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Non-Syndromic Deafness Associated with a Mutation and a Polymorphism in the Mitochondrial 12S Ribosomal RNA Gene in a Large Zairean Pedigree

Key Words

Mitochondrial DNA
Hereditary deafness
12S ribosomal RNA
Mutation
Polymorphism
Aminoglycosides

Abstract

Hereditary non-syndromic deafness has been associated with a point mutation in the mitochondrial 12S rRNA gene. We present data from deaf individuals in 12 nuclear families originating from a small village in Zaire. The patients have a sudden-onset and profound, bilateral sensorineural deafness with a highly variable age of onset. Inheritance is compatible with a mitochondrial DNA defect. Sequencing of the mitochondrial 12S rRNA gene revealed the presence of a homoplasmic 1555 A to G mutation in the patients and their normal siblings. The mutation is invariably associated with a T to C transition at 1420 in the same gene. Additional (mitochondrial or autosomal) genetic defect(s) or an environmental factor must be implicated in the expression of the defect. In Epstein-Barr-virus-transformed lymphocytes harbouring the normal or mutant mitochondrial DNA, no differential effect of aminoglycosides on protein translation was observed.

Introduction

Mitochondrial defects are often associated with sensorineural deafness [for a review see ref. 1]. Indeed, the cochlea and the neuronal conductance pathways of the inner ear appear to be very sensitive to reduced oxidative phosphorylation or to an altered energy metabolism. Two different mitochondrial mutations have been found to cause non-syndromic deafness. The first observed was a homoplasmic A to G mutation at position 1555 of the mitochondrial DNA (mtDNA) in pedigrees in the Far East with familial aminoglycoside-induced deafness [2–4] and in a single large Arab-Israeli pedigree with congenital, hereditary deafness [4–6]. This mutation is considered a ‘predisposing’ mutation because it causes deafness either in combination with aminoglycosides or with a second, nuclear defect. The probable nuclear defect, however, has

not yet been identified. A homoplasmic A to G mutation at position 7445 in the tRNA^{ser} gene may also be associated with cochlea-specific damage, as it was the most drastic alteration of the mtDNA in a single maternal pedigree with non-syndromic sensorineural deafness [7].

Here we report a series of families, all originating from a small village in Zaire, in which deafness affected several generations in the maternal lineage, suggestive of a mtDNA mutation.

Subjects, Materials and Methods

Subjects and Demography

Verbal tradition describes a profound, sudden-onset deafness which around 1954 suddenly fell as a curse on the members of a large family originating from a small village in Mayombe, in the south-west of Zaire. They are all descendants of a single female ancestor

who founded the village at least 150 years ago, according to verbal tradition. The deafness occurs both in inhabitants of the village and in their relatives living abroad. Samples and data from affected and normal members of 12 nuclear families were collected. At the same time, a limited population study was undertaken to assess the number of deaf inhabitants in this and in surrounding villages and to determine the environmental and demographic conditions in this isolated region. Control samples were obtained from a student population of mixed black ethnicity living in Kinshasa. Samples from Caucasian deaf children were obtained from the School for Deaf Children in Brugge, Belgium.

Mitochondrial 12S rRNA Sequence

The mitochondrial 12S rRNA gene was amplified from 300 ng total DNA with 25 pmol of each primer (5'-CCCCTCCATAC-TACTAATCTC-3' and 5'-biotin-CTCAGAGCGGTCAAGTTAAG-T-3'), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM dNTPs and 1U Taq DNA polymerase (Perkin Elmer/Cetus) in a volume of 100 μl, with an initial 5 min denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 70°C for 1 min. 50 μl PCR product were used for solid-phase dideoxy-sequencing on the Pharmacia ALF automated sequencer using 3 fluorescent primers to cover the entire 12S rRNA gene: 5'-FITC-CCTCAAAG-CAATACATG-3', 5'-FITC-CCAATAAAGCTAAAAGCTC-3' and 5'-FITC-AGCCTATATACCGCCATC-3'. The entire 12S rRNA gene was sequenced in 8 deaf and 1 non-deaf family member.

