ORIGINAL ARTICLE

Mitochondrial DNA alterations involving position 961 are not sufficient to explain sensorineural hearing loss

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OBJECTIVE: We present the results of screening for the m.961delTinsC(n) and m.961T>G alterations among children with SNHL in Turkey.

PATIENTS AND METHODS: We screened 210 unrelated Turkish children with congenital or prelingual-onset sensorineural nonsyndromic hearing loss for the mtDNA m.961delTinsC(n) and m.961T>G alterations, which are related to hearing loss.

RESULTS: One child with m.961delTinsC(n) and 2 children with m.961T>G were identified. Autosomal recessive (rather than mitochondrial) inheritance of the hearing impairment, with multiple affected sibs with normal hearing parents, was the most likely explanation in 2 of these families. There was only one affected child in the third family. Other autosomal recessive deafness genes or loci were not causative DNA changes in one family with the m.961delTinsC(n) mutation.

CONCLUSION: Because hearing subjects carry both changes, the two changes alone are not sufficient to cause deafness, although involvement of nuclear modifiers is also possible.

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Congenital or prelingual-onset sensorineural hearing loss [SNHL] affects at least 1 in 800 newborns, and genetic factors play causative roles in half of these children. There are more than 100 different candidate loci for SNHL in the nuclear genome. Mutations in the GJB2 gene at the DFNB1 locus are the most common genetic cause in many populations[1].

Several mutations in the mitochondrial DNA (mtDNA) resulting in SNHL have also been discovered[2]. The m.1555A>G mutation was the first identified change in the mitochondrial 12S rRNA gene, which causes SNHL with or without aminoglycoside ototoxicity[3]. Deletion of T961 and insertion of varying numbers of nucleotide C [the m.961delTinsC(n) mutation] was later described in the same gene, and was similarly proposed to cause aminoglycoside-induced deafness[4]. This mutation was initially found in 1 out of 35 sporadic Chinese individuals with histories of aminoglycoside-induced deafness, yet was negative in 799 controls[4]. It was later detected in an Italian family in which 5 maternally related individuals became deaf after aminoglycoside exposure[5]. An additional patient carrying this mutation and a history of aminoglycoside-induced deafness from Japan has recently been reported[6]. The m.961delTinsC(n) mutation was identified in 7 of 1,173 anonymized newborn screening samples in the United States[7], suggesting that its frequency is considerably higher in the US.

The m.961T>G alteration (T to G change at position 961 in mtDNA) was first described by Tang and colleagues[7] and detected in 6 of 1,173 newborn samples. It was later found in 5 of 164 children with SNHL without aminoglycoside exposure, but was negative in 226 hearing controls[8]. The latter study proposed that the m.961T>G alteration might have a role in the pathogenesis of hearing loss.

In this study, we present the results of screening for the m.961delTinsC(n) and m.961T>G alterations among children with SNHL in Turkey.

MATERIALS AND METHODS

This study included a total of 210 probands from unrelated families, including 100 females and 110 males ranging from 3 to 20 years (mean ± SD = 12.0 ± 4.4 years), who were diagnosed with severe to profound congenital or prelingual-onset SNHL. Syndromic forms of deafness were not included. Ninety one children were the only affected member in their families (simplex cases), whereas affected relatives were noted in the remaining 119 children (multiplex cases). Pedigrees suggested autosomal recessive or dominant inheritance patterns in 115 and 4 families, respectively. Exposure to aminoglycosides during infancy was noted in 3 sporadic children. There was no clear example of maternal (mitochondrial) inheritance. All samples had been earlier screened for the presence of mutations in the GJB2 and m.1555A>G mutation in the mtDNA 12S rRNA genes. The study group included 19 individuals with heterozygous c.35delG in GJB2 and 2 with homoplasmic (having the same genotype in all mitochondria) m.1555A>G mutations. Both probands with m.1555A>G were children and had histories of antibiotic exposure before the onset of hearing loss, although families did not specifically mention aminoglycosides. All other 189 samples were negative for these mutations. One hundred DNA samples of hearing subjects were studied as a control group.

The study was approved by the Ethics Committee of Ankara University in 2001 and the families were recruited between 2001 and 2004. After signed informed consent forms were obtained, anticoagulated blood samples were drawn and DNA was extracted using a standard phenol-chloroform method.

