ONLINE MUTATION REPORT

Molecular and clinical characterisation of three Spanish families with maternally inherited non-syndromic hearing loss caused by the 1494C→T mutation in the mitochondrial 12S rRNA gene

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Mutations in the 12S rRNA gene of the mitochondrial genome are responsible for maternally inherited non-syndromic hearing loss (NSHL), and for increased susceptibility to the ototoxicity of aminoglycoside antibiotics. Among these mutations, 1555A→G is the most prevalent in all populations tested so far. Recently, the 1494C→T mutation was reported in two large Chinese pedigrees with maternally inherited NSHL. In this study, sequencing of the 12S rRNA gene in a Spanish family with maternally inherited NSHL showed the presence of the 1494C→T mutation. An additional screening of 1339 unrelated Spanish patients with NSHL allowed the authors to find two other families with the mutation. Audiological data were obtained from 17 confirmed 1494C→T carriers, which showed that the hearing loss was sensorineural, bilateral and symmetrical, with a remarkable variability in age of onset and severity. Three carriers were asymptomatic. Three affected carriers had a history of treatment with aminoglycoside antibiotics. The mitochondrial genome of one affected person from each of these three families was entirely sequenced, and it was established that they belong to different mitochondrial haplogroups (H, U5b, U6a). The study results further support the pathogenic role of 1494C→T on hearing, and show that this mutation can be found in different Caucasian mitochondrial DNA backgrounds.

Mutations in the 16 569-bp human mitochondrial DNA (mtDNA) result in a variety of diseases affecting many organs and tissues.[1 2] Among the different clinical presentations caused by mtDNA mutations, hearing loss is remarkable, as it can be part of defined syndromes such as mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS; MIM 540000), myoclonus epilepsy and ragged-red fibres (MERRF; MIM 545000), and maternally inherited diabetes mellitus and deafness (MIDD; MIM 520000), or it can constitute the only clinical sign (non-syndromic hearing loss). Mutations in the mtDNA resulting in non-syndromic hearing loss (NSHL) are clustered in the genes encoding the 12S rRNA and the $tRNA^{Ser(UCN)}$.[3–5]

Mutations affecting the 12S rRNA gene are responsible for NSHL and increased susceptibility to the ototoxicity of aminoglycoside antibiotics.[6 7] The spectrum of pathogenic sequence variants includes 961delT+C(n),[8–10] 1095T→C,[11–14] 1494C→T[15] and 1555A→G,[16] as well as other variants whose pathogenicity requires confirmation by further studies.[17 18] The 1555A→G mutation was the first to be described,[16] and since then it has been reported in many pedigrees, of different ethnicities, segregating NSHL with or without exposure to aminoglycoside antibiotics.[7] Remarkably, in Spain, this mutation may account for about 15–20% of all familial cases of NSHL, irrespective of their mode of inheritance and age of onset.[19 20] Recently, two large Chinese families with maternally inherited NSHL, including affected people with and without previous exposure to aminoglycosides, were reported to segregate the 1494C→T mutation.[15 21] This mutation had been found previously in a Chinese person whose mtDNA was entirely sequenced as part of a study on the East Asian mtDNA phylogeny.[22] Both 1555A→G and 1494C→T lie in the conserved decoding region of the 12S rRNA, and they are structurally equivalent. Furthermore, biochemical studies support the pathogenic potential of 1494C→T.[23] However, no families of other ethnicities with this novel mutation have been reported so far. Here, we provide further evidence for the pathogenicity of the 1494C→T mutation, through the clinical and molecular characterisation of three Spanish families with maternally inherited hearing loss, segregating the 1494C→T mutation in different mitochondrial genetic backgrounds.

PATIENTS AND METHODS

A total of 1340 unrelated Spanish patients with NSHL were enrolled in this study. They included 835 multiplex families (with two or more affected patients) and 505 simplex families (with only one affected patient). After obtaining written informed consent, peripheral blood samples were obtained from all participants. DNA extraction was carried out by standard procedures.

