**Frequency of mitochondrial mutations in non-syndromic hearing loss as well as possibly responsible variants found by whole mitochondrial genome screening**

Takuya Yano[1], Shin-ya Nishio[1], Shin-ichi Usami[1], the Deafness Gene Study Consortium[2]

[1]Department of Otorhinolaryngology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano, 390-8621 Japan

[2] Participating institutions: see Acknowledgements

Correspondence:

Shin-ichi Usami

Department of Otorhinolaryngology

Shinshu University School of Medicine

3-1-1 Asahi, Matsumoto 390-8621, Japan

Tel: +81-263-37-2666

Fax: +81-263-36-9164

E-mail: usami@shinshu-u.ac.jp

**Keywords**

Mitochondrial mutation /non-syndromic hearing loss

**Abstract**

Mutations in mitochondrial DNA (mtDNA) are reported to be responsible for the pathogenesis of maternally inherited hearing loss. Complete mtDNA sequencing may detect pathogenic mutations, but whether they are indeed pathogenic can be difficult to interpret because of normal ethnic-associated haplogroup variation and other rare variations existing among control populations. In this study, we performed systemic mutational analysis of mtDNA in 394 Japanese patients with hearing loss. Two different cohorts were analyzed in this study: Cohort 1, 254 maternally inherited patients; and Cohort 2, 140 patients with various inheritance modes. After screening of the entire mtDNA genome with direct sequencing, we evaluated the frequency of previously reported mutations and the frequency and pathogenicity of the novel variants. As a result, the 'Confirmed' mitochondrial mutations were found predominantly in Cohort 1 rather than in Cohort 2 (14.6 vs 0.7%). 1555A>G (n=23) is the most common mutation, followed by the 3243A>G (n=11) mutations. On the basis of prediction analysis, we detected10 novel homoplasmic mitochondrial variants. After further classification, the 3595A>G and 6204A>G variants were found to be new candidate mutations possibly associated with hearing loss.

## Introduction

Hearing impairment is one of the most common sensory handicaps, with a frequency of at least 1/1000 at birth, and 50% of these cases can be attributed to genetic causes. Furthermore, causative mitochondrial DNA (mtDNA) mutations have been found in 5–10% of patients with postlingual non-syndromic hearing loss.[1]

Among mitochondrial mutations, 1555A>G mutations in the mitochondrial 12S rRNA are found frequently (0.6–5.3%, depending on the ethnic group) in aminoglycoside-induced and late-onset non-syndromic hearing loss. [2–4] A 1494C>T mutation in 12S rRNA is also associated with aminoglycoside-induced and non-syndromic hearing loss.[5] A 3243A>G mutation in the $tRNA^{Leu(UUR)}$ is associated with maternally inherited diabetes combined with deafness, [6] and mitochondrial myopathy, encephalopathy, lactic acidosis and stroke- like episodes (MELAS), which frequently present with hearing loss. 7445A>C/G/T, [7,8] 7472insC and 7510T>C [9] mutations in the $tRNA^{Ser(UCN)}$ are also associated with aminoglycoside-induced or non-syndromic hearing loss.

Moreover, additional mutations in 12S rRNA (827A>G, [10] 961T>C, 961delT+Cn, 1005T>C and 1095T>C [11]) have been reported as mitochondrial hearing loss mutations. Although there were growing numbers of reports of various novel mtDNA mutations associated with hearing loss, most focused on a few limited nucleotide positions or only the 12SrRNA region. [12] Therefore, we conducted a whole mitochondrial genome mutational analysis by direct sequencing using samples from 254 maternally inherited and 140 non-syndromic Japanese hearing loss probands with various

inheritance modes, and summarized the frequencies of the mutations, as well as the spectrum and phenotypes found in the hearing loss patients with mtDNA mutations.

## Materials and methods

### Subjects

Two cohorts were used in this study: Cohort 1, 254 Japanese maternally (or possibly autosomal dominant with affected mother and one or more affected children) inherited sensorineural hearing loss (SNHL) subjects; and Cohort 2, 140 Japanese SNHL subjects with various inheritance modes (14 autosomal dominant or mitochondrial inherited, 126 autosomal recessive inherited or sporadic cases), both collected from 33 ENT departments nationwide in Japan. All subjects gave prior written informed consent for participation in the project, which was approved by the ethical committee of each hospital. The control group consisted of 192 unrelated Japanese healthy individuals with normal hearing evaluated by auditory testing.

### Mutation analysis

Whole mtDNA from each patient was amplified into two long fragments, A and B, by LA Taq DNA polymerase (TaKaRa BIO, Shiga, Japan) as described elsewhere [13]. In brief, each genomic DNA sample was amplified by long PCR for 1 min at 94˚C, followed by 30 three-step cycles of 94˚C for 30 s, 60˚C for 30 s and 72˚C for 6 min, with a final extension at 72˚C for 5 min, ending with a holding period at 4˚C.

