Association of nuclear and mitochondrial genes with audiological examinations in Iranian patients with nonaminoglycoside antibiotics-induced hearing loss

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Abstract: Mitochondrial DNA mutations play an important role in causing sensorineural hearing loss. The purpose of this study was to determine the association of the mitochondrial genes *RNR1*, *MT-TL1*, and *ND1* as well as the nuclear genes *GJB2* and *GJB6* with audiological examinations in nonfamilial Iranians with cochlear implants, using polymerase chain reaction, DNA sequencing, and RNA secondary structure analysis. We found that there were no novel mutations in the mitochondrial gene *12S rRNA (MT-RNR1)* in patients with and without *GJB2* mutation (*GJB2*⁺ and *GJB2*⁻, respectively), but a total of six polymorphisms were found. No mutations were observed in *tRNA^{Leu(UUR)} (MT-TL1)*. Furthermore, eight polymorphisms were found in the mitochondrial *ND1* gene. Additionally, no mutations were observed in the nuclear *GJB6* gene in patients in the *GJB2*⁻ and *GJB2*⁺ groups. The speech intelligibility rating and category of auditory perception tests were statistically assessed in patients in the *GJB2*⁻ and *GJB2*⁺ groups. The results indicated that there was a significant difference ($P < 0.05$) between the categories of auditory perception score in the *GJB2*⁻ group compared to that in the *GJB2*⁺ group. Successful cochlear implantation was observed among individuals with *GJB2* mutations (*GJB2*⁺) and mitochondrial polymorphisms compared to those without *GJB2* mutations (*GJB2*⁻). In conclusion, the outcome of this study suggests that variation in the mitochondrial and nuclear genes may influence the penetrance of deafness. Therefore, further genetic and functional studies are required to help patients in making the best choice for cochlear implants.

Keywords: mitochondrial *12S rRNA (MT-RNR1)* gene, mitochondrial *tRNA^{Leu(UUR)} (MT-TL1)* gene, MT-ND1, nuclear *GJB2* and *GJB6* genes, cochlear implant, sensorineural hearing loss

Introduction

Hearing loss (HL) is one of the most common sensory disorders in humans affecting one to three of every 1,000 newborns.¹ The onset of HL usually occurs in childhood, is predominantly postlingual, and may be accompanied by vertigo² and tinnitus.^{3,4} There is a high variability in severity, ranging from normal hearing to profound deafness.^{5,6} This may be due to the fact that the phenotypic effects are a result of several factors and can develop gradually.⁷ HL occurs in both syndromic and nonsyndromic deafness caused by mitochondrial DNA (mtDNA) mutations,⁸ where both environmental and genetic factors are also involved, such as noise pollution, use of aminoglycoside drugs, and genomic diversity.⁹ mtDNA mutations are responsible for both maternally inherited syndromic and nonsyndromic HL and play a role in predisposition to aminoglycoside-induced ototoxicity.¹⁰ In Italy, at least 5% of cases of postlingual, non-syndromic hearing impairment may be attributed to mtDNA mutations.¹ Furthermore,

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it has been estimated that in up to 67% of patients with and without GJB2 mutations (GJB2⁺ and GJB2⁻, respectively), mtDNA disorders also manifest as sensorineural hearing loss (SNHL).¹¹ SNHL associated with mtDNA mutations is generally progressive with high frequency.^{12–15} This may be explained by the high oxidative phosphorylation demands in cochlear cells, as conveyed by mtDNA mutations.¹

Human mtDNA is a 16,569-bp, circular, double-stranded molecule that encodes 37 genes, including 13 subunits of the respiratory chain complexes, two ribosomal RNAs, and 22 transfer RNAs. Each nucleated human cell contains a few thousand copies of mtDNA. The somatic mutation rate of mtDNA is presumed to be 10–20 times higher than that of nuclear DNA (nDNA).¹⁶ Mitochondria are essentially double-membraned subcellular organelles present in all nucleated mammalian cells. Their primary function is to support aerobic respiration, that is, the production of adenosine triphosphate through oxidative phosphorylation.¹⁷ In addition, mutations and/or polymorphism variance in mitochondrial genes play important roles and are related to many diseases, such as Leber's hereditary optic neuropathy,¹⁸ Friedreich's ataxia,¹⁹ autism,²⁰ Alzheimer's disease,²¹ oculocutaneous albinism type 1,²² recurrent pregnancy loss,²³ and different cancers, such as gastric,^{24,25} bladder,²⁶ colorectal,²⁷ and breast.²⁴