A dot blot system with specific oligomers (for the 1555 A to G mutation: 5'-CGAAGGTTGGATTTAGCA-3' and 5'-TCGAAG-GCGGATTAGC-3'; for the 1420 T to C mutation: 5'-ATAG-AGGAGACAAGTCGTA-3' and 5'-ATAGAGGAGGCAAGTCG-TA-3') was used to screen for both mutations in other family members, in 30 black control samples and in 30 unrelated Belgian children with non-syndromic deafness.

Nucleotide numbers refer to the 'Cambridge' sequence [8].

Mitochondrial D-Loop Sequencing

The two hypervariable regions SI and SII of the human mtDNA D-loop [9] were amplified by PCR using primers located in the adjacent non-variable regions (5'-CTCCACCATTAGCACCCAAAGC-3' and 5'-biotin-TGATTTACGGAGGATGGTG; and 5'-GTCC-TTTGTGCTACTGATCACAGGTCTATCACCTA (linked to a universal primer) and 5'-biotin-CTGTAAAAGTGCATACCCG-CA). The regions were sequenced as above, with the primers 5'-FITC-GCACCCAAAGCTAGATTC-3' and the universal primer, 5'-FITC-GTCCTTTGTCGATACTG-3', respectively.

Metabolic Labelling of Epstein-Barr-Virus (EBV)-Transformed Lymphocytes

EBV-transformed lymphocytes from non-deaf and deaf family members were grown in DME-F12 medium supplemented with 15% fetal calf serum but without any antibiotics, at 37°C under standard conditions. Mitochondrial protein synthesis was studied in intact cells. Approximately 10⁷ cells were resuspended in methionine-free medium with 100 μg/ml emetine and starved for 1 h at 37°C. The cells were pelleted, resuspended in 5 ml medium without methionine but with emetine and with 200 μCi L-³⁵S-methionine (NEN), in the presence or absence of 40 μg/ml chloramphenicol, 100 μg/ml neomycin, gentamicin or streptomycin (all chemicals were from Sigma). After 2 h labelling, mitochondria were isolated, essentially as described by Bourgeron et al. [10].

Samples containing 50 μg total protein were subjected to SDS-PAGE (15% acrylamide [37.5:1 bisacrylamide] and 0.1% SDS) in 200 mM Tris-HCl (pH 8.8) at 250 V for 4 h and at 4°C. Gels were treated for fluorography with 2,5-diphenyloxazole (PPO) in DMSO and exposed at -70°C for 2-3 days. Because the gel system differs slightly from those used by Ching and Attardi [11], no attempt was made to assign the different mitochondrial proteins to the respective bands.

Results

Family Data and Clinical Presentation

The village contains 53 deaf people among 348 inhabitants. Our 12 families containing descendants of the same matriline but most of whom never lived in the village, include 68 deaf family members (among 152 sibs in a biased sample). The slight preponderance of affected males in the village (34 versus 19 females) is not present in our families, and is probably caused by the traditional emigration of female rather than male sibs. The matriarchal society is typical for this region.

Figure 1 shows two representative pedigrees. In some families, all siblings from an affected mother are deaf (e.g. II-2 in family 1). However, in families 1 and 3 (fig. 1), not all children or siblings of deaf mothers are affected. On the other hand, a reportedly unaffected mother (II-2 in family 3, fig. 1), maternally related to deaf family members, has affected children. There is no paternal transmission of the deafness in any of the families.

A clinical evaluation was made of 25 family members, either deaf and/or maternally related to deaf individuals (details not shown). Twenty-one were reported deaf, 4 were considered normal. Audiometry in the patients indicated severe to profound sensorineural deafness of at least 40 dB at 0.25 kHz and 60 dB at 4 kHz. Audiograms were available from 2 non-deaf individuals: one member of family 3 (III-11, fig. 1), with a deaf mother and deaf siblings and born in 1950, had quasi-normal values (20 dB at 0.25 kHz, 35 dB at 4 kHz). The other, in family 7 (not shown), also born in 1950, with deaf siblings, maternal uncles and aunts, and with a diabetic mother, had a bilateral hearing loss of 50 dB at high frequencies. He is the single person in the entire sample with a record of streptomycin use. There is no audiometric data available from other reportedly normal family members. Physical examination of deaf subjects showed no major health problems. There is no evidence for any neuromuscular or central nervous problem possibly associated with mitochondrial disease. A congenitally deaf girl (family 3, IV-5, fig. 1) was born with jaundice and is mentally retarded. Diabetes was

