbo*II [13,14].

Quantitative analysis of the T7511C mutation was carried out by allele-specific termination of primer extension [8]. For this purpose, the PCR-amplified fragments (positions 7148–8095) were used as templates, and the 5′-end ^{32}P-labeled HO7458 oligodeoxynucleotide (positions 7485–7508) was used as a primer in 1:1 molar ratio. Nucleoside triphosphate concentrations were 100 μM for dTTP and 300 μM for ddCTP. The mixtures were heated to 95 °C for 3 min, and then cooled to 45 °C for 5 min, to 37 °C for 10 min, and finally chilled on ice. After addition of 1 μl of 1:8 diluted Sequenase (USB), the mixtures were incubated at 45 °C for 5 min. The reaction products were denatured and separated on a 50-cm long 20% polyacrylamide–6 M urea gel. Quantification of the intensity of the bands was done by using a PhosphorImager (Molecular Dynamics) and the IMAGEQUANT program.

Mutational analysis of GJB2 gene. The DNA fragments spanning the entire coding region of *GJB2* gene were amplified by PCR using the following oligodeoxynucleotides: forward-5′TATGACACTCCCCA GCACAG3′ and reverse-5′GGGCAATGCTTAAACTGGC3′. PCR-amplification and subsequent sequencing analysis were performed as detailed elsewhere [22]. The results were compared with the wild type *GJB2* sequence (GenBank Accession No.: M86849) to identify the mutations.

Results and discussion

As shown in Fig. 1, this familial history is consistent with maternal inheritance. Fourteen of 21 matrilineal relatives in this Japanese family exhibited early or late-onset/progressive but not congenital, hearing impairment. All affected individuals showed the loss of high frequencies. In 10 of the 14 affected subjects, hearing impairments were symmetric. Audiometric data revealed the variable severity of hearing impairment in the maternal kindred: two subjects suffered mild hearing impairment, seven subjects showed moderate hearing impairment, four subjects exhibited a severe hearing impairment, and seven subjects had normal hearing. Their hearing loss shared some common features: bilateral and sensorineural. In addition, there was a wide range in the age at onset of hearing impairment, varying from 3 to 30 years. Furthermore, clinical data showed that all matrilineal relatives did not have the history of exposure to aminoglycosides.

To further determine the presence and amount of T7511C mutation in these matrilineal relatives, the DNA fragments spanning the entire mitochondrial $tRNA^{Ser(UCN)}$ gene were first PCR-amplified and subsequently analyzed for the existence of the T7511C mutation by digesting with the *Mbo*II. PCR fragments derived from both affected matrilineal relatives and unaffected matrilineal relatives have been partially digested by the *Mbo*II but not 141 controls from East-Asian background, confirming the presence of the homoplasmic T7511C mutation in those individuals (data not shown). However, the incomplete digestion of PCR products by the *Mbo*II makes it difficult to quantify the level of the T7511C mutation in matrilineal relatives and controls. Thus, a more sensitive experiment, involving allele-specific termination of primer extension, was carried out. As can be seen in Fig. 2, there is a small amount (<10%) of detectable wild type DNA in some matrilineal relatives, indicating that the T7511C mutation appears to be a high proportion of heteroplasmy or near homoplasmy. However, as shown in Table 2, the levels of T7511C mutation in those matrilineal relatives did not correlate with the severity and age-of-onset of hearing impairment.

To determine the role of mitochondrial haplotypes in the phenotypic expression of the T7511C mutation, the DNA fragments spanning the entire mitochondrial genome of an affected patient III-1 were PCR-amplified, and each fragment was purified and subsequently analyzed by direct sequence. The comparison of the resultant sequence with the Cambridge consensus sequence [20] identified a number of nucleotide changes as shown in Table 1. Sequence analysis confirmed the presence of the T7511C mutation in matrilineal relatives of this