HL is caused by genetic or nongenetic factors. The nongenetic risk factors for HL during the neonatal period include treatment in a neonatal intensive care unit, craniofacial anomalies, meningitis,^{28,29} and cytomegalovirus infections.³⁰ mtDNA variants, including mutations, deletions, and insertions, particularly in the *MT-RNR1* gene, have been identified to play an important role in patients with SNHL associated with or without a history of aminoglycoside therapy, suggesting that this locus in particular is a hotspot for deafness-associated mutations.^{31,32} The *MT-TL1* gene encoding mitochondrial *tRNA*^{Leu(UUR)} is a hotspot for pathogenic mtDNA mutations,³³ and a previous study reported the possible role of *MT-TL1* in nonsyndromic disease.⁹ Mutations in this region cause severe myopathy with respiratory insufficiency,^{33,34} as this region is highly conserved among mammals.³⁵ mtDNA mutations, in particular T3308C, have been identified to induce a significant decrease in the levels of the *MT-ND1* gene, suggesting that mutations in this region can increase the penetrance of deafness in patients with HL.^{1,36} Mutations in the nuclear *GJB2* and *GJB6* genes on the DFNB1 locus at chromosome 13q11-q12 are responsible for up to 50% of the most common causes of prelingual onset, recessively inherited nonsyndromic SNHL in humans, encoding the gap junction proteins connexin 26 (Cx26) and connexin 30, respectively,^{37,38} and play a role in cochlear

homeostasis.³⁹ Recessive mutations in the *GJB2* gene are the most common cause of hearing impairment⁴⁰ affecting both paternal and maternal alleles.^{40,41} Thus, in order to estimate the incidence ratio of mutation in the next generation, the frequency of the mutation is to be ascertained.^{41,42} The *GJB2* gene is the most common cause of the congenital HL,⁴³ and the mutation spectra are different among different ethnic groups.⁴² It is essential to investigate the carrier frequency and mutation spectrum of each genetic background in order to provide more precise genetic counseling. The *GJB2* gene encodes Cx26, a member of the connexin family of proteins that are constituents of the intercellular gap junction.⁴⁴ The first *GJB2* mutation was reported by Kelsell et al,⁴⁵ in which c.35delG is the most common mutation in the Caucasian population while c.235delC and p.Val37Ile are the most common mutations in the Asian population.^{42,46} In this study, we aimed to compare the impact of mutations in the mitochondrial *12S rRNA* (*MT-RNR1*), *tRNA*^{Leu(UUR)} (*MT-TL1*), and *ND1* on Iranian patients with nuclear *GJB2* mutation (GJB2⁺) and without GJB2 mutation (GJB2⁻) undergoing cochlear implants.

Materials and methods

Specimen collection and ethical statement

In this study, blood samples from 84 patients who had prelingual deafness with normal cochlear structures were obtained from the Rasoul Akram Hospital, Tehran, Iran. The patients did not show any syndromic symptoms or other clinical abnormalities, including muscular diseases, diabetes, visual dysfunction, or neurological disorders. Eighty-four patients with cochlear implant were categorized into two groups including 24 patients without *GJB2* (GJB2⁻) mutations and 60 patients with *GJB2* (GJB2⁺) mutations. Written informed consent, including consent to participate in this study and consent to submission and publish, was obtained from the parents on behalf of their children in accordance with the Medical Ethics Committee of Rasoul Akram Hospital, Tehran, Iran (Approval No 375/105/D/93).

Audiological examinations

Categories of auditory performance (CAP)⁴⁷ and speech intelligibility rating (SIR)⁴⁸ were used to assess hearing ability and speech intelligibility, respectively. The infants were screened at 2 months of age and were then referred to an audiologist after 2 years of age for audiometric tests, including CAP and SIR tests, where profound HLs (>90 dB) were identified in the patients. In the CAP test, the rating of auditory ability consists of the following eight categories:⁴⁷ Score 0 – no awareness of environmental sounds; Score 1 – awareness