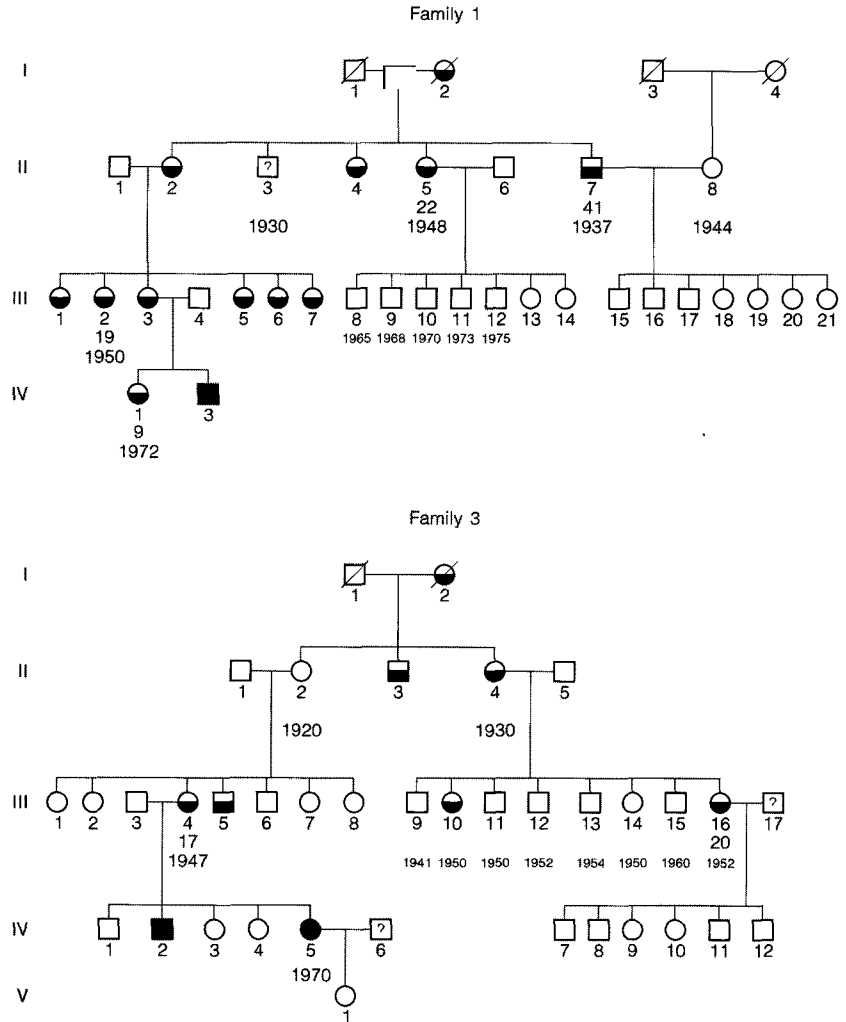


Fig. 1. Representative pedigrees of families with hereditary deafness. Filled symbols represent patients with congenital deafness, half-filled symbols stand for onset at a later age. The ? indicates that the disease status of the member is not certain. The age of onset and the birth year are given for a few members of these families. Patients I-2 in family 1 and I-2 in family 3 were deaf, but the age of onset is not known.

reported in one family member's mother (see above). Vision problems are reported by only two of 21 affected members.

Among the 21 deaf individuals in this group, 5 were congenitally deaf. Other patients had experienced a painless, sometimes rapidly progressive loss of hearing preceded by tinnitus in most cases. Six became deaf before the age of 15, 7 between 15 and 30 years of age, and 3 at still later ages (35, 39 and 41 years). The sample may not exactly reflect the actual distribution in the families. Of the 53 deaf inhabitants of the village, 15 were congenitally deaf, 5 before the age of 15, 7 between 15 and 30, and 17 after 30 years of age (no data on age of onset for 9 patients).