After the PCR amplification, resulting products were purified and direct sequenced with ABI Big Dye terminators and ABI 3130 autosequencer (Applied Biosystems). Sequencing reaction was

performed with 50 primers for the whole mitochondrial genome, designed with mitoSEQr™ Resequencing System (Applied Biosystems).

Sequencing data were analyzed by SeqScape ver2.6 and SeqAnalysis (Applied Biosystems). The sequencing result from each patient was compared with the rCRS (Reversed Cambridge Reference Sequence) to identify mtDNA mutations. Mitochondrial DNA mutations included in the mtSNP [http://mtsnp.tmig.or.jp/mtsnp/index.shtml], MITOMAP [http://www.mitomap.org/MITOMAP], or Uppsala mtDB [http://www.genpat.uu.se/mtDB/] databases were excluded as non-pathogenic variants.

We evaluated mutations according to evaluation criteria derived from a previous report by Zaragoza et al [14].

**Prediction of pathogenicity of mtDNA mutations**

Initially, we measured the frequencies of each mutation found in healthy controls in our study (n= 192) and in the mtSNP database (n= 2153, including: centenarians in Gifu and Tokyo, type 2 diabetes mellitus patients (with or without vascular disorders), overweight and non-overweight young adult males, Parkinson's disease patients, and Alzheimer's disease patients in Japan). The nucleotide conservation in each gene from humans and 60 mammalian species (*Artibeus jamaicensis*, NC_002009; *Balaenoptera musculus*, NC_001601; *Balaenoptera physalus*, NC_001321; *Bos Taurus*, NC_006853; *Canis familiaris*, NC_002008; *Cavia porcellus*, NC_000884; *Cebus albifrons*,

NC_002763; *Ceratotherium simum*, NC_001808; *Chalinolobus tuberculatus*, NC_002626; *Dasypus novemcinctus*, NC_001821; *Didelphis virginiana*, NC_001610; *Dugong dugon*, NC_003314; *Echinops telfairi*, NC_002631; *Echinosorex gymnura*, NC_002808; *Equus asinus*, NC_001788; *Equus caballus*, NC_001640; *Erinaceus europaeus*, NC_002080; *Felis catus*, NC_001700; *Gorilla gorilla*, NC_001645; *Halichoerus grypus*, NC_001602; *Hippopotamus amphibious*, NC_000889; *Hylobates lar*, NC_002082; *Isoodon macrourus*, NC_002746; *Lama pacos*, NC_002504; *Loxodonta Africana*, NC_000934; *Macaca sylvanus*, NC_002764; *Macropus robustus*, NC_001794; *Mus musculus*, NC_005089; *Myoxus glis*, NC_001892; *Nycticebus coucang*, NC_002765; *Ochotona collaris*, NC_003033; *Ornithorhynchus anatinus*, NC_000891; *Orycteropus afer*, NC_002078; *Oryctolagus cuniculus*, NC_001913; *Ovis aries*, NC_001941; *Pan paniscus*, NC_001644; *Pan troglodytes*, NC_001643; *Papio hamadryas*, NC_001992; *Phoca vitulina*, NC_001325; *Physeter catodon*, NC_002503; *Pongo pygmaeus*, NC_002083; *Pongo pygmaeus* abelii, NC_002083; *Pteropus dasymallus*, NC_002612; *Pteropus scapulatus*, NC_002619; *Rattus norvegicus*, NC_001665; *Rhinoceros unicornis*, NC_001779; *Sciurus vulgaris*, NC_002369; *Soriculus fumidus*, NC_003040; *Sus scrofa*, NC_000845; *Tachyglossus aculeatus*, NC_003321; *Talpa europaea*, NC_002391; *Tarsius bancanus*, NC_002811; *Thryonomys swinderianus*, NC_002658; *Trichosurus vulpecula*, NC_003039; *Tupaia belangeri*, NC_002521; *Ursus americanus*, NC_003426; *Ursus arctos*, NC_003427; *Ursus maritimus*, NC_003428; *Volemys kikuchii*, NC_003041; *Vombatus ursinus*, NC_003322) was evaluated by the ClustalW method or the mtSNP database (mtSAP

Evaluation;http://mtsnp.tmig.or.jp/mtsnp/search_mtSAP_evaluation.html). The mutations were considered to be possibly pathogenic if the original amino acid or base was conserved in more than 50% of the species (31 or more of 61 species) [15].