of environmental sounds; Score 2 – response to speech sounds; Score 3 – recognition of environmental sounds; Score 4 – discrimination of at least two speech sounds; Score 5 – understanding of common phrases without lip reading; Score 6 – understanding of conversation without lip reading with a familiar talker; and Score 7 – use of a telephone with a familiar talker. The SIR test consists of five categories.⁴⁸ Scores 1–5 depend on the spoken ability rating scale criteria, which are as follows: 5 – connected speech is intelligible to all listeners and the child is understood easily in everyday contexts; 4 – connected speech is intelligible to a listener who has a little experience of a deaf person's speech; 3 – connected speech is intelligible to a listener who concentrates and lip-reads; 2 – connected speech is unintelligible, where intelligible speech is developing single words when context and lip-reading cues are available; and 1 – connected speech is unintelligible, where prerecognizable words in spoken language, the primary mode of communication, may be manual. All children received early intervention services within an average of 2 months after identification. This study was conducted in the Cochlear Implant Centre of the Hazrat Rasoul Akram Medical Complex, Tehran, Iran. The CAP and SIR assessments were conducted by two speech therapists during the follow-up period before implantation and at 6 months, 1 year, and 2 years after implantation. All 84 subjects had scores for both CAP and SIR before and after implantation. The children had to orally repeat the words/sentences they heard from the test conductor who produced the stimuli with his/her mouth covered (all the subjects were tested by the same conductor) and were scored based on the number of (key)words they correctly repeated. The answers were then recorded.

DNA extraction and polymerase chain reaction

Total DNA was extracted from the peripheral blood sample of each individual using a QIamap DNA Micro Kit (QIAGEN

no 56304). The DNA was amplified for the mitochondrial *12S rRNA*, *tRNA^{Leu(UUR)}*, and *ND1* genes (located near the *MT-TL1* gene) and also for the nuclear *GJB2* and *GJB6* genes, using specific primers (Table 1).

Briefly, polymerase chain reaction (PCR) was performed in 25 µL of reaction volumes containing 50–100 ng of genomic DNA, 2.5 µL of 10× PCR buffer, 10 mM of each dNTP, 1 mM of MgCl₂, 10 pmol of each primer, and 5 U of *Taq* polymerase (CinnaGen, Tehran, Iran) to a final volume of 25 µL, topped up using distilled water. The reactions were performed in a thermal cycler (Eppendorf, Hamburg, Germany), and the PCR products were examined on 1.5% agarose gel electrophoresis. The PCR conditions for amplification of the mitochondrial *12S rRNA* were as follows: initial DNA denaturation at 95°C for 5 minutes, then 35 cycles of denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute, followed by final extension at 72°C for 10 minutes. The PCR conditions for amplification of the mitochondrial *tRNA^{Leu(UUR)}* were as follows: initial DNA denaturation at 94°C for 5 minutes, then 35 cycles of denaturation at 94°C for 50 seconds, annealing at 55°C for 50 seconds, and extension at 72°C for 45 seconds, followed by final extension at 72°C for 10 minutes.

The primer sequences for the *GJB6* gene used in this study were in accordance with previous studies.^{49–51} However, neither mutations nor deletions have been identified in the *GJB6* gene in our patients. The PCR conditions for amplification of the nuclear *GJB6* gene were as follows: initial DNA denaturation at 95°C for 5 minutes, then 35 cycles of denaturation at 95°C for 1 minute, annealing at 58.5°C for 1 minute, and extension at 72°C for 1 minute, followed by final extension at 72°C for 10 minutes. The PCR conditions for amplification of the nuclear *GJB2* gene were performed in two steps, after initial DNA denaturation at 93°C for 3 minutes. The first step was done with five cycles of denaturation at 95°C for 1 minute, annealing at 59°C for 1 minute, and then final extension at 72°C for 1 minute.