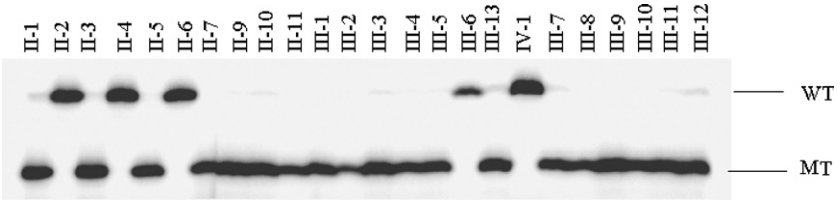

Fig. 2. Quantification of T7511C mutation in the $tRNA^{Ser(UCN)}$ gene of mutants (MT) and wild type (WT) subjects derived from the Japanese family. Allele-specific termination of primer extension was carried out as detailed in Materials and methods. The products were separated on a 20% polyacrylamide–7 M urea sequence gel.

Table 1
mtDNA mutations in the Japanese pedigree

Gene	Position	Replacement	Conservation[a] H/B/M/X	Previously reported[b]
D-loop	73	A to G		Yes
	152	T to C		Yes
	263	A to G		Yes
	310	T to CTC		Yes
	16,129	G to A		Yes
	16,223	C to T		Yes
	16,362	T to C		Yes
	16,519	T to C		Yes
12S rRNA	750	A to G	A/A/G/—	Yes
	1438	A to G	A/A/A/G	Yes
16S rRNA	2706	A to G	A/G/A/A	Yes
	3010	A to G	G/G/A/A	Yes
	3206	C to T	C/A/T/A	No
ND2	4769	A to G		Yes
	4883	C to T		Yes
	5178	C to A (Leu to Met)	L/T/T/T	Yes
CO1	7028	C to T		Yes
$tRNA^{Ser(UCN)}$	7511	T to C	T/T/T/T	Yes
A6	8414	C to T (Leu to Phe)	L/F/M/W	Yes
	8473	T to C		Yes
	8701	A to G (Thr to Ala)	T/S/L/Q	Yes
CO3	9540	T to C		Yes
ND3	10,398	A to G (Thr to Ala)	T/T/T/A	Yes
	10,400	C to T		Yes
$tRNA^{Arg}$	10,410	T to C	T/C/A/G	Yes
ND4	10,873	T to C		Yes
	11,719	G to A		Yes
ND5	12,705	C to T		Yes
cytochrome *b*	14,783	T to C		Yes
	14,979	T to C (Ile to Thr)	I/I/L/L	Yes
	15,043	G to A		Yes
	15,301	G to A		Yes
	15,236	A to G (Thr to Ala)	T/M/I/I	Yes

[a] Conservation of amino acid for polypeptides or nucleotide for RNAs, in human (H), bovine (B), mouse (M), and *Xenopus laevis* (X).
[b] See http//www.mitomap.org.

family. Of other nucleotide changes, there are two variants in the 12S rRNA gene, three variants in the 16S rRNA gene, and one variant in the $tRNA^{Arg}$ gene. Those variants, except the C3206T variant, were previously identified in the control population [23]. These variants in the RNA genes were further evaluated by phylogenetic analysis of these mtDNA variants and mtDNAs from other organisms including human [20], mouse [24], bovine [25], and *Xenopus laevis* [26]. All variants in the RNA genes were not highly evolutionarily conserved. Those variants appear to be the homoplasmy in this affected matrilineal relative. Of other nucleotide changes in this mitochondrial genome, 8 polymorphisms in the D-loop region and 21 variants in protein encoding genes were previously identified in the control population [23]. As shown in Table 1, six amino acid substitutions caused by corresponding mtDNA variants occurred in different polypeptides in this matrilineal relative. However, none of variants in the polypeptides were highly evolutionarily conserved. Interestingly, in these matrilineal relatives, there was the absence of the ND1 T3308C and $tRNA^{Ala}$ T5655C mutations, which were found in the African family carrying the T7511C mutation with a high penetrance (36/43) [13]. Thus, the presence of the ND1 T3308C and $tRNA^{Ala}$ T5655C mutations in the African family [13] but the absence of these mtDNA mutations in the Japanese family seems to account for different penetrance between two pedigrees. Our previous biochemical study from the African family carrying the T7511C mutation, in conjunction with the ND1 T3308C and $tRNA^{Ala}$ T5655C mutations, showed that the T5655C mutation produces ~50% reduction in the $tRNA^{Ala}$ level and the T3308C mutation causes a significant decrease both in the amount of ND1 mRNA and co-transcribed $tRNA^{Leu(UUR)}$ in mutant cells [17]. These biochemical data strongly support the genetic and clinical findings that the T3308C and T5655C mutations contribute to the higher penetrance of deafness in the African pedigree than the Japanese family carrying the T7511C mutation.