**Results**

Direct sequence screening of the 254 probands of Japanese maternally inherited SNHL families and 140 non-syndromic hearing loss probands with various severities of hearing loss revealed 634 SNPs in whole mitochondrial genome. Among those SNPs, 19 were previously reported as associated with hearing loss: 792C>T (n=1), 827A>G (n=10), 856A>G (n=3), 961T>C (n=3), 1005T>C (n=2), 1095T>C (n=1), 1310C>T (n=3), 1494C>T (n=1), 1555A>G (n=23), 3243A>G (n=11), 3398T>C (n=1), 3421G>A (n=2), 5628T>C (n=1), 7511T>C (n=3), 8108A>G (n=1), 8348A>G (n=1), 11696G>A (n=4), 14693A>G (n=1), and 15927G>A (n=4) (Tables 1, 2). In this study, based on the MITOMAP database, status was considered to be "Confirmed" if at least two or more independent laboratories had published reports on the pathogenicity of a specific mutation (Table 1). More ambiguous substitutions were categorized as "Unclear," "Reported," or "point mutation/polymorphism" (Table 2). "Reported" status indicates that one or more reports have considered the mutation as possibly pathologic. "point mutation/polymorphism" status indicates that some reports have determined the mutation to be a non-pathogenic polymorphism. 14.6% (37/254) of the patients in Cohort 1 (maternally inherited patients) were associated with the "Confirmed" mutations. Only 0.7% (1/140) of the patients had the "Confirmed" mutations in Cohort 2 (patients with various inherited modes)(Table 1). Ambiguous-status substitutions were associated in 7.5% (19/254) of Cohort 1, in contrast to 13.6% (19/140) of Cohort 2 (Table 2).

With regard to the audiogram configuration, various types were found.

69% (79% in Cohort 1, 59% in Cohort 2) of the patients had progressive hearing loss and 59% (74% in Cohort 1, 45% in Cohort 2) had tinnitus, while 34% (39% in Cohort 1, 30% in Cohort 2) of the patients were associated with vertigo (Table 1, 2). Concerning clinical symptoms other than hearing loss, 80% (8/10) of the patients with the 3243A>G mutation had diabetes mellitus, but no other clinical symptoms were noticed (Table 1).

Ten novel variants which were not included in the public mitochondrial DNA databases were found in this study and they were located in the *16S rRNA*, *ND1, COI, ATPase6, ND4L, ND5,* and *Cytb* regions (Table 3). All new variants were found in only one different family each.

Four of the novel variants were found in the *16S rRNA* gene: 2069T>C, 2285T>G, 2285T>C, and 2634T>C. Although the 2634T>C variant had high conservation rate (66.7%), the 2069T>C, 2285T>G, and 2285T>C variants had low conservation rates: 31.4%, 43.1%, and 43.1%, respectively.

The remaining six novel variants were located in the protein coding regions: 3595A>G in *NADH dehydrogenase 1* gene (MTND1 (MIM 516000), 6204A>G in *cytochrome oxydase I* gene (MTCOI (MIM 516030)), 9124A>G in *ATPase 6* gene (MTATP6 (MIM 516060)), 10680G>A in *NADH dehydrogenase 4L* gene (MTND4L (MIM 516004)), 13153A>G in *NADH dehydrogenase 5* gene (MTND5 (MIM 516005)) and 15003G>C in *cytochrome b* gene (MTCYB (MIM 516020)). These variants are found in very well conserved gene positions (57.4~100%).

The conservation rates in all “Confirmed” mtDNA mutations were high (Table 4).

However, as in Table 3, the 9124A>G, 10680G>A, 13153A>G, and 15003G>C variants were found in sporadic cases which are not generally compatible with mitochondrial deafness. Based on the above evaluations, we categorized 3595A>G, and 6204A>G as possibly pathogenic mutants, and the remaining eight others as uncertain pathogenic mutants.

The homoplasmic mutation 3595A>G in the *ND1* was found in a 4-year-old male patient with prelingual, severe hearing loss of high frequencies (Figure 1). He was suspected to have hearing impairment when he was about 1 year old, but ABR testing and Computed Tomography resulted in a diagnosis of normal hearing. However, when he was 3 years old, his mother again suspected he had hearing impairment and testing confirmed it. The mother, who had the same mutation, also had hearing impairment as well as progressive bilateral tinnitus and occasional vertigo from childhood. The homoplasmic mutation 6204A>G in the *COI* gene was found in a 62-year-old male with mild hearing loss of high frequencies (Figure 2). He noticed his hearing loss at the age of 50 and suffered from tinnitus, and mild diabetes mellitus. His mother also had hearing impairment that gradually progressed with age. DNA samples were not obtained from other family members.