Screening for mutations in the DFNB1 locus was carried out as described previously.[24 25] A specific detection test for the 1555A→G mutation was carried out as previously reported.[20] Sequencing of the entire mtDNA was carried out on 24 overlapping segments as described elsewhere.[26] The entire mtDNA was sequenced on both strands in one proband from each family. Regions containing putative novel polymorphisms were sequenced again on both strands from different members of the same family, to exclude that they were polymerase chain reaction (PCR) artefacts. Sequences were screened for mutations by eye and by using the ChromasPro 1.33 software. The screening of people with

Abbreviations: rCRS, revised Cambridge reference sequence; MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonus epilepsy and ragged-red fibres; MIDD, maternally inherited diabetes mellitus and deafness; mtDNA, mitochondrial DNA; NSHL, non-syndromic hearing loss; PCR, polymerase chain reaction

normal hearing for polymorphisms at positions 4769, 8860 and 15326 was carried out by digestion with restriction endonucleases *Alu*I and *Hpy*CH4III, and by DNA sequencing, respectively, of PCR fragments obtained with primers already reported.[26]

Specific amplifications of the 1494C and 1494T alleles were carried out in two parallel multiplex PCR reactions by using four primers in each reaction, as follows: 5′-CGCCGCAGGCCCCTTCGCCC-3′ combined with 5′-GACGGGTTGGGCCAGGGGATTA-3′ for amplification of a 543-bp mtDNA segment (positions 3951–4493) as a control of the efficiency of the reaction; and 5′-AGGTTTAGCTCAGAGCGGTCAAG-3′, combined with either 5′-GTACACACCGCCCGTGACC-3′ or 5′-GTACACACCGCCCGTGACT-3′ for specific amplification of the 1494C and 1494T alleles, respectively (204-bp fragments). These reverse primers differ only in the mutated nucleotide at their 3′-end. We introduced a C→G change in the sequence of these reverse primers (the modified nucleotide is underlined), to produce an additional mismatch that contributes to increase the allele specificity of the reaction. PCR was carried out in a Perkin–Elmer GeneAmp PCR System 9700, using the following programme: one cycle of denaturation at 95℃ for 5 min; five touch-down cycles of denaturation at 94℃ for 40 s, annealing for 40 s at 73℃ for the first cycle and a 1℃ reduction per cycle; 25 cycles of denaturation at 94℃ for 40 s and annealing at 68℃ for 40 s; and a final extension step of 72℃ for 7 min. The reaction was carried out in a final volume of 15 µl, at final concentrations of 1 or 2.5 mM magnesium chloride for the 1494C and 1494T alleles, respectively, using Fast Start Taq DNA polymerase (Roche, Basel, Switzerland). PCR products were separated by agarose gel electrophoresis (1.5% agarose gels; fig 1).

RESULTS

A Spanish family (S372) segregating hearing loss with a pattern highly suggestive of matrilineal inheritance (fig 2) was ascertained through the ENT Department, Hospital Ramón y Cajal, Madrid, Spain. Once the presence of the 1555A→G mutation was excluded, the genes for the mitochondrial 12S rRNA and $tRNA^{Ser(UCN)}$ were sequenced, showing the 1494C→T mutation. Subsequently, the presence of the mtDNA 1494C→T mutation was investigated in a cohort of 834 multiplex (familial) and 505 simplex (sporadic) unrelated Spanish families with NSHL, by testing one proband from each family by allele-specific PCR. We detected the mutation in two of the familial cases (S003 and S422) and in none of the simplex cases. The 1494C→T mutation in the two families was confirmed by DNA sequencing. The mutation was not found in 894 Spanish controls with normal hearing. The presence of mutations in the DFNB1 locus, a common cause of non-syndromic hearing impairment, was excluded in the three families carrying the 1494C→T mutation.