Table 1 PCR primers of selected nuclear and mitochondrial genes

Name	Gene	Primer name	Primer sequences	TM (°C)	Amplicon size (bp)
<i>12S rRNA</i>	<i>MT-RNR1</i>	NG 78-F	F: 5'-CAGCACACACACACCGCTGC-3'	57.2	950
		NG 81-R	R: 5'-GCTAAGGTTGTCTGGTAGTA-3'		
<i>tRNA^{Leu(UUR)}</i>	<i>MT-TL1</i> ^a	ONP 82-F	F: 5'-CTCAACTTAGTATTATACCC-3'	57.2	300
		ONP 164-R	R: 5'-GATGGTGAGAGCTAAGGTCG-3'		
Connexin 26	<i>GJB2</i>	Cxn 26-F	F: 5'-CTCCCTGTTCTGTCCTAGCT-3'	59	800
		Cxn 26-R	R: 5'-CTCATCCCTCTCATGCTGTC-3'		
Connexin 30	<i>GJB6</i>	Cxn 30-F	F: 5'-CACTTCCCAAGGCCTCTTC-3'	58.5	850
		Cxn 30-R	R: 5'-GGTTGGTATTGCCTTCTGGA-3'		

Note:^a*ND1* gene is covered by *MT-TL1* primers.

Abbreviations: PCR, polymerase chain reaction; F, forward; R, reverse; TM, temperature.

The second step was carried out with 26 cycles of denaturation at 94°C for 45 seconds, annealing at 59°C for 45 seconds, and extension at 72°C for 45 seconds, followed by final extension at 72°C for 8 minutes.

DNA sequencing and analysis of variants

The PCR products were sequenced with the respective forward or reversed primers on an ABI 3700 sequencer (Takapo Zist Company, Tehran, Iran) and compared with the revised Cambridge Reference Sequence and the NCBI Reference Sequence Database (<http://www.ncbi.nlm.nih.gov/refseq/>) using the FinchTV program version 1.4.0. Identification of the nucleotide changes was verified through MITOMAP (<http://mitomap.org/MITOMAP>) and the Human Gene Mutation Database, 2007.

RNA secondary structure analysis

The RNA fold software from the Institute for Theoretical Chemistry, University of Vienna (rna@tbi.univie.ac.at;

<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>), was used to predict the RNA secondary structure based on minimum energy requirements and base pairing (Figures 1 and 2).

Statistical analysis

The independent *t*-test using Statistical Package for the Social Sciences, version 13, was used to analyze the relationship between CAP and SIR with cochlear implantation in the GJB2⁻ and GJB2⁺ groups; *P*-values <0.05 were regarded as statistically significant (Table 2).

Results

In this study, the *MT-RNR1* (*12sRNA*) and *MT-TL1* (*tRNA^{Leu(UUR)}*) genes were analyzed. Polymorphisms that were detected in *MT-RNR1* included G709A in eight patients (9.6%), T1243C in two patients (2.4%), T980C in three patients (3.6%), T1406C in one patient (1.2%), G930A in one patient (1.2%), and T1189C in three patients (3.6%). No mutation was found in the *MT-TL1* (*tRNA^{Leu(UUR)}*) gene.

Figure 1 (Continued)

Figure 1 (Continued)

Figure 1 Comparison of sequence analysis of the polymorphism in *MT-RNR1* and *MT-ND1* and heterozygous *GJB2* genes among patients with GJB2 mutation (GJB2⁺) and without GJB2 mutation (GJB2⁻).
Note: The arrows indicate the location of the nucleotide changes in wild type vs polymorphism.

Figure 2 (Continued)

Figure 2 Comparison of RNA secondary structure analysis of wild type and polymorphic predicted in *MT-RNR1* and *MT-ND1* genes among patients with GJB2 mutation (GJB2⁺) and without GJB2 mutation (GJB2⁻).
Note: The arrows indicate the location of the nucleotide changes based on RNA secondary structure analysis in wild type vs polymorphism.

Additionally, eight polymorphisms in the *MT-ND1* (NADH dehydrogenase I) gene were found, including A3360G (1.2%), A3505G (2.4%), A3339G (1.2%), G3337A (2.4%), G3392C (1.2%), G3483A (1.2%), A3480G (3.6%), and G3421A (2.4%). Here, all mtDNA mutations were homoplasmic, while only one – A3339G (1.2%) – was heteroplasmic mtDNA

ND1 polymorphism. Two GJB2⁺ patients with V153I and R184P did not show any mitochondrial RNR1 and ND1 mutations. In addition, all GJB2⁻ and GJB2⁺ patients revealed the presence of the A750G and A1438G polymorphisms in the *MT-RNR1* gene. Sequence analysis in the nuclear *GJB2* and *GJB6* genes demonstrated that no mutation in nuclear *GJB6*

Table 2 Statistical analysis of SIR and CAP in the GJB2⁻ and GJB2⁺ patients using independent *t*-test