## Discussion

Nineteen known mitochondrial mutations were found predominantly in the maternally inherited group (Tables 1, 2). Clarification of pathogenecity of mitochondrial substitutions was hampered by low penetrance (probably due to heteroplasmy). Therefore, based on the MITOMAP database, they were classified as "Confirmed" "Ambiguous" status substitutions (Table 1, 2). The "Confirmed" mitochondrial mutations were found predominantly in Cohort 1 rather than in Cohort 2 (14.6% vs 0.7%), supporting the pathogenicity of these mutations. Frequencies of 1555A>G and 3243 A>G mutations were significantly high, indicating that these two mutations are important causes of maternally inherited hearing loss. In general, patients with these mitochondrial mutations showed more or less similar clinical characteristics, i.e., progressive hearing loss with tinnitus (Table 1). Among the ten novel variants (Table 3), two, the *ND1* mutation 3595A>G and *COI* mutation 6204A>G, are thought to be possibly pathogenic, because 1) they are found in autosomal or maternal inheritance (some of the others are found as sporadic cases); 2) the conservation rate of the variation at the position among mammals is at least over 50%, as is the conservation rate in all confirmed mtDNA mutations associated with phenotypes (Table 4); and 3) they are associated with high frequency hearing loss; the characteristic hearing type of mitochondrial hearing loss. These mutations affected a conserved nucleotide in the mitochondrial gene in primates and other species and had a conservation index of more than 50% (88.5% and 100%, respectively). None of these mutations were found in the controls nor in the databases, further indicating that they are associated

with hearing loss, however, no conclusion can be drawn without enzymatic analysis. Unfortunately, this study was a retrospective study using collected DNA samples from 1995-2012, so it was impossible to contact the patients and to get muscle or living samples from them. Therefore, enzymatic analysis of these mtDNA samples was not feasible.

In this study, we found one novel possibly pathogenic mutation in the ND1 hydrophobic arm region, in a patient with a homoplasmic 3595A>G mutation and hearing loss of the high frequencies from age 3 without complications. The family members of this patient did not have diabetes mellitus. On the other hand, the novel possibly pathogenic mutation 6204A>G was located in the *COI* gene. The amino acid conservation rate of this position was 100% (61/61 mammals). In previous reports, more than 20 pathogenic mutations in the *MT-ND1* gene were reported in patients with LHON (Leber's hereditary optic neuropathy) and MELAS. Also, *ND1* mutation-related hearing impairment has been reported: 3308T>C causing MELAS with deafness [16], 3395A>G causing hypertrophic cardiomyopathy with profound sensorineural hearing loss [17], and 3396T>C and 3421G>A causing maternally inherited diabetes and deafness [18,19]. Three COI mutations related to hearing loss have also been reported (7443A>G [20], 7444G>A [21], and 7445A>G [7,8]). Our results taken with these previous reports support the possibility that mutations in the ND1 and COI regions are associated with hearing impairment.

Most of the mitochondria DNA mutations associated with hearing loss indicate low penetrance explained as a mild biochemical defect indicating that the mutation itself is not sufficient to produce

the clinical phenotype. Thus, other modifying factors including nuclear backgrounds, environmental factors, and mitochondrial haplotypes are necessary for the phenotypic manifestation of the mutation. The degree of hearing loss from mtDNA mutation can be similar within individual families but varied among different family groups, probably due to the modifier effect by nuclear genes [22].

## Conclusion

In addition to the previously reported mitochondrial mutations, we detected ten novel homoplasmic mutations in the mitochondrial genes related to hearing loss by direct sequencing of whole mitochondrial genomes in Japanese patients. Two of them, 3595A>G and 6204A>G, are possibly associated with hearing loss.

## Acknowledgements

We thank the participants of the Deafness Gene Study Consortium: Drs. Norihito Takeichi and Satoshi Fukuda (Hokkaido University), Drs. Atsushi Namba and Hideichi Shinkawa (Hirosaki University), Drs. Yumiko Kobayashi and Hiroaki Sato (Iwate Medical University), Drs. Tetsuaki Kawase and Toshimitsu Kobayashi (Tohoku University), Drs. Tomoo Watanabe, Tsukasa Ito and Masaru Aoyagi (Yamagata University), Drs. Hiroshi Ogawa and Koichi Omori (Fukushima Medical University), Drs. Kotaro Ishikawa and Keiichi Ichimura (Jichi Medical University), Drs. Kyoko Nagai and Nobuhiko Furuya (Gunma University), Drs. Shuntaro Shigihara, Yasuyuki Nomura and Minoru Ikeda (Nihon University School), Drs. Tetsuo Ikezono and Toshiaki Yagi (Nippon