Our previous data showed that the expression of clinical phenotype of nonsyndromic deafness-associated mtDNA mutation(s), such as A1555G mutation [3–5,27,28] and C1494T mutation [8], requires for the

Table 2
Summary of molecular and clinical data for some members of the Japanese pedigree

Subject	Level of hearing impairment	Age-of-onset (years)	Level of T7511C mutation (%)	G79A (Ile to Val) in *GJB2*	G109A (Ile to Val) in *GJB2*
II-1	Severe	5	91.30	+/−	−/−
II-2	Normal	—	0.00	+/−	−/−
II-3	Severe	8	91.23	+/−	−/−
II-4	Normal	—	0.00	−/−	+/−
II-5	Moderate	n/a	93.70	+/−	−/−
II-6	Normal	—	0.00	+/−	−/−
II-7	Moderate	28	96.98	+/−	−/−
II-9	Mild	n/a	95.74	+/−	−/−
II-10	Normal	—	94.15	+/−	−/−
II-11	Severe	30	95.80	+/+	−/−
III-1	Moderate	3	96.99	+/+	−/−
III-2	Moderate	8	95.23	+/−	−/−
III-3	Normal	—	94.20	+/−	−/−
III-4	Normal	—	94.33	+/+	−/−
III-5	Moderate	10	94.37	+/−	+/−
III-6	Normal	—	0.00	+/−	−/−
III-7	Moderate	14	92.32	+/−	+/−
III-8	Severe	n/a	97.37	−/−	+/+
III-9	Normal	—	97.37	+/−	−/−
III-10	Mild	n/a	98.78	+/−	+/−
III-11	Normal	n/a	94.98	+/+	−/−
III-12	Moderate	9	91.66	+/+	−/−
III-13	Normal	—	94.34	+/−	−/−
IV-1	Normal	—	0.00	+/−	−/−

contribution of modulating factors including aminoglycosides or nuclear modifier genes. The connexin 26 (*GJB2*) is a potential candidate modifier gene as mutations in this gene are the most common cause of hereditary hearing loss [29]. To examine the role of *GJB2* gene in the phenotypic expression of the T7511C mutation, we performed the mutational screening of the *GJB2* gene in 24 maternal members of this Japanese family. As shown in Table 2, two known variants, G79A changing valine 27 into isoleucine and G109A replacing valine 37 with isoleucine in the *GJB2* gene [30,31], were found in some family members with hearing impairment and normal hearing. These variants were then examined to determine the allele frequency in the East-Asian control population by sequencing the PCR fragment spanning the *GJB2* coding region derived from 141 East-Asian control individuals. Nine control subjects carry the homozygous G79A variant, while 14 individuals have the heterozygous G79A variant. This translates to ~19.1% allelic frequency of this variant in the control population. Similarly, the allelic frequency of G109A variant is ~9.2% in the control population. These data strongly suggest that G79A and G109A are polymorphisms in the East-Asian population. Indeed, the lack of correlation in the severity and age-of-onset in hearing impairment with the homozygous or heterozygous G79A or G109A or combination of both variants in the *GJB2* gene in those subjects with hearing impairment and normal hearing indicates that those variants of *GJB2* gene may not be a modifier of the phenotypic effects of the T7511C mutation in those subjects. Thus, other modifier factors such as environmental factors or nuclear modifier genes may also contribute to the penetrance of the T7511C mutation, specifically in late-onset cases in this family.

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