Previously described primers[7] were used to amplify the 438 bp region in the mitochondrial 12S rRNA gene, including position 961. The PCR products were digested with the Mnl1 restriction enzyme (New England BioLabs, Beverly, Mass, USA) [7]. Deletion of nucleotide T or a change from T to G at position 961 yields 362 bp and 76 bp bands. The wild-type PCR product results in 216 bp, 146 bp, and 76 bp bands. The results were visualized on a 2% ethidium bromide-stained agarose gel under UV light. When this initial study suggested a mutation in a sample, the PCR product was directly sequenced. Cycle sequencing kits (Beckman Coulter, Fullerton, Calif USA) were used in

cycle sequencing reactions following purification of the PCR products (PCR purification kit, Metis, Ankara, Turkey). Well-read attached sequence products were run on an automated sequencer (Beckman Coulter CEQ 2000XL) and analyzed using Beckman Coulter Software (Version 1.1).

The coding exons and surrounding regions of the previously cloned deafness genes, GJB6, GJB3, OTOF, and SLC26A4, in addition to GJB2, were screened in one family with the m.961delTinsC(n) mutation using polymerase chain reaction – single stranded conformational polymorphism protocols [10-13]. Primer sequences used in these studies are available on request. The PCR products were denatured at 95ºC, loaded on 7% nondenaturing polyacrylamide gels, run overnight at 4ºC with a vertical gel electrophoresis system (BioRad Protean II Xi Cell, Hercules, Calif, USA), and visualized with silver staining. Samples showing band changes were directly sequenced as previously described. The Δ (GJB6-D13S1830) deletion in the GJB6 gene was screened using previously described PCR protocols [9].

Microsatellite markers were used in the family with m.961delTinsC(n) to explore co-segregation of deafness with known recessive loci (Table 1). Following standard PCR reactions, PCR products were denatured, loaded on 6% denaturing polyacrylamide gels, and run on a vertical polyacrylamide gel system (Life Technologies Model S2001, Gibco BRL, Gaithersburg, MD, USA). Silver staining was used for visualization. Two reference DNA samples (from Centre du Etude Polymorphisme Humain families), whose genotypes had previously been determined, were run simultaneously to size the PCR products.

Since mutations in mtDNA modify the deafness phenotype associated with an mtDNA mutation at another site[14], the following mitochondrial mutations (in addition to m.1555A>G) were investigated in the family with m.961delTinsC(n): m.7445A>G, m.3243A>G, m.7510T>C, and m.7511T>C. Previously described PCR-RFLP [polymerase chain reaction- restriction fragment length polymorphism] methods were used[15-18].

Each subject underwent audiometric evaluation in a sound-treated booth using the Interacoustics AC-40 audiometer (Assens, Denmark) at 0.25, 0.5, 1, 2, 4, and 6 kHz using a down 10/up 5 paradigm .(American Speech-Language-Hearing Association, 1977) Middle ear pressure and stapedial reflex thresholds were also measured.

Categorical values between the groups were compared with X^2 analysis. Differences were considered to be significant if the p value was less than 0.05.

RESULTS

Three samples were positive for a change at position 961 in the initial screening with PCR-RFLP. There was no indication of heteroplasmy (mutant and wild-type mtDNA sequences were not detected in the same

Table 1. Used microsatellite markers for testing cosegregation of hearing loss with previously identified deafness loci

Locus	Gene (s)	Markers
DFNB1	GJB2, GJB6	D13S141, D13S250, D13S1275
DFNB3	MYO15	D7S122, D7S807
DFNB7	TMC1	D9S301, D9S166
DFNB8	TMPRSS3	D21S1225, D21S1259
DFNB9	OTOF	D2S158, D2S174, D2S2223, D2S2350
DFNB10	CDH23	D10S532, D10S580
DFNB18	USH1C	D11S527
DFNB21	TECTA	D11S925, D11S4107, D11S4111
Nuclear modifier for m.1555A>G		D8S277, D8S561, D8S1819

individual). None of the control samples was found to have screened DNA alterations. The differences in the frequency of the 961 sequence variations were not statistically significant. Two of the identified changes were the m.961T>G alteration (Figure 1a), and 1 sample was m.961delTinsC(n) (Figure 1b).