Medical School), Dr. Shunichi Tomiyama (Nippon Medical School Tama Nagayama Hospital), Drs. Hiromi Kojima, Yuika Sakurai and Hiroshi Moriyama (Jikei University), Dr. Kozo Kumakawa (Toranomon Hospital), Drs. Hajime Sano and Makito Okamoto (Kitasato University), Dr. Satoshi Iwasaki (Hamamatsu Medical University), Dr. Kazuhiko Takeuchi (Mie University), Dr. Masako Nakai (Shiga Medical Center for Children), Drs. Masahiko Higashikawa and Hiroshi Takenaka (Osaka Medical College), Drs. Yuko Saito, Masafumi Sakagami (Hyogo College of Medicine), Dr. Yasushi Naito (Kobe City Medical Center General Hospital), Drs. Keiji Fujihara, Akihiro Sakai and Noboru Yamanaka (Wakayama Medical University), Drs. Kunihiro Fukushima, and Kazunori Nishizaki (Okayama University), Drs. Kazuma Sugahara and Hiroshi Yamashita (Yamaguchi University), Drs. Naoto Hato and Kiyofumi Gyo (Ehime University), Drs. Yasuhiro Kakazu and Shizuo Komune (Kyushu University), Drs. Mayumi Sugamura and Takashi Nakagawa (Fukuoka University), Dr. Haruo Takahashi (Nagasaki University), Dr. Yukihiko Kanda (Kanda ENT Clinic), Drs. Hirokazu Kawano and Tetsuya Tono (Miyazaki Medical College), Drs. Ikuyo Miyanohara andYuichi Kurono (Kagoshima University), Drs. Akira Ganaha and Mikio Suzuki (Ryukyus University), for providing samples of their patients.

We thank all the families that participated in the present study. We would also like to thank Ms. S. Matsuda for technical assistance, and Ms. A. C. Apple-Mathews for help in preparing the manuscript.

## References


1. H.T. Jacobs, T.P. Hutchin, T. Kappi, G. Gillies, K. Minkkinen, J. Walker, et al., Mitochondrial DNA mutations in patients with postlingual, nonsyndromic hearing impairment. *Eur. J. Hum. Genet.* **13**, 26–33. (2005).

2. Prezant TR, Agapian JV, Bohlman MC et al. Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. *Nat Genet*. **4**, 289 – 294 (1993).

3. Fischel-Ghodsian, N., Prezant, T. R., Bu, X., Oztas, S. et al. Mitochondrial ribosomal RNA gene mutation in a patient with sporadic aminoglycoside ototoxicity. *American Journal of Otolaryngology*. **14 (6)**, 399-403 (1993).

4. Usami S, Abe S, Akita J, Namba A, Shinkawa H, Ishii M, et al. Prevalence of mitochondrial gene mutations among hearing impaired patients. *J Med Genet.* **37,** 38-40 (2000).

5. Zhao H, Li R, Wang Q, Yan Q, Deng JH, Han D, et al. Maternally inherited aminoglycoside-induced and nonsyndromic deafness is associated with the novel C1494T mutation in the mitochondrial 12S rRNA gene in a large Chinese family. *Am J Hum Genet*. **74,** 139-152 (2004).

6. van den Ouweland, J. M., Lemkes, H. H. P., Ruitenbeek, W., Sandkjujl, L. A., deVijlder, M. F. et al. Mutation in mitochondrial tRNALeu(UUR) gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. *Nature Genetics*. **1 (5)**, 368-371 (1992).

7. Reid FM, Vernham GA, Jacobs HT. A novel mitochondrial point mutation in a maternal pedigree with sensorineural deafness. *Hum Mutat*. **3**: 243–247 (1994).

8. Fischel-Ghodsian N, Prezant TR, Fournier P, Stewart IA, Maw M. et al. Mitochondrial mutation associated with nonsyndromic deafness. *Am J Otolaryngol*; **16**, 403–408 (1995).

9. Hutchin TP, Parker MJ, Young ID, Davis AC, Pulleyn LJ, Deeble J. A novel mutation in the mitochondrial tRNA(Ser(UCN)) gene in a family with non-syndromic sensorineural hearing impairment. *J Med Genet*. **37,** 692-694 (2000).

10. Li, R., Greinwald, J. H., Jr., Yang, L., Choo, D. I., Wenstrup, R. J. et al. Molecular analysis of the mitochondrial 12S rRNA and tRNASer(UCN) genes in paediatric subjects with

non-syndromic hearing loss. *Journal of Medical Genetics*. **41(8)**, 615-620 (2004).

11. Thyagarajan, D., Bressman, S., Bruno, C., Przedborski, S., Shanske, S. A novel mitochondrial 12SrRNA point mutation in parkinsonism, deafness, and neuropathy. *Annals of Neurology*. **48 (5),** 730-736 (2000).

12. Mutai H, Kouike H, Teruya E, Takahashi-Kodomari I, Kakishima H, Taiji H, et al. Systematic analysis of mitochondrial genes associated with hearing loss in the Japanese population: dHPLC reveals a new candidate mutation. *BMC Medical Genetics*. **12**, 135 (2011)

13. Bonneux S, Fransen E, Van Eyken E, Van Laer L, Huyghe J, Van de Heyning P, et al. Inherited mitochondrial variants are not a major cause of age-related hearing impairment in the European population. *Mitochondrion.* **11**, 729-34 (2011).

14. Zaragoza MV, Brandon MC, Diegoli M, Arbustini E, Wallace DC. Mitochondrial cardiomyopathies: how to identify candidate pathogenic mutations by mitochondrial DNA sequencing, MITOMASTER and phylogeny. *Eur J Hum Genet.* **19**, 200-7 (2011).