Sequence Analysis of 12S rRNA: A Homoplasmic Mutation at 1555 and a Novel Polymorphism at 1420

Sequencing the entire 12S rRNA gene revealed the presence of a number of deviations from the 'Cambridge' sequence (table 1). The 1555 A to G transition [4] was found in all deaf patients tested and in their mothers and siblings (if available). Of 32 family members with the mutation, 24 were deaf and 8 were reportedly normal (the group available for DNA analysis was biased towards deaf individuals and differed from the group from which full clinical data was available). A middle-aged 'carrier' of the mutation shows a decreased perception as documented by audiometry. The mutation was not found in the 30 black controls or in a sample of 30 Belgian deaf children.

	143	147	182	186	189	195	198	200	247	272	297	316	16074	16145	16187	16189	16209	16213	16234	16263	16265	16278	16286	16291	16292	16294	16295	16309	16311		
1555G/1420C	G	C	T	A	C	C	T	A	A	A	G	A	T	A	T	C	T	G	C	C	T	C	A	C							Deaf
1555A/1420C	G	C	T	A	C	C	T	A	A	A	G	A	C	A	T	C	T	G	C	C	T	C	A	C							Normal
1555A/1420T(1)	G	C	C	C	G	T	C	G	G	G	A	G	C	G	C	T	A	C	C	C	T	C	T	A	C						Normal
1555A/1420T(2)	A	C	C	C	A	C	C	A	G	A	A	G	C	G	C	T	T	G	C	C	A	T	C	T	C	G	T				Normal
Cambridge	G	C	C	C	A	T	C	A	G	A	A	G	C	G	C	T	T	G	C	T	A	C	C	C	C	C	A	T			Reference

Fig. 2. The two hypervariable regions of the mitochondrial D-loop were sequenced in a patient with both the 1555 and 1420 mutations, and in 3 normal individuals with or without the polymorphism at position 1420. The sequences are compared to the reference sequence obtained from Anderson et al. [8]. Only positions that differ between any of the sequences are shown, and their position in the mtDNA is given above the sequence.

Table 1. Mutations in the 12S ribosomal RNA gene in the Zairean pedigree

Position	Base change	Conservation ^a	Arab-Israeli pedigree ^b
750	(A->) G	A in B, M, R, X	present
769	(G->) A	A in B, no homologous sequence in M, R, or X	present
825	(T->) A	A in B, M, R, X	present
1018	(G->) A	A in B, M, R, X	present
1420	(T->) C	A in B, M R, X	absent
1438	(A->) G	A in B, M, R; G in X	present
1555	(A->) G	A in B, M, R; G in X	present

^a Nucleotide found at this location in bovine (B), mouse (M), rat (R) and *Xenopus* (X) 12S rRNA.

^b See Prezant et al. [4].

invariably present at 1420. This is probably a polymorphism, because it was also detected in one maternally unrelated female and in 1 of 30 Zairean controls. The 1420 mutation is phylogenetically less conserved than its neighbouring bases and the 1555 mutation [12].

Origin of the 1555 and 1420 Mutations

All affected members had identical D-loop sequences (fig. 2). A control individual, harbouring the 1420 but not the 1555 mutation (1555A/1420C), had a polymorphic sequence that differed from the 'deafness type' (1555A/1420T) but less than from other controls (fig. 2). The latter suggests that the 1555 mutation probably occurred in a mtDNA that already carried the 1420 mutation.

Hair-Cell-Specific Alterations in Mitochondrial Ribosomal Activities? Lack of Effect of Aminoglycosides on Protein Synthesis in Lymphocyte Cultures

It has been speculated that the 1555 mutation results in greater susceptibility to the effects of aminoglycosides on translational fidelity in the mitochondrial ribosome [2, 4, 6]. However, the effects of aminoglycosides on lymphocytic cells in culture have not been reported. Furthermore, it remained to be investigated whether the substitution at 1420 contributes to the ribosomal malfunction and the disease phenotype. EBV-transformed lymphocytes from deaf patients and from normal family members were metabolically labelled in the absence and presence of streptomycin, neomycin and gentamicin, and the mitochondrial translation products were examined on SDS-PAGE (fig. 3). There was no (differential) inhibition of protein synthesis in mitochondria of either deaf or