One proband with m.961T>G was a simplex case. She had bilateral profound hearing loss with normal middle ear pressures. She had 3 hearing siblings. The other proband with m.961T>G had a sibling with SNHL. The sibling was later demonstrated to have the m.961T>G change . Both siblings were also heterozygous for the c.35delG mutation in the GJB2 gene, but no other mutations in the coding exon of GJB2 or the Δ (GJB6-D13S1830) deletion was detected[9]. Both probands denied aminoglycoside exposure.

The family with the m.961delTinsC(n) alteration showed autosomal recessive inheritance of SNHL (Figure2).

The pure tone audiograms of evaluated family members are shown in Figure 3. Two affected children with the m.961delTinsC(n) mutation showed severe to profound sensorineural hearing loss (individuals V:37 and V:38). Measurements of middle ear pressures were normal.

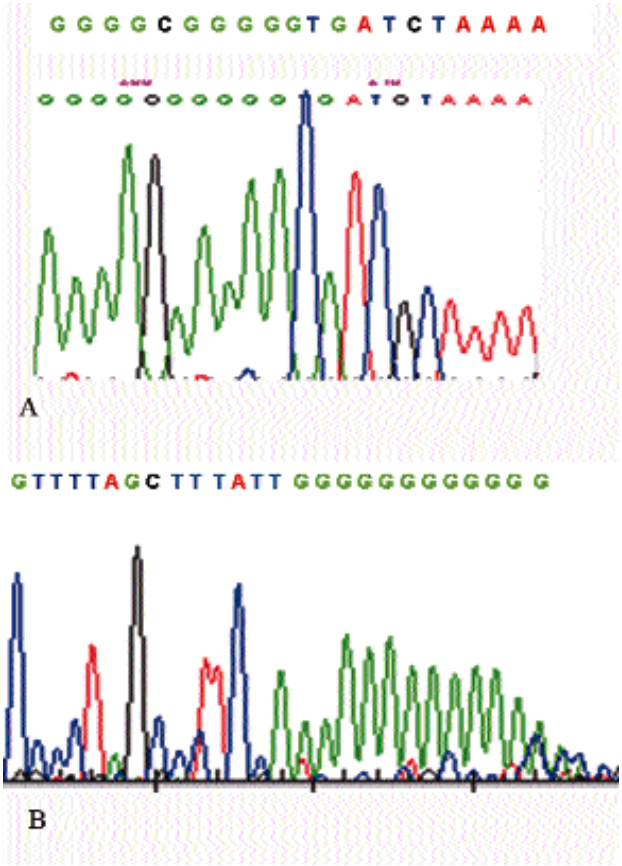

Figure 1-A) The m.961T>G change on a reverse sequence. The arrow indicates position 961; **B)** The m.961delTinsC(n) change on a reverse sequence. The arrow indicates position 961. The DNA sequence following the guanine residues is unreadable owing to variable number of inserted guanines.