15. Leveque M, Marlin S, Jonard L, Procaccio V, Reynier P, Amati-Bonneau P, et al. Whole mitochondrial genome screening in maternally inherited non-syndromic hearing impairment using a microarray resequencing mitochondrial DNA chip. *Eur J Hum Genet,* **15**, 1145-1155. (2007)

16. Li, X., Fischel-Ghodsian, N., Schwartz, F., Yan, Q., Friedman, R. A., et al. Biochemical characterization of the mitochondrial tRNASer(UCN) T7511C mutation associated with nonsyndromic deafness. *Nucleic Acids Research*. **32**, 867-77 (2004).

17. Chamkha, I., Mkaouar-Rebai, E., Aloulou, H., Chabchoub, I., Kifagi, C., Fendri-Kriaa, N, et al. A novel m.3395A>G missense mutation in the mitochondrial ND1 gene associated with the new tRNA(Ile) m.4316A>G mutation in a patient with hypertrophic cardiomyopathy and profound hearing loss. *Biochem Biophys Res Commun*. **404**, 504-510 (2011).

18. Mkaouar-Rebai, E., Tlili, A., Masmoudi, S., Belguith, N., Charfeddine, I., Mnif, M, et al. Mutational analysis of the mitochondrial tRNALeu(UUR) gene in Tunisian patients with mitochondrial diseases. *Biochem Biophys Res Commun*. **355,** 1031-1037 (2007).

19. Chen, F. L., Liu, Y., Song, X. Y., Hu, H. Y., Xu, H. B., Zhang, X. M, et al. A novel mitochondrial DNA missense mutation at G3421A in a family with maternally inherited diabetes and deafness. *Mutation Research*. **602**, 26-33. (2006)

20. A.Pandya, X.J. Xia, R. Erdenetungalag, M. Amendola, B. Landa, J.Radnaabazar, B, et al. Heterogenous point mutations in the mitochondrial tRNASer(UCN) precursor coexisting with the A1555G mutation in deaf students from Mongolia. *Am. J. Hum. Genet*. **65,** 1803–1806 (1999).

21. Brown, M.D., Voljavec, A.S., Lott, M.T., Torroni, A., Yang, C.C., Wallace, D.C. Mitochondrial DNA complex I and III mutations associated with Leber's hereditary optic neuropathy. *Genetics*. **130 (1),** 163-173 (1992) .

22. Torroni A, Campos Y, Rengo C, Sellitto D, Achilli A, Magri C, et al. Mitochondrial DNA haplogroups do not play a role in the variable phenotypic presentation of the A3243G mutation. *Am J Hum Genet.* **72,** 1005-1012 (2003).

Titles and legends to figures

Figure 1 Clinical features of the proband carrying the homoplasmic 3595A>G variant

A: Family pedigree. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband.

B: Audiograms of the proband and mother.

C: Electropherogram depicting the 3595A>G sequence and its flanks.

Arrow indicates the position of the 3595A>G variant.

Figure 2 Clinical features of the proband carrying the homoplasmic 6204A>G variant

A: Family pedigree. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband.

B: Audiogram of the proband.

C: Electropherogram depicting the 6204A>G sequence and its flanks.

Arrow indicates the position of the 6204A>G variant.

Table 1 "Comfirmed" mitochondrial mutations associated with sensorineural hearing loss found in this study

| Allele | Locus | Status * | Disease | Total (/394) | Cohort 1 (/254) | Cohort 2 (/140) | Control (/192) | Case | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Hearing characteristics | Progression of hearing loss | Tinnitus | Vertigo | Associated symptom | |
| C1494T | 12S rRNA | Confirmed | SNHL | 1 | - | 1 | - | High frequency | 1/1 | 1/1 | 0/1 | - | 5 |
| A1555G | 12S rRNA | Confirmed | SNHL | 23 | 23 | - | - | High frequency | 15/21 | 13/16 | 6/16 | - | 2 |
| A3243G | $tRNA^{Leu\ (UUR)}$ | Confirmed | SNHL/ DM/ FSGS/ Cardiac dysfunction | 11 | 11 | - | - | Flat | 10/10 | 6/10 | 6/10 | Diabetes mellitus (8/10) | 6 |
| T7511C | $tRNA^{Ser\ (UCN)}$ | Confirmed | SNHL | 3 | 3 | - | - | High frequency | 1/2 | 3/4 | 0/4 | - | 35 |
| | | | | | 37/254 (14.6%) | 1/140 (0.7%) | | | 27/34 | 23/31 | 12/31 | | |

*Based on the MITOMAP database; "Confirmed" status indicates that at least two or more independent laboratories have published reports on the pathogenicity of a specific mutation.

