Molecular analysis of mitochondrial gene mutations in Korean patients with nonsyndromic hearing loss

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Abstract. Mutations in mitochondrial DNA (mtDNA) are a major cause of hearing loss. In this study, we performed a systematic mutational screening of the 12S rRNA, $tRNA^{Ser(UCN)}$, $tRNA^{Lys}$ and $tRNA^{Leu(UUR)}$ genes in 227 unrelated patients with nonsyndromic hearing impairment for the first time in a Korean population. We found two individuals with an A1555G mutation, which is a frequency (0.9%) lower than that of other East Asians. Furthermore, two novel variants (C895T and 961-CC insertion) in the 12S rRNA gene were identified in the affected individuals, but were absent in 217 controls, indicating that they may play a role in the pathogenesis of hearing loss. Notably, 961delT and T1005C mutations were identified at similar frequencies in both patients and control subjects. Our data suggest that these variants seem to be polymorphisms rather than causes of disease. On the other hand, we did not find any of the known deafness-associated mutations in these tRNA genes. These data suggest that the 12S rRNA gene may be a hot spot for mitochondrial mutations causing hearing loss in the Korean population.

Introduction

Congenital hearing loss is one of the most common sensory disorders in humans, affecting 1 in 1,000 newborns, and genetic causes are thought to be responsible for over 50% of cases in developed countries (1,2). Of the hearing loss disorders attributable to genetic causes, approximately 70% are classified as nonsydnromic since hearing impairment is the only symptom, while 30% are classified as syndromic and are associated with other clinical features.

Mutations in mitochondrial DNA (mtDNA) have been associated with both syndromic and nonsyndromic deafness (3-5). In particular, the mitochondrial genes encoding 12S ribosomal RNA (rRNA) (MTRNR1) and the transfer RNA $(tRNA)^{Ser(UCN)}$ gene have been found to be associated with nonsyndromic hearing loss. Different mutations in the 12S rRNA gene, the A1555G, A827G, T1095C, C1494T, and 961 mutations, can cause maternally inherited, nonsyndromic hearing loss, which in most cases is induced or aggravated by ototoxic aminoglycosides (6-15). Among these mutations, the A1555G mutation seems to be the most common cause of aminoglycoside-induced hearing loss, especially in those cases with family history (8,16). It has also been demonstrated to cause hearing loss even without an aminoglycoside injection, and consequently may be related to inner ear susceptibility. Therefore, it may be that the mutation manifests itself as a diminished ability to repair cochlear damage from a variety of causes, including, for example, noise and aging (8,17,18).

Furthermore, five nonsyndromic deafness-associated mutations, A7445G, G7444A, 7472insC, T7510C, and T7511C, have been identified in the $tRNA^{Ser(UCN)}$ gene (19-29). These mutations often occur in homoplasmy or in high levels of heteroplasmy, indicating a high threshold for pathogenicity. It is believed that mutations in this gene can cause a failure in tRNA metabolism, thereby leading to a decrease in the amount of affected tRNAs, which subsequently results in insufficient mitochondrial protein synthesis and respiration defects (30). However, nonsyndromic deafness-associated mtDNA mutations, such as the A1555G or A7445G mutations, are often insufficient to produce clinical deafness, since some individuals carrying these mutations have normal hearing (6,8,18,19,31). Thus, other factors including other mtDNA mutations, nuclear background, and environmental factors modulate the phenotypic variability and penetrance of deafness associated with these mtDNA mutations. In addition, A3243G, T3171C in the $tRNA^{Leu(UUR)}$ gene and G8363A in the $tRNA^{Lys}$ gene are also known to be associated with maternally inherited syndromic hearing loss (4,32,33).

However, less is known about the incidence of the deafness-associated mtDNA mutations in the Korean population.

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With the aim of identifying mtDNA mutations associated with hearing loss, we undertook a systematic and extended mutation screening of mitochondrial genes including the 12S rRNA, $tRNA^{Ser(UCN)}$, $tRNA^{Lys}$ and $tRNA^{Leu(UUR)}$ genes in 227 unrelated Korean patients with nonsyndromic hearing impairment. To examine the role of the *GJB2* and *GJB6* genes in the phenotypic expression of the known or putative deafness-associated mtDNA mutation, we also performed mutation screening of the *GJB2* gene and PCR assay for the 342-kb deletion of the *GJB6* gene.