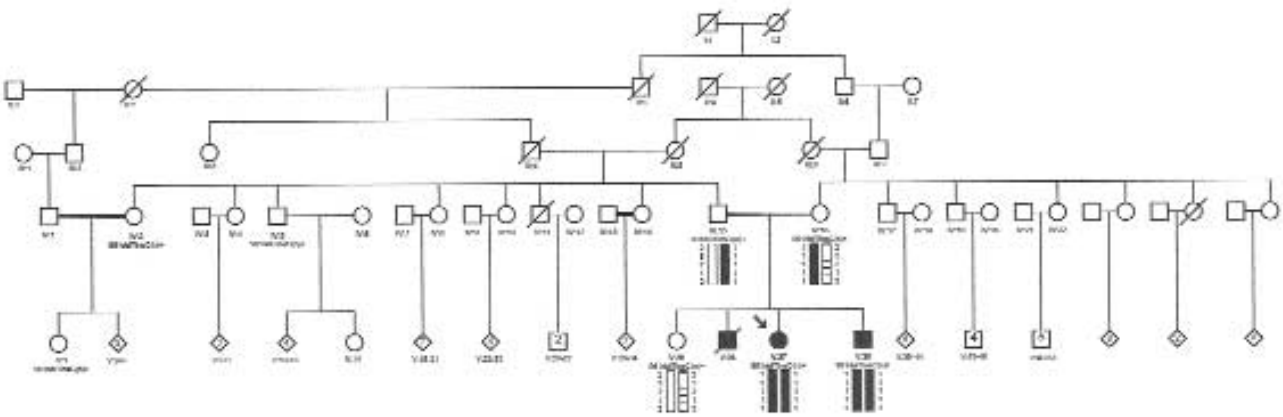

Figure 2: Pedigree of the family with the m.961delTinsC(n) change. The allele numbers in the OTOF locus are arbitrarily assigned. Markers, from top to bottom: D2S158, D2S2223, OTOF 5'UTR 3C/5C polymorphism, D2S2350, and D2S174. Closed circle and squares indicate deafness.

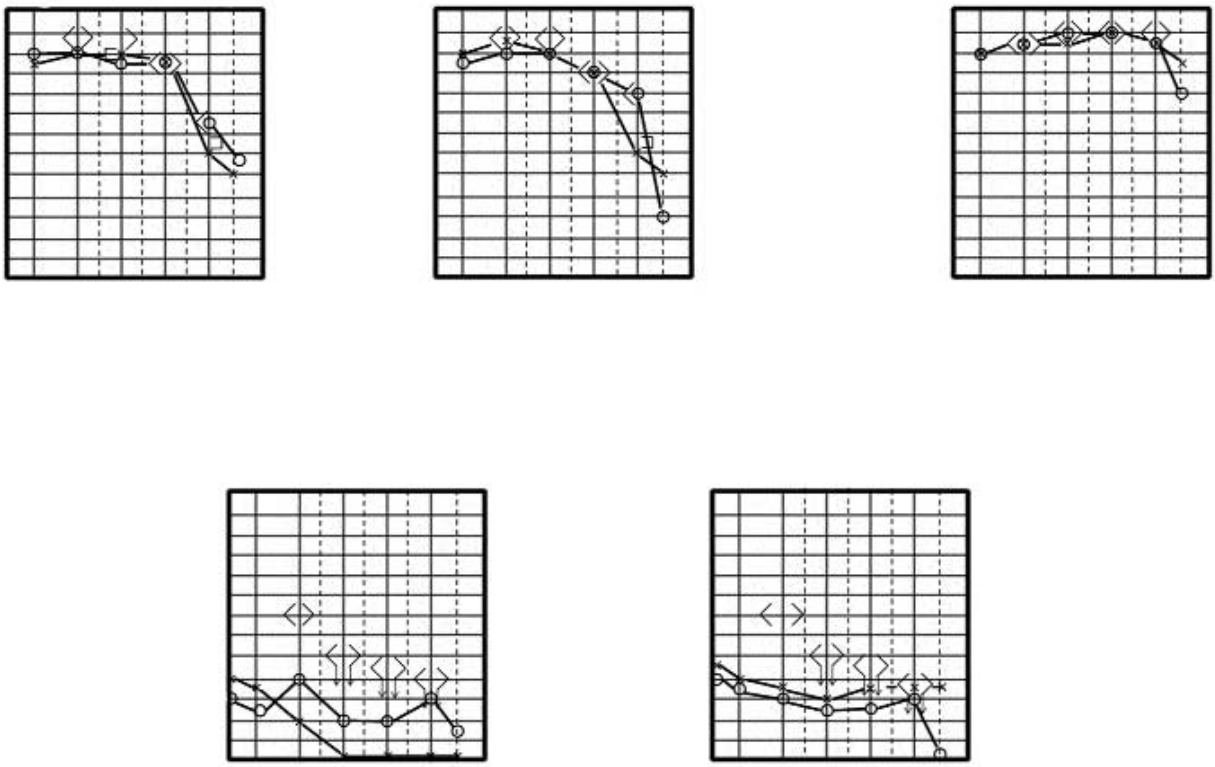

Figure 3: Pure tone audiograms of individuals IV:5 at 49 years (A), IV:15 at 39 years (B), IV:16 at 41 years (C), V:37 at 16 years (D), and V:38 at 8 years (E).