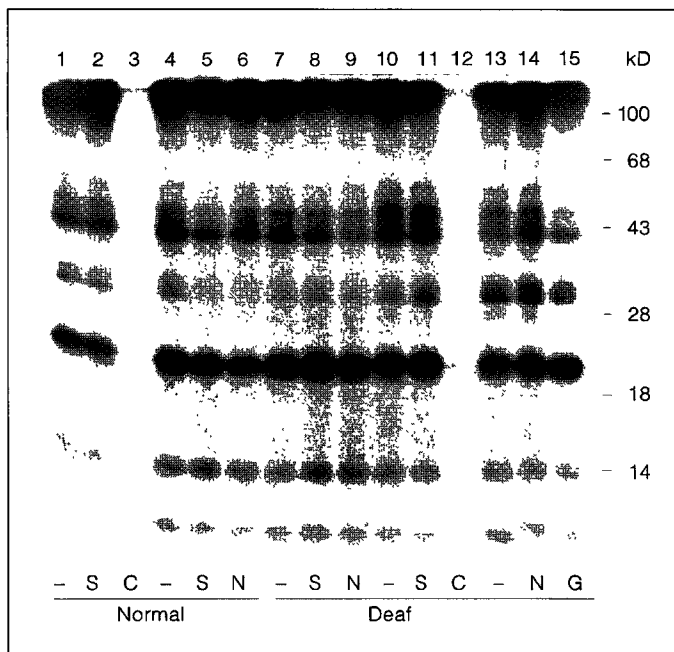


Fig. 3. Mitochondrial translation in EBV-transformed lymphocytes as assayed by electrophoresis of mitochondrial proteins synthesized in the presence of ^{35}S -L-methionine. Ten million cells were metabolically labelled for 2 h in the presence of 100 $\mu\text{g}/\text{ml}$ emetine, in the absence or presence of aminoglycosides. Proteins from the mitochondrial extract were separated on a 15% SDS-PAGE gel. Lanes 1–3 and 4–6 contain samples from two normal individuals (1555A, 1420T); lanes 7–9, 10–12 and 13–15 from deaf (1555G, 1420C) patients. Streptomycin (S, 100 $\mu\text{g}/\text{ml}$) was added in lanes 2, 5, 8 and 11, neomycin (N, 100 $\mu\text{g}/\text{ml}$) in lanes 6, 9 and 14, gentamicin (G, 100 $\mu\text{g}/\text{ml}$) in lane 15 and chloramphenicol (C, 50 $\mu\text{g}/\text{ml}$) in lanes 3 and 12. Protein size markers are indicated on the right.

normal people. Chloramphenicol completely inhibited translation in mitochondria from deaf and normal persons.

Discussion

A non-syndromic, bilateral symmetrical, sensorineural deafness, transmitted maternally, is present in 12 families belonging to the same large pedigree originating from a small village in south-west Zaire. The hearing loss results in an inability to understand speech. Prezant et al. [4] have previously identified an A to G substitution at position 1555 in the mitochondrial 12S rRNA gene associated with deafness, either in an early onset form with a postulated recessive nuclear defect for phenotypic expression, or in conjunction with an otherwise non-toxic dose of

aminoglycosides. The 1555 A to G transition is found in all deaf persons and their siblings tested in this study. However, the age of onset in this family is extremely variable, from congenital deafness in an estimated 25–35% of the cases to onset after the age of 40. Nevertheless, the 1555 mutation is homoplasmic in all maternally related members of this family.

Analysis of the mitochondrial D-loop sequence also allows us to suggest that the 1555 mutation occurred in a mtDNA that already carried the 1420 mutation. According to the verbal tradition in this family, four sisters founded, probably more than one and a half centuries ago, four maternal pedigrees with deafness associated with only one of them. Even though descendants of each of these four branches are said to be living today, due to the lack of a written genealogy, it will be hard to reconstruct the entire pedigree to find out where or when the mutation sneaked into the pedigree.