SNHL: sensorineural hearing loss, DM: siabetes mellitus, FSGS: focal segmental glomerulosclerosis

Table 2 Ambiguous-status mitochondrial substitutions associated with sensorineural hearing loss found in this study

| Allele | Locus | Status * | Disease | Total (/394) | Cohort 1 ( /254 ) | Cohort 2 (/140) | Control (/96) | Case | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Hearing characteristics | Progression of hearing loss | Tinnitus | Vertigo | Associated symptom | |
| C792T | 12S rRNA | Reported | SNHL | 1 | 1 | - | - | Flat | 1/1 | 1/1 | 1/1 | - | 28 |
| A827G | 12S rRNA | Conflicting reports | SNHL | 10 | 5 | 5 | 1 | High frequency | 4/11 | 6/11 | 2/11 | - | 10 |
| A856G | 12S rRNA | Reported | SNHL/ LHON/ AD | 3 | 3 | - | - | Flat | 1/1 | 1/1 | 1/1 | - | 29 |
| T961C | 12S rRNA | Unclear | SNHL/ LVNC | 3 | 3 | - | 2 | Profound | 1/1 | 1/1 | 1/1 | - | 30 |
| T1005C | 12S rRNA | Unclear | SNHL | 2 | 1 | 1 | 1 | Low frequency | 2/2 | 1/1 | 1/1 | - | 30 |
| T1095C | 12S rRNA | Unclear | SNHL | 1 | 1 | - | - | Flat | 1/1 | 1/1 | 1/1 | - | 11 |
| C1310T | 12S rRNA | Reported | SNHL | 3 | - | 3 | - | unknown | 1/3 | 0/3 | 0/3 | - | 31 |
| T3398C | ND1 | Reported | SNHL / DM/ HCM / GDM / LVNC / Cardiomyopathy | 1 | 1 | - | - | Profound | 1/1 | 1/1 | 0/1 | - | 32 |
| G3421A | ND2 | Reported | SNHL | 2 | 1 | 1 | - | Profound | 1/1 | 1/1 | 0/1 | - | 33 |
| T5628C | $tRNA^{Ala}$ | Reported | SNHL/ CPEO | 1 | 1 | - | 1 | Profound | 1/1 | 0/1 | 1/1 | - | 34 |
| A8108G | CO2 | Reported | SNHL | 1 | 1 | - | - | Low frequency | 1/1 | 1/1 | 1/1 | - | 36 |
| A8348G | $tRNA^{Lys}$ | Reported | SNHL/ Cardiomyopathy/ HT | 1 | - | 1 | - | Low frequency | 1/1 | 0/1 | 1/1 | - | 37 |
| G11696A | ND4 | Reported | SNHL/ LHON/ LDYT/ HT | 4 | - | 4 | 2 | Profound | 1/4 | 1/4 | 0/4 | - | 38 |
| A14693G | $tRNA^{Glu}$ | Reported | SNHL/ MELAS/ LHON/ HT | 1 | - | 1 | 1 | Profound | 0/1 | 0/1 | 0/1 | - | 39 |
| G15927A | $tRNA^{Thr}$ | Point mutation/ Polymorphism | SNHL/ MS | 4 | 1 | 3 | 4 | High frequency | 3/4 | 0/4 | 0/4 | - | 38 |
| | | | | | 19/254 (7.5%) | 19/140 (13.6%) | | | 20/34 | 15/33 | 10/33 | | |

*Based on the MITOMAP database; "Reported" status indicates that one or more reports have considered the mutation as possibly pathologic.

"Point mutation/Polymorphism" status indicates that some published reports have determined the mutation to be a non-pathogenic polymorphism.

SNHL: sensorineural hearing loss, LHON: Leber hereditary optic neuropathy, AD: Alzheimer's disease, LVNC: left ventricular noncompaction, MIDD:maternally inherited diabetes and deafness, DM: diabetes mellitus, MIDD: maternally inherited diabetes and deafness, FSGS: focal segmental glomerulosclerosis, HT: hypertension, LDYT: Leber's hereditary optic neuropathy and dystonia, MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes, MS: multiple sclerosis