Materials and methods

Subjects. A total of 227 nonsyndromic hearing loss patients were recruited from the Soree Ear Clinic, Seoul, Korea and a local school for deafness. There were 107 male and 120 female patients, with an age range of 7 months to 65 years (mean age, 13.7 years). In the case of patients who attended the Soree Ear Clinic, audiological studies were carried out including pure tone audiometry, tympanometry, or auditory brainstem response test (ABR) in a sound-treated room. Pure-tone average (PTA) was calculated as an average of the threshold measured at 0.5, 1.0, 2.0 and 3.0 KHz for comparing subgroups of patients. One hundred and fifty-seven patients from the Soree Ear Clinic were chosen for this study. All of the patients had severe hearing loss over 95 dB of PTA or over 90 dB in ABR. The medical records of deaf individuals in the school were reviewed, and 70 patients with profound hearing loss above 95 dB were chosen for the study. None of the subjects showed unilateral hearing loss, past history of meningitis, head trauma, noise trauma, infectious disease associated with hearing loss, or other acquired hearing loss. Two control groups were employed: i) 75 Korean subjects who had no hearing loss and were evaluated with pure tone audiometry (group 1), and ii) 142 normal healthy Korean controls who were not examined by audiometric evaluation (group 2).

All participants provided written informed consent according to the protocol approved by the Ethics Committee of Kyungpook National University Hospital prior to the study.

Mutational analyses of the mitochondrial 12S rRNA, $tRNA^{Ser(UCN)}$, $tRNA^{Lys}$ and $tRNA^{Leu(UUR)}$ genes. Genomic DNA was extracted from peripheral blood using a FlexiGene DNA extraction kit (Qiagen, Hilden, Germany) or from buccal swab specimens using Puregene Buccal Cell Core Kit A (Qiagen).

The mitochondrial 12S rRNA, $tRNA^{Ser(UCN)}$, $tRNA^{Lys}$ and $tRNA^{Leu(UUR)}$ genes were amplified by polymerase chain reaction (PCR) using the appropriate intronic primer sets. PCR was performed in a total of 25 μl reaction, containing 0.2 mM of each deoxynucleotide, 15 pmol of each forward and reverse primer, 1.0-1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.75 U of Taq DNA polymerase (Solgent, Korea), and 25 ng of genomic DNA. PCR conditions were as follows: 35 cycles of denaturation at 95˚C for 30 sec; annealing at 55˚C or 57˚C, depending on the primers for 30 sec; and extension at 72˚C for 1 min. The first denaturation step and the last extension step were at 95˚C for 2 min and 72˚C for 10 min, respectively. Five microliters of the PCR products were separated and visualized on a 2% agarose gel. Fifteen microliters of this PCR product were then treated with 0.3 U of shrimp alkaline phosphatase (USB) and 3 U of exonuclease I (USB) at 37˚C for 1 h, followed by incubation at 80˚C for 15 min. This was diluted with an equal volume of dH_2O, and 6 μl was used for the final sequencing reaction. Sequencing reactions were performed in both directions on the PCR products in reactions containing 5 pmol of primer, 0.25 μl of ABI Big Dye Terminator v3.1 Cycle Sequencing Kit, and 1 μl of 5X dilution buffer (400 mM Tris-HCl, pH 9.0, and 10 mM $MgCl_2$). Cycling conditions were 95˚C for 2 min followed by 35 cycles of 94˚C for 20 sec, 55˚C for 20 sec, and 60˚C for 4 min. Sequencing reaction products were ethanol precipitated, and the pellets were resuspended in 10 μl of formamide loading dye. An ABI 3130XL DNA sequencer was used to resolve the products, and data were analyzed by using ABI Sequencing Analysis (v.5.0) and Lasergene-SeqMan software. The resultant sequences were compared with the updated Cambridge sequence (GenBank Accession No. NC_001807) (34).

The samples were also tested for the presence of mutations in the coding region of *GJB2* by direct sequencing and the 342-kb deletion of the *GJB6* gene by a specific PCR assay as previously reported by del Castillo *et al* (35).

Secondary structure prediction. Structures for the wild-type and mutated mitochondrial 12S rRNAs were generated using the RNAfold software from the Vienna RNA package (36). RNAfold predicts RNA secondary structure based on minimum energy requirements and pair probabilities.