The pattern of transmission of NSHL was consistent with maternal inheritance in all three families (fig 2). In total, 20 people were confirmed to be 1494C→T carriers. In all of them, the mutation was homoplasmic, considering the detection limits of our allele-specific PCR assay (>99% mutant copies). Audiological data were obtained from 17 confirmed carriers. Auditory impairment was observed in 14 carriers, whereas 3 other carriers had normal hearing. Interestingly, all asymptomatic carriers belonged to family S003 and they were younger than 22 years old, suggesting an age-dependent penetrance of the 1494C→T mutation at least in this family. In affected patients, no syndromic features were observed. Three patients from family S003 (II:3, III:4 and III:5) had been treated with streptomycin in early childhood. Another patient (S372-III:3) had pneumonia shortly after birth, and received treatment with antibiotics whose identity could not be established, but they probably included aminoglycosides. In these four patients, the onset of the hearing impairment coincided with the treatment. Environmental factors were excluded as causes of hearing loss in the remaining affected patients. Conductive hearing loss was ruled out by otoscopic examination, tympanometry with acoustic reflex testing and use of the tuning fork tests. Pure-tone audiometry, testing for air and bone conductions in frequencies 125–8000 Hz, showed that the hearing impairment was sensorineural, bilateral and symmetrical in all cases, with more severe hearing losses in the high frequencies (fig 3). Four people maintained normal hearing for low and middle frequencies. In the 10 affected people, apparently not exposed to aminoglycosides, onset was postlingual, ranging from childhood (family S422, patients II:3, II:7 and II:11) to the fourth decade (family S422, patients I:2 and II:5), or even the sixth decade (patient S003-II:1), where it cannot be distinguished from presbycusis. Most of the patients referred progression of their hearing loss, but this was confirmed by serial audiograms in only two of them. Severity ranged from moderate to profound. No symptoms of vestibular dysfunction were noticed. This remarkable intrafamilial and interfamilial phenotypic variation is often observed in mitochondrial NSHL.[4 5]

To investigate the role of mitochondrial haplotypes in the phenotypic manifestation of the 1494C→T mutation, we sequenced the entire mtDNA of one carrier from each of the

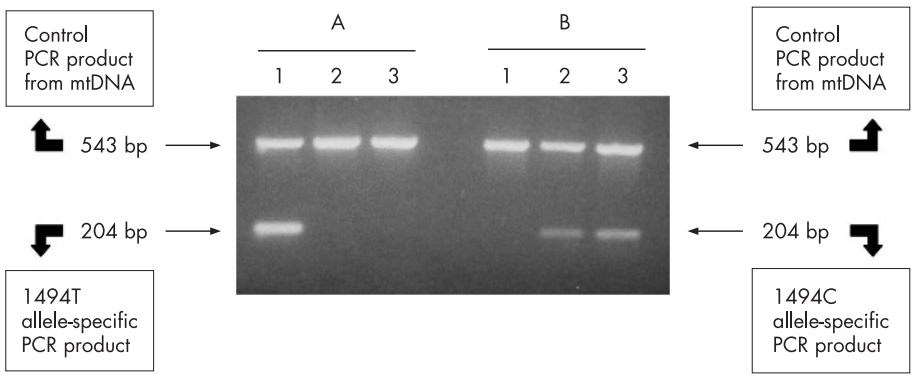

Figure 1 Allele-specific polymerase chain reaction (PCR) for the 1494C and 1494T sequence variants in the mitochondrial DNA (mtDNA). PCR fragments were separated by electrophoresis in a 1.5% agarose gel. (A) 1494T-specific PCR; (B) 1494C-specific PCR. Sample 1, 1494C→T carrier; samples 2 and 3 are wild type.