The mutation does not seem to cause deafness on its own. The 1420 mutation is not a candidate as it was also invariably homoplasmic in the Zairean family. To further rule out the possibility that another, heteroplasmic mitochondrial mutation causes the disease, scrutiny of the entire mitochondrial genome in affected and normal family members is required. However, the question was indirectly addressed by Prezant et al. [4] who found no heteroplasmy at any other position of the mtDNA in their Arab-Israeli pedigree.

The recent and coincident appearance of the deafness in this family – the first cases were reported at about the same time in the early 1950s – suggests an environmental factor. Mitochondrial inheritance requiring environmental factors for expression was shown for aminoglycoside-induced deafness. However, there has been no documented use of aminoglycosides in these patients except one. It is unlikely that the population in this region would have received antibiotics and other drugs on a large scale in the 1950s. Moreover, the homogenous environment in the village on the one hand, along with the occurrence of deafness in family members that have never lived in the village [for instance, family 3 (fig. 1) left the village at least three generations ago], would have required the introduction since the early fifties of a drug or other external factor on a large scale. If the mutation occurred in the founder mother about a century ago, it could have been enriched to homoplasmy within a few generations, which would explain the initial outburst during one generation.

Although consanguineous marriages in the matrilineal descent are considered incestuous in this matriarchal society, consanguinity in the paternal lineage is frequent (a

man commonly marries a niece of his father). We therefore favour the existence of a nuclear defect as a modifying factor in these Zairean families, but it is not clear whether a single dominant or recessive defect or a multi-genic defect would explain the inheritance pattern. The establishment of the entire pedigree is a prerequisite for a segregation analysis in this family. Jaber et al. [5] have analyzed the inheritance pattern in their family with mitochondrial deafness for a Mendelian genetic component and excluded an X-linked and an autosomal dominant defect. The segregation of the deafness in the Israeli-Arab pedigree is compatible with the existence of a recessive modifying gene [4, 5]. There is insufficient ascertainment in our families to make similar predictions.

The damaging effect is specific for the cochlea, because no major differences in mitochondrial protein synthesis were seen in lymphocytes from deaf and normal individuals [see also ref. 6]. Moreover, aminoglycosides did not detectably affect the electrophoretic pattern of in vivo labelled mitochondrial proteins. An interaction between the altered mitochondrial 12S ribosomal subunit and a cochlea-specific isoform of one of the nuclear encoded proteins with a role in mitochondrial translation or oxidative phosphorylation, could explain the selectively devastating effect of this mutation on hearing.

In summary, we have described here patients with a bilateral sensorineural deafness with an extremely variable age of onset. All affected members and their non-affected siblings carry a homoplasmic 1555 A to G muta-

tion and a previously undescribed polymorphism at position 1420 in the mitochondrial 12S rRNA gene. The inheritance pattern and the environmental conditions suggest the presence of an autosomal trait in conjunction with the mitochondrial defect, but there is insufficient ascertainment of nuclear families to further substantiate this hypothesis at the moment. Because of the 1555 mutation, the clinical implication for this family is that none of its members, maternally related to deaf patients, should use aminoglycosides.

Acknowledgements

We wish to thank M. Willems, G. Deferme and L. Mekers for establishing EBV-lymphocytes and skin fibroblast cultures, and R. Decorte and E. Jehaes for assistance in sequencing the D-loop variable regions. We are indebted to Prof. R. De Wachter for the alignments of 12S rRNA genes of about 50 species, and to Dr. M. Jorissen for commenting on the audiograms. We thank Dr. E. Legius and Prof. P. Marynen for critically reading the manuscript. Dr. L. Standaert from the Spermalie Instituut in Brugge collected the samples from deaf children for us. The help of many collaborators in Kinshasa and in Kai Singini, and the contribution of the families are gratefully acknowledged. The families have expressed a great wish towards the understanding and the cure of this mysterious disease. G.M. is a 'Post-doctoraal Onderzoeker' and S.C. an 'Aspirant' of the National Foundation for Scientific Research, Belgium. This investigation was supported by grant 'S2/5-ID. E103' of the Fonds voor Geneeskundig Wetenschappelijk Onderzoek, Belgium and by the Inter-University Network for Fundamental Research sponsored by the Belgian Government (1991-1996).

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