Results

Mitochondrial 12S rRNA and tRNA gene analyses. Mutational screening of the entire mitochondrial 12S rRNA gene in 227 subjects with nonsyndromic hearing loss and 217 hearing individuals revealed the presence of 29 nucleotide deviations from the Cambridge sequence as shown in Table I (34). Of these changes, three were novel and 26 had been previously described. The A1555G mutation, which is the most common cause of hearing loss linked to mutations in mitochondrial DNA, was found in two patients, and in one of these it was present in heteroplasmy. Both of the patients had familial history, and showed profound hearing loss in audiological evaluation. This result indicates a frequency of about 0.9% for the A1555G mutation in this group. One novel variant, C895T in the 12S rRNA gene, was present in homoplasmy in one patient and this substitution was not observed in controls (Fig. 1A). This variant was further evaluated by phylogenetic analysis of this mtDNA variant and mtDNAs from other organisms. As shown in Table I, the C895 is highly conserved in humans, mouse, bovine, and *Xenopus laevis* (34,37-39). Since the A1555G mutation was predicted to alter the secondary structure of the 12S rRNA molecule which affects both transcriptional fidelity and interaction of the ribosome with aminoglycoside antibiotics, a prediction of the secondary structure of the 12S rRNA molecule was carried out to study the possible functional effect of this novel change (36). In this model, the C895T change resulted in a structural change compared to the wild-type prediction (Fig. 1B).

Table I. Variants in the mitochondrial 12s rRNA gene in Korean subjects with nonsyndromic hearing loss.[a]

Position	Replacement	Frequency			Conservation (H/B/M/X)[e]	Previously reported[f]
		In patients[b]	In normal subjects[c]	In normal subjects[d]		
709	G to A	48/227	16/75	28/142	G/A/A/-	Yes
750	A to G	227/227	75/75	142/142	A/A/A/-	Yes
752	C to T	5/227	3/75	2/142	C/C/A/-	Yes
769	G to A	1/227	0/75	0/142	G/A/T/-	Yes
789	T to C	0/227	1/75	0/142	T/T/T/A	Yes
827	A to G	4/227	4/75	2/142	A/A/A/A	Yes
869	C to T	1/227	0/75	0/142	C/T/T/A	Yes
895	**C to T**	**1/227**	**0/75**	**0/142**	**C/C/C/C**	**No**
904	C to T	1/227	0/75	0/142	C/C/C/T	Yes
958	C to T	1/227	0/75	0/142	C/C/A/T	Yes
961	C ins	2/227	0/75	1/142	T/T/A/A	Yes
961	**CC ins**	**1/227**	**0/75**	**0/142**	**T/T/A/A**	**No**
961	T del + Cn	9/227	3/75	10/142	T/T/A/A	Yes
1005	T to C	3/227	1/75	2/142	T/T/T/T	Yes
1009	C to T	1/227	0/75	0/142	C/T/C/T	Yes
1041	A to G	3/227	3/75	3/142	A/A/T/T	Yes
1048	C to T	10/227	2/75	7/142	C/C/T/C	Yes
1095	T to C	0/227	0/75	1/142	T/T/T/T	Yes
1107	T to C	19/227	5/75	8/142	T/C/T/T	Yes
1116	A to G	1/227	0/75	0/142	A/A/A/T	Yes
1118	A to T	1/227	1/75	0/142	A/A/A/C	No
1119	T to C	10/227	2/75	2/142	T/T/T/G	Yes
1310	C to T	1/227	0/75	1/142	C/G/C/T	Yes
1382	A to C	7/227	5/75	7/142	A/A/A/G	Yes
1391	T to C	0/227	1/75	0/142	T/A/T/A	Yes
1438	A to G	224/227	70/75	139/142	A/A/A/G	Yes
1442	G to A	1/227	0/75	1/142	G/A/A/C	Yes
1555	**A to G**	**2/227**	**0/75**	**0/142**	**A/A/A/A**	**Yes**
1598	G to A	9/227	2/75	2/142	G/A/T/T	Yes

[a]Known and putative pathogenic mutations are indicated in boldface. [b]Number of patients with the substitution/total patients analyzed. [c]Number of controls with the variant/total controls with audiological evaluation. [d]Number of controls with the variant/total controls without audiological evaluation. [e]Conservation of nucleotide for the 12S rRNA gene in humans (H), bovine (B), mouse (M), and *Xenopus laevis* (X). [f]See the online mitochondrial genome database Mitomap (http://www.mitomap.org), mtDB-Human Mitochondrial Genome Database (http://www.genput.uu.se/mtDB/) or Human Mitochondrial Genome Polymorphism Database (http://mtsnp.tmig.or.jp/mtsnp).

Notably, three different variants at position 961 including two known variants, 961-C insertion, delT961Cn, and one novel variant, 961-CC insertion were identified in this study and all of them were present in homoplasmy. As shown in Table I, the delT961Cn mutation was detected in 9/227 patients (3.9%). This mutation was also found in 3/75 (4%) of control group 1 and 10/142 (7%) of control group 2. The 961-C insertion was detected in two patients and found in one individual in control group 2. In contrast, the 961-CC insertion was detected in one of the patients, but was not identified in either of the control groups. In addition, the T1005C variant was first identified in three Chinese patients and suggested to be a pathogenic mutation (40). This variant was also detected in three patients as well as in 1/75 (0.1%) of control group 1 and in 2/142 (4.3%) of control group 2. Sequencing analyses of the tRNA genes were performed and no mutation associated with hearing loss was identified in those genes in this study.