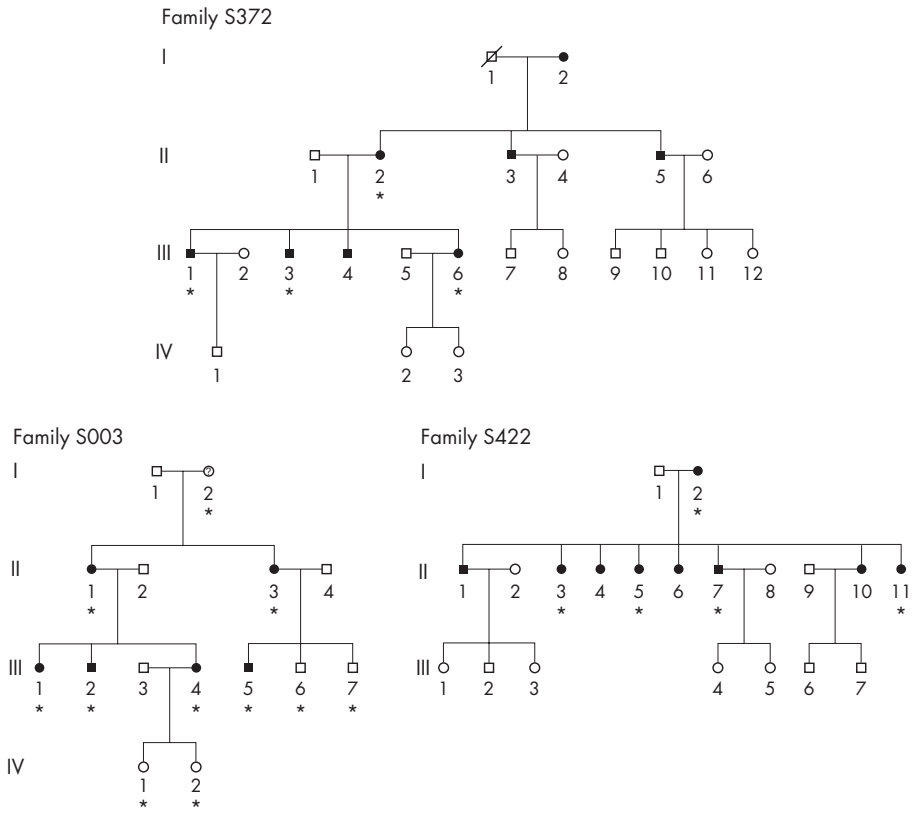

Figure 2 Pedigrees of Spanish families S003, S372 and S422, segregating non-syndromic hearing loss (NSHL) and the 1494C→T mitochondrial DNA (mtDNA) mutation. Asterisks indicate confirmed carriers of the 1494C→T mutation. A question mark inside a symbol is used to represent people whose clinical status is unknown.

three families. Comparison of these sequences with the revised Cambridge reference sequence (rCRS)[27] showed, in addition to 1494C→T, a total of 32, 25 and 17 variants in families S003, S372 and S422, respectively, all but one homoplasmic (table 1). They included three novel polymorphisms (table 1).[28 29] The analysis of these sequence variants allowed us to classify these mtDNA into mitochondrial haplogroups.[30 31] Family S003 carries the 3348G, 11467G, 12308G, 12372A, 16172C and 16219G diagnostic sequence variants, which are characteristic of haplogroup U6, as well as 7805A and 14179G, which allow further classification into haplogroup U6a.[31] Family S372 carries 3197C, 9477A, 11467G, 12308G, 12372A, 13617C and 16270T, which are indicative of haplogroup U5, as well as 150T, 7768G, and 14182C, which allow further classification into haplogroup U5b.[31] Family S422 carries 14766C and 7028C, characteristic of haplogroup H. Therefore, the mtDNAs of these three novel families with the 1494C→T mutation belong to different mitochondrial haplogroups, which are also different from those of the two Chinese families previously reported (haplogroup A). The three Spanish and two Chinese families carrying 1494C→T share only three other sequence variants in their mtDNAs with respect to the rCRS, 4769G, 8860G and 15326G. These variants are shared by most of the mtDNAs all over the world, as the rCRS carries rare polymorphisms in those positions. We determined their frequencies in 52 unrelated Spanish people with normal hearing. As expected, the 4769G–8860G–15326G haplotype was found in 50 of the 52 controls (96.15%).

DISCUSSION

Several mutations in the mitochondrial 12S rRNA gene have been associated with an increased susceptibility to the ototoxicity of aminoglycoside antibiotics, but a molecular explanation of this effect has been proposed only for 1555A→G and 1494C→T.[6 7] The nucleotides in the equivalent positions in the homologous bacterial 16S rRNA have been shown to interact with the aminoglycoside molecule, and mutations of these nucleotides result in bacterial resistance to these antibiotics. Mutations 1555A→G and 1494C→T allow the formation of an additional base pair (C–G and U–A, respectively) in a stem structure in the conserved decoding region of the 12S rRNA.[7] This new base pair makes the secondary structure of this rRNA more closely resemble the homologous region in the bacterial 16S rRNA; hence, it facilitates the binding of aminoglycosides, as shown in vitro.[23 32]

The 1555A→G mutation was first described in 1993,[16] and since then it has been reported in many families all over the world, with hearing loss associated with or without exposure to aminoglycoside. By contrast, the 1494C→T mutation was first reported to be associated with hearing loss in a Chinese family in 2004,[15] and only recently has another report with the mutation been published, also of a Chinese family and belonging to the same mtDNA haplogroup A.[21] In this work, the report of three Spanish families segregating maternally inherited non-syndromic or aminoglycoside-induced hearing loss, and carrying 1494C→T in different mtDNA genetic backgrounds (haplogroups H, U5b and U6a), further supports the pathogenicity of this mutation.