Table 3. 10 novel mitochondrial SNPs

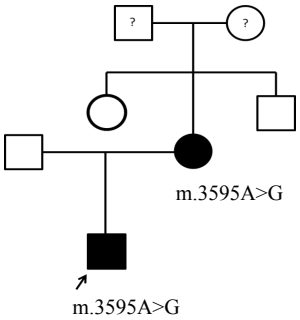
| Location | Mutation | Consevation rate (base) (/61) | Conservation rate (base) (%) | Amino acid change | Consevation rate (amino acid) (/61) | Conservation rate (amino acid) (%) | Amino acid number / all amino acid of locus | Control (/192) | Mode of inheritance | Type of hearing loss |
|---|---|---|---|---|---|---|---|---|---|---|
| 16S rRNA | 2069T>C | 16 | 31.4 | - | - | - | - | - | AD or Mit* | high frequency |
| 16S rRNA | 2285T>C | 22 | 43.1 | - | - | - | - | - | AD or Mit* | high frequency |
| 16S rRNA | 2285T>G | 22 | 43.1 | - | - | - | - | - | Sporadic | dish shaped |
| 16S rRNA | 2634T>C | 34 | 66.7 | - | - | - | - | - | Sporadic | profound |
| ND1 | 3595A>G | 54 | 88.5 | Asn>Asp | 54 | 88.5 | 97/318 | - | AD or Mit* | high frequency |
| COI | 6204A>G | 61 | 100 | Ser>Gly | 61 | 100 | 101/513 | - | AD or Mit* | high frequency |
| ATPase6 | 9124A>G | 60 | 98.4 | Thr>Ala | 59 | 96.7 | 200/226 | - | Sporadic | unilateral |
| ND4L | 10680G>A | 59 | 96.7 | Ala >Thr | 59 | 96.7 | 71/98 | - | Sporadic | unknown |
| ND5 | 13153A>G | 44 | 72.1 | Ile >Val | 35 | 57.4 | 273/603 | - | Sporadic | high frequency |
| Cytb | 15003G>C | 61 | 100 | Gly >Ala | 61 | 100 | 86/380 | - | Sporadic | profound |

*AD or Mit; autosomal dominant inheritance or maternal inheritance

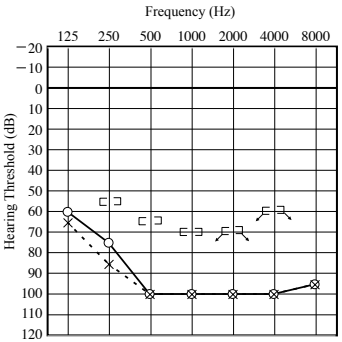
Table 4. Conservation rate of "Confirmed" mitochondrial mutations

| Location | Mutation | Consevation rate (base) (/61) | Conservation rate (base) (%) |
|---|---|---|---|
| 12S rRNA | 1494A>G | 61 | 100.0 |
| 12S rRNA | 1555A>G | 56 | 91.8 |
| tRNA $^{Leu\ (UUR)}$ | 3243A>G | 60 | 98.4 |
| tRNA $^{Leu\ (UUR)}$ | 3291T>C | 58 | 95.0 |
| tRNA $^{Ser\ (UCN)}$ | 7445A>G | 42 | 68.9 |
| tRNA $^{Ser\ (UCN)}$ | 7511T>C | 60 | 98.4 |
| tRNA $^{Lys}$ | 8363G>A | 49 | 80.3 |
| tRNA $^{His}$ | 12147G>A | 61 | 100.0 |
| tRNA $^{Glu}$ | 14709T>C | 58 | 95.0 |

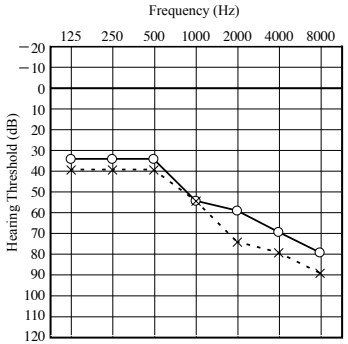
A




B



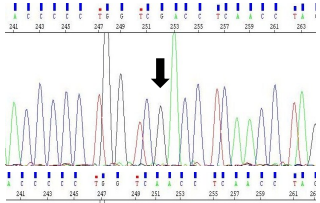

Mother's audiogram



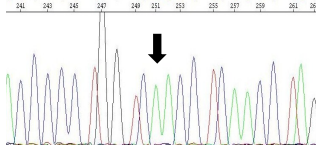

Proband's audiogram

C

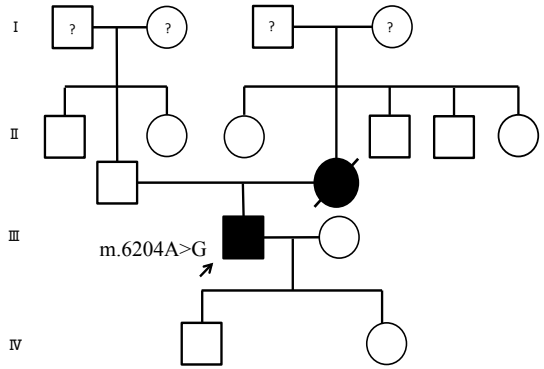
Mutation (m.3595A>G)



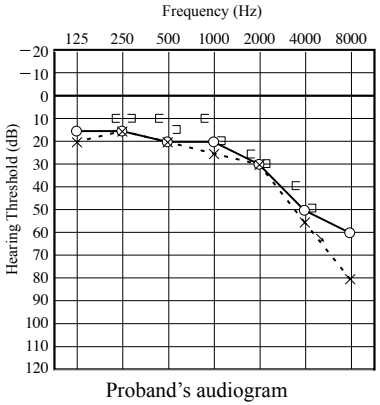
Wild Type



A




B




Proband's audiogram

C