Mutational analyses of GJB2 and 342-kb deletion of GJB6 genes. Several studies have shown that modifiers encoded by nuclear genes are one of the major modulators of the phenotypic expression of the deafness-associated 12S rRNA gene mutations (22,41,42). To examine the phenotypic heterogeneity observed in the known or putative mtDNA mutations, we performed mutation screening of the *GJB2* gene and PCR assay for the 342-kb deletion of the *GJB6* gene. Sequencing analysis revealed no mutations in the *GJB2* gene in subjects

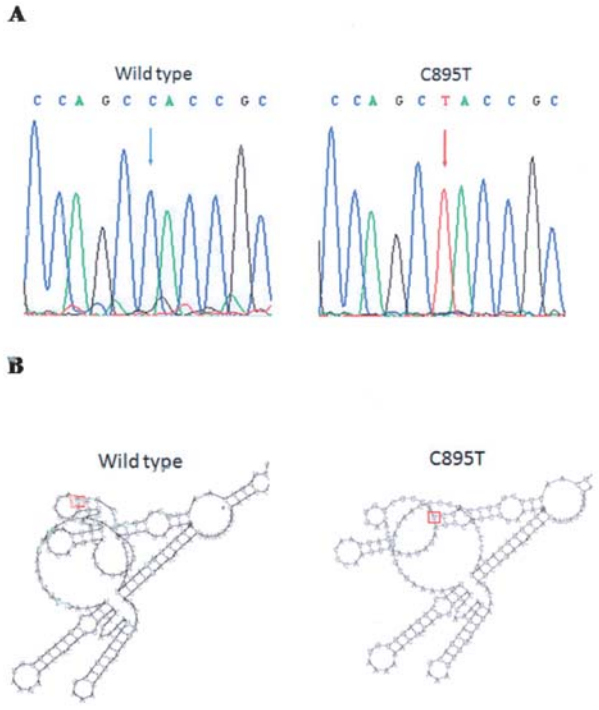

Figure 1. (A) Sequence chromatograms showing the novel C895T variation detected in the mitochondrial 12S rRNA gene. (B) The predicted secondary structures of the C895T variant compared to the wild-type prediction.

carrying the A1555G and 961-CC insertion in the 12S rRNA gene. Of nine subjects carrying the delT961Cn mutation, the homozygous 235delC mutation in the *GJB2* gene was found in one patient with profound hearing loss, whereas the other eight subjects with mild or profound hearing loss lacked the mutation in the same gene (data not shown). In addition, one of the subjects showing the 961-C insertion was homozygous for the 235delC mutation, but another subject with the same mutation did not have any mutation in the *GJB2* gene. Both of these patients showed congenital profound hearing loss in audiological evaluation. In addition, one patient with the C895T mutation was heterozygous for the 235delC mutation in the gene. The *GJB6* gene was not detected in any subject in this study.

Discussion

Mutations in mtDNA are a major cause of hearing loss and most of the molecular defects responsible for mitochondrial disorder-associated hearing loss are mutations in the 12S rRNA and tRNA genes (3,8). In the present study, we performed a mutation screening of 12S rRNA and tRNA genes in 227 Korean subjects with nonsyndromic hearing loss to examine the prevalence and characterization of mtDNA mutation causing hearing loss in the Korean population. As a result, we identified 29 nucleotide changes including three novel variants in the 12S rRNA gene. Of these, the A1555G mutation was found in two patients, accounting for 0.9% of the study group. Since this mutation was first described in a large Arab-Israeli pedigree, the relevance of the A1555G mutation has been investigated in different hearing-impaired populations (6-8,18,43,44). This mutation was found in 0.6% of a Caucasian pediatric population, and in 2.4% in Danish, 1.8% in Turkish, less than 2.4% in German, Polish, and Hungarian, and 17% in Spanish populations (45-48). In Asian nonsyndromic hearing-impaired populations, the incidence of the A1555G mutation appears to be higher than that in Caucasians: 2.9% in Chinese, 3% in Japanese, and 5.3% in Indonesian populations (40,49,50). In Korea, Jeong *et al* (51) evaluated the frequency of the A1555G mutation by restriction fragment length polymorphism (RFLP) from 129 unrelated sensorineural hearing loss (SNHL) patients and it was found in 2.3% (3/129) of the group (51). Combined with the results of that study, our data indicate that the A1555G mutation occurs in approximately 1.4% of the Korean population. The prevalence of the A1555G mutation in Koreans seems to be lower than in other East Asians. This difference would result from different genetic backgrounds among Asians.