In the absence of exposure to aminoglycoside, both 1555A→G and 1494C→T have weak in vivo pathogenic potentials, as most of the of non-exposed carriers show moderate hearing losses, and both mutations are found in homoplasmy in affected patients, except for a few cases carrying 1555A→G in heteroplasmy.[20] Both mutations result

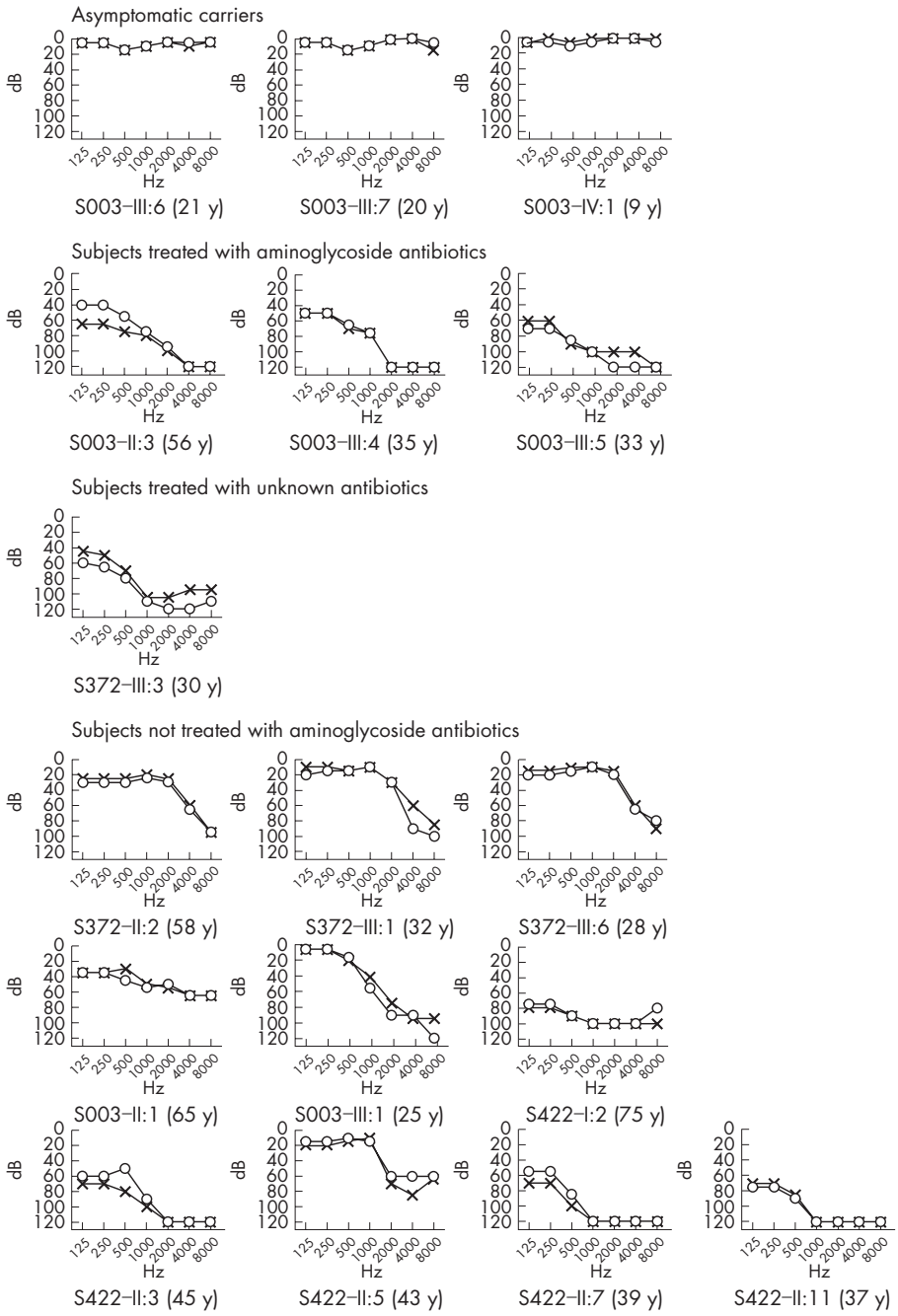

Figure 3 Air conduction audiograms (hearing level in dB *v* frequency in Hz) of 17 carriers of the 1494C→T mutation from families S003, S372 and S422. All these audiograms were obtained during this study, and hence represent the most recent record for each patient, whose age is indicated in years (y). Circles, right ear; crosses, left ear.