Two other mutation carriers in this family (individuals IV:5 and IV:15) had high-frequency sensorineural hearing loss without previous noise exposure. A normal audiogram was obtained in 1 member with the mutation (IV:6), and no problems with hearing were reported in 3 other carrier family members (IV:2, V:1, and V:35). Individuals II:5, III:5, and III:6 reportedly had no hearing problems, although they were inferred to carry the m.961delTinsC(n) mutation. Moreover, reportedly none of the inferred mutation carriers in generations IV and V, based on the maternal (mitochondrial) inheritance of m.961delTinsC(n), had hearing problems. Exposure to aminoglycosides was denied in all members studied. Mutations in the coding regions of the recessive deafness genes, GJB2, GJB3, GJB6, and SLC26A4 were excluded. There were no other mutations in the mtDNA (m.1555A>G, m.7445A>G, m.3243A>G, m.7510T>C, and m.7511T>C).

Deafness in this family did not cosegregate with screening markers for DFNB1(GJB2,GJB6), DFNB3 (MYO15), DFNB7/11 (TMC1), DFNB8/10 (TMPRSS3), DFNB12 (CDH23), DFNB18 (USH1C), or DFNB21 (TECTA) loci, or with the nuclear modifier locus for the mtDNA m.1555A>G mutation. Microsatellite markers for DFNB9 (OTOF) showed cosegregation in the nuclear family, and the 3C/5C polymorphism in the 5'-UTR of the OTOF gene (19) also segregated with deafness (Figure 2). Further screening of the coding exons and intron-exon boundaries of the OTOF gene did not reveal mutations.

DISCUSSION

The m.961T>G change was detected in 2 unrelated probands with SNHL in this study. However, its pathogenicity remains unclear. The first proband with this alteration did not have any relatives with hearing loss, although presumably she has maternal (mitochondrial) relatives with the same mtDNA change. The other proband had a sibling who was a carrier of the

same mutation and was also deaf. However, we found the c.35delG mutation in GJB2 in these 2 siblings, which is the most common genetic cause of recessive nonsyndromic SNHL in the Turkish population[13]. Although c.35delG was heterozygous, there was no other mutation in this gene. It has recently been shown that if a person with SNHL is found to have a heterozygous mutation in the GJB2 gene (which is not sufficient to explain the hearing loss in that person because heterozygotes have normal hearing), there is still a greater chance of having another GJB2-related, yet unidentified, DNA chance in that person[20].

Our study demonstrates, for the first time, the presence of the m.961delTinsC(n) change in a family with deafness not related to aminoglycoside exposure. Its absence in 100 controls suggests that it is not a common polymorphism in the Turkish population. Our pedigree with m.961delTinsC(n), although small, indicates autosomal recessive inheritance, with 3 affected children born to first-degree cousin hearing parents. Therefore, the most likely explanation for hearing loss in this family is the presence of an unrelated mutation in the nuclear DNA. The presence of m.961T>G or m.961delTinsC(n) in hearing subjects in this family demonstrates that this change alone cannot induce deafness. Based on the maternal inheritance of this mitochondrial change, we inferred that m.961delTinsC(n) was introduced into this family by individual II:5. There should be 70 carriers of this alteration, both detected and inferred, in generations II, III, IV, and V. However, congenital or prelingual SNHL is present in only 3 persons, V:36, V:37, and V:38. It is possible that the high frequency hearing loss in two carriers of m.961delTinsC(n) (individuals IV:5 and IV:15) is a mild manifestation of this mitochondrial alteration. This finding could also be simple presbycusis.

Nuclear genes modify hearing loss associated with mitochondrial DNA mutations. A contemporary example is the discovery of a putative modifier locus at chromosome 8, which modifies the deafness phenotype associated with the m.1555A>G mutation in families without histories of aminoglycoside exposure [21]. A similar mechanism may explain SNHL in our family with m.961delTinsC(n). During the search for a recessive mutation in the nuclear genome, we observed cosegregation of polymorphic markers for a locus including the OTOF gene. However, the size of the nuclear family did not allow us to obtain statistical significance; nor did we identify a mutation in the coding region of this gene. Therefore, it is not very likely that an alteration in OTOF plays a modifier role in this family for the hearing loss associated with the mtDNA m.961delTinsC(n) mutation.

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