Audiological examinations	Before cochlear implantation (mean ± SD)		After cochlear implantation (mean ± SD)		P-value
	GJB2 ⁻ patient (N=60)	GJB2 ⁻ patient (N=24)	GJB2 ⁺ patient (N=60)	GJB2 ⁺ patient (N=24)	
SIR	1±0	1±0	2.93±0.54	2.92±0.88	0.663
CAP	1±0	1±0	6.75±1.36	5.58±0.79	0.002*

Notes: There is a significant difference ($P<0.05$) between the CAP score of GJB2⁻ patients compared to GJB2⁺ patients, while there is no significant difference in the SIR score between GJB2⁻ and GJB2⁺ patients. The score of CAP in GJB2⁺ patients is higher than that of GJB2⁻ patients, which shows that this group comprises better candidates for cochlear implant than the other group. CAP and SIR assessments were done 2 years after cochlear implant. *Statistically significant ($P<0.05$).
Abbreviations: SIR, speech intelligibility rating; CAP, category of auditory performance; SD, standard deviation.

Table 3 Summary of clinical data and mitochondrial *RNR1* and *ND1* and nuclear *GJB2* and *GJB6* gene alterations

Category	Mutation	Homo/hetero	Gene	Before cochlear implant		After cochlear implant		Family history of HL	Sex		S/NS	GJB2 gene	GJB6 gene
				CAP		SIR			M, n (%)	F, n (%)			
GJB2 ⁺ patients	T980C	Homo	MT-RNR1	I	I	7	3	No	2 (2.4%)	1 (1.2%)	NS	wt	wt
	G709A	Homo	MT-RNR1	I	I	7	3	No	2 (2.4%)	1 (1.2%)	NS	wt	wt
	T1243C	Homo	MT-RNR1	I	I	7	3	No	1 (1.2%)	1 (1.2%)	NS	wt	wt
	T1406C	Homo	MT-RNR1	I	I	7	3	No	1 (1.2%)	–	NS	wt	wt
	A3339G	Hetero	MT-ND1	I	I	6	2	No	–	1 (1.2%)	NS	wt	wt
	A3505G	Homo	MT-ND1	I	I	7	3	No	1 (1.2%)	1 (1.2%)	NS	wt	wt
	G3337A	Homo	MT-ND1	I	I	3	2	No	1 (1.2%)	1 (1.2%)	NS	wt	wt
	G3392C	Homo	MT-ND1	I	I	8	1	No	–	1 (1.2%)	NS	wt	wt
	G3483A	Homo	MT-ND1	I	I	8	2	No	–	1 (1.2%)	NS	wt	wt
	A3360G	Homo	MT-ND1	I	I	3	2	No	–	1 (1.2%)	NS	wt	wt
	G930A	Homo	MT-RNR1	I	I	7	3	No	1 (1.2%)	–	NS	35delG hetero ^a	wt
GJB2 [−] patients	T1189C	Homo	MT-RNR1	I	I	8	3	No	2 (2.4%)	1 (1.2%)	NS	35delG hetero	wt
	A3480G	Homo	MT-ND1	I	I	6	3	No	2 (2.4%)	1 (1.2%)	NS	R143V hetero	wt
	G3421A	Homo	MT-ND1	I	I	6	3	No	2 (2.4%)	10 (12%)	NS	M163V hetero	wt
	–	–	–	I	I	6	3	No	10 (12%)	3 (3.6%)	NS	V153I hetero	wt
	–	–	–	I	I	6	3	No	1 (1.2%)	–	NS	R184P hetero	wt
	G709A	Homo	MT-RNR1	I	I	7	3	No	3 (3.6%)	2 (2.4%)	NS	hetero	G380A

Note: ^aOf the 60 patients with *GJB2* mutation (GJB2⁺), 31 patients indicated the 35delG heterozygous mutation, where only four patients showed MT-RNR1 mutations and the remaining 27 patients did not show any MT-ND1 or MT-RNR1 mutations.

Abbreviations: CAP, category of auditory performance; SIR, speech intelligibility rating; HL, hearing loss; M, male; F, female; S, syndromics; NS, nonsyndromics; wt, wild type; homo, homoplasmic; hetero, heteroplasmic.