in a wide variability in age of onset and severity of the hearing loss, which indicates that other genetic or environmental factors must be involved in their phenotypic manifestation. Interestingly, none of the three Spanish families carry the 961insC sequence variant, which was hypothesised to play a role in the phenotypic manifestation of 1494C→T in the two Chinese families.[15 21] Our data suggest that the role of *cis*-acting genetic factors in the modulation of the phenotype caused by the 1494C→T mutation, if any, would be minor. On the contrary, our data further support the hypothesis of the involvement of nuclear modifiers in the phenotypic manifestation of this mutation.

The global prevalence of pathogenic mtDNA sequence variants among patients with NSHL is about 5% in most populations, and only shows higher figures, up to 20%, in populations highly exposed to aminoglycoside antibiotics, as in China and Spain.[4 5] This effect, however, seems to be mainly due to the high prevalence of the 1555A→G mutation among patients with hearing impairment in China and Spain. In our study, 1494C→T was found in only 0.3% of the familial cases, and in none of the sporadic cases in our Spanish cohort. Further studies are needed to establish the prevalence of this mutation in other populations. As the three Spanish families belong to different mtDNA haplogroups, the 1494 C→T mutation in the Spanish population would have multiple origins, as also occurs with the 1555A→G mutation.[33]

The identification of the genetic causes of hearing impairment has not yet resulted in specific treatments. Mutations in the mitochondrial 12S rRNA gene result in an increased susceptibility to aminoglycoside ototoxicity, and also in a hearing loss of variable onset, but mostly

Table 1 Sequence variants found in the mitochondrial DNA (mtDNA) of 1494C→T carriers

	Spanish family		
Gene	**S003**	**S372**	**S422**
12S rRNA	1438A→G	1438A→G	1438A→G
	1472G→A*		
	1494C→T	**1494C→T**	**1494C→T**
16S rRNA	2706A→G	2706A→G	
		3197T→C	
ND1	3348A→G		
		3507C→A*	
		(Thr67Asn)†	
	3969C→T		
ND2	4769A→G	4769A→G	4769A→G
tRNA-Ala			5618T→C*
CO1	7028C→T	7028C→T	
CO2		7768A→G	
	7805G→A		
ATP6			8723G→A
			8812A→G
	8860A→G	8860A→G	8860A→G
	8994G→A		
CO3		9477G→A	
ND4L			10707T→G
ND4	11467A→G	11467A→G	
	11719G→A	11719G→A	
tRNA-Leu(CUN)	12308A→G	12308A→G	
ND5	12372G→A	12372G→A	
		13617T→C	
	13759G→A		
ND6	14179A→G		
		14182T→C	
CytB	14766C→T	14766C→T	
	14927A→G		
	15217G→A		
	15326A→G	15326A→G	15326A→G
DNA loop			16093T→C
	16108C→T		
	16111C→T		
	16172T→C		
	16189T→C		
		16192C→T	
			16193C→T
	16219A→G		
			16258A→G
		16270C→T	
			16311T→C
		16319G→A	
	16519T→C		16519T→C
DNA loop	73A→G	73A→G	
		150C→T	
	152T→C		
			195T→C
	251G→A		
	263A→G	263A→G	263A→G
	309C→CCC		309C→CC
			309C→CCC
			heteroplasmic
	315C→CC	315C→CC	315C→CC
		533A→G	

*Novel mitochondrial DNA sequence variants found in this work.
†Conservative amino acid substitution.

postlingual. If genetic testing for these mutations is carried out before aminoglycoside administration, it would prevent an earlier onset and an immediate worsening of the hearing impairment in mutation carriers, a knowledge that should be translated into clinical practice.

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