The C895T change was identified in homoplasmy in one case, and it was not found in any of the controls. Moreover, evaluation of this position in different species showed that the C at nucleotide 895 was conserved throughout evolution. In addition, the prediction using RNAfold software showed changes in the secondary structure of the 12S rRNA in this case. Moreover, this patient was a heterozygote for the 235delC mutation in the *GJB2* gene. Several studies have indicated that the A1555G mutation may be a predisposing mutation, which requires the combined action of environmental factors and/or nuclear modifying genes to cause hearing impairment (42,52-55). To fully understand the pathogenic mechanisms of this mtDNA variant, it would be necessary to perform functional studies of cell lines derived from this patient, but from the data presented here it seems clear that C895T is a mtDNA disease-causing mutation.

The 961delT mutation was more frequently found than other mutations in this study. The 961delT mutation was first reported in one out of 35 patients with a history of aminoglycoside exposure. Since then, several mutations at position 961 such as 961-C insertion, T961G, and T961C mutations have been found in isolated individuals and genetically unrelated families with aminoglycoside-induced and/or nonsyndromic SNHL (15,28,45,56,57). Recently, Kobayashi *et al* (58) demonstrated that a similar frequency of the 961delT mutation was found in control subjects, and that the hearing loss phenotype did not segregate with this mutation in families. In our study, the 961delT variant was the most frequent one in the patients, but it was also found in both control groups. In particular, subjects with this variant in group 1 showed a normal hearing level below 20 dB in both ears. This is consistent with the finding of the abovementioned study that the 961delT mutation was not the cause of hearing loss (58). In addition, the C insertion(s) at the position 961 were detected in the patient group, but one of these variants (961-C insertion) was also detected in control group 2. T1005C was present in homoplasmy in five cases and in three normal subjects. Yao *et al* (61) reported that these variants, 961-C insertion and T1005C, were in the list of characteristic mutations of haplogroup B51b and haplogroup F2 (59-61). Since the haplogroup data were based on phylogenetic information from the populations, these variants may be rare polymorphisms rather than mutations. However, it still remains

possible that the changes at position 961 are associated with disease as the subject showing the 961-CC insertion variant had a family history showing progressive hearing loss, and the change was not found in any of the controls tested. Therefore, further investigation such as functional analysis is necessary in order to define this variant as pathogenic, and a careful assessment will be needed to determine whether these changes in the vicinity of nucleotide 961 cause hearing loss.

Mitochondrial tRNA genes are a hot spot for mutations in maternally inherited SNHL, as a number of deafness-associated mutations have been identified in three of the 22 tRNA genes including $tRNA^{Ser(UCN)}$, $tRNA^{Lys}$, and $tRNA^{Leu(UUR)}$. In this study, we failed to detect the presence of mutation in these genes. This suggests that mutations in these mitochondrial tRNA genes are not a common cause of deafness in the Korean population.

Usually, a single mutation in the mitochondrial 12S rRNA gene or tRNA genes is not sufficient to cause hearing loss, but requires the contribution of other modulating factors such as nuclear modifier gene(s), environment factor(s), or mitochondrial haplotypes for the phenotypic expression. Among the nuclear genes responsible for SNHL, mutations in the *GJB2* gene have been of particular interest because they have been found to account for about 50% of inherited nonsyndromic deafness cases (62). Also, the 342-kb deletion disrupting the *GJB6* gene (delGJB6-D13S1830) is common in patients from Spain, France, Germany, the United Kingdom and Brazil, and in patients of Ashkenazi Jewish origin (35,63-65). We studied the potential relationship between mtDNA mutations and mutations in the *GJB2* or *GJB6* genes for phenotypic expression. A possible interaction was only found in the subject carrying the C895T mutation with heterozygous 235delC mutation. Since the prevalence of mutations in the *GJB2* gene responsible for SNHL was only about 10% in the Korean population, the *GJB2* gene does not seem to be a major modifier gene affecting the severity of hearing loss in this population.

In summary, we observed the frequency of the A1555G mutation to be 1.4% in the Korean nonsyndromic hearing loss population, and we found the novel C895T and 961-CC insertion variants of the 12S rRNA gene that may be associated with SNHL. These data suggest that the 12S rRNA gene is the mitochondrial hot spot for mutations causing hearing loss. Further studies are necessary to determine whether there are other genes which also account for a significant proportion of hearing loss in the Korean population.

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