Figure 3 Frequency of polymorphism *MT-RNR1* and *MT-ND1* genes in patients without GJB2 mutation (GJB2⁻) and patients with GJB2 mutation (GJB2⁺).
Notes: (A) *MT-ND1* gene and (B) *MT-RNR1* gene.

gene was detected in all GJB2⁻ and GJB2⁺ groups. Of the 84 individuals, no *GJB2* mutation was observed in 24 patients (GJB2⁻), but different types of *GJB2* mutations were observed among the 60 GJB2⁺ patients (Table 3; Figure 3).

Discussion

In this study, the impact of the mitochondrial *12S rRNA* (*MT-RNR1*), *tRNA^{Leu(UUR)}* (*MT-TL1*), and *ND1* variations among 84 random Iranian patients were compared between patients with GJB2 mutation (GJB2⁺) and without GJB2 mutation (GJB2⁻) undergoing cochlear implants, which had not been examined by earlier Iranian researchers. These hotspots are recognized to be among the most frequent causes of hearing impairment.⁵² Mitochondria play an important function in metabolism, thus mutations in mitochondrial genes result in many metabolic diseases.⁵³ In the present study, we analyzed three fragments of mtDNA, namely *12S rRNA* (*MT-RNR1*), *tRNA^{Leu(UUR)}* (*MT-TL1*), and *ND1*, and two fragments of nDNA, namely *GJB2* (Cx26) and *GJB6*, in 84 subjects with nonsyndromic HL. All patients (GJB2⁻ and GJB2⁺) were found to have the A750G and A1438G polymorphisms in the *MT-RNR1* gene, which is in agreement with previous studies that reported that the A750G, A1438G, A4769G, A8860G, and A15326G polymorphisms are common to mtDNA sequences from Africans, Asians, and Europeans.⁵⁴ Therefore, the polymorphisms in the *MT-RNR1* gene are not the cause of deafness and hearing impairment in Iranian patients. However, among the eight polymorphisms in the *MT-ND1* (NADH dehydrogenase I) gene, A3505G and A3480G are associated with prostate cancer, while G3337A and G3421A

are associated with cardiomyopathy⁵⁵ and pancreatic cancer,⁵⁶ respectively.

It should be noted that late-onset and gradual worsening of hearing impairment may reflect the tendency of the mitochondrion to accumulate mutations with aging, due to its genomic instability. As previously reported,⁵⁷ mitochondrial mutation plays an important role in cochlear implantation, whereas in our study, mitochondrial polymorphisms also indicate candidacy for cochlear implantation. Additionally, RNA secondary structure analysis demonstrated that the RNA at G930A, T1189C, and A3480G had different structures to compare the wild type, where it is possible that these polymorphisms and their correlation with the GJB2 mutation have more effect on deafness.

The association between mitochondrial and nuclear mutations with aminoglycoside exposure is quite variable, where aminoglycosides are probably only one of the factors interacting with the mutation in determining the deafness phenotype and play a role only in ~20% of patients.^{1,58–61} Several studies have reported that patients carrying the A1555G mutation exposed to aminoglycosides develop more severe deafness with an earlier onset.^{31,59,60,62–64} However, consistent with the other studies,^{58–61,65,66} our study showed that even in the absence of aminoglycoside exposure, mitochondrial polymorphisms could be responsible for hearing impairment. Previous studies revealed the mitochondrial m1555A>G,⁶⁷ m.1005T>C,⁶⁸ and m.921T>C⁶⁸ mutations among Iranian patients, while these mentioned mutations were not detected in our patients with HL. Additionally, a study indicated the high carrier frequency of the nuclear GJB2 mutation (35delG) in the north of Iran.⁶⁹

Conclusion

In conclusion, our finding indicates that a combination of polymorphisms in mitochondrial and nuclear genes may increase the penetrance of deafness and that there may be an association between nuclear gene variation and mitochondrial deafness with cochlear implant in Iranian patients. Therefore, the pathogenicity impact of mtDNA and nDNA variants and their correlation with other conditions should be established. Therefore, further genetic and functional studies are required in order to help individuals decide whether to undergo cochlear implantation.

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Author contributions

Conceived and designed the experiments: MH. Performed the experiments: MB. Analyzed the data and contributed the reagents/materials/analysis tools: MDA, MRH, HA, and MF. Wrote the manuscript, contributed to the discussion, and reviewed the article: BK and FA. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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