Mitochondrial 12S rRNA gene mutations affect RNA secondary structure and lead to variable penetrance in hearing impairment

Ester Ballana a, Estela Morales b, Raquel Rabionet a, Bàrbara Montserrat a, Marina Ventayol a, Olga Bravo c, Paolo Gasparini d, Xavier Estivill a,∗

a Genes and Disease Program, Centre for Genomic Regulation (CRG), Universitat Pompeu Fabra (UPF), Barcelona Biomedical Research Park, Barcelona, Catalonia, Spain
b Centro Nacional de Genética Médica, Ciudad de la Habana, Cuba
c Otolaryngology Department, Ciutat Sanitària i Universitària de Bellvitge, L’Hospitalet de Llobregat, Catalonia, Spain
d Telethon Institute of Genetics and Medicine, Naples, Italy

Received 9 January 2006
Available online 24 January 2006

Abstract

Mutations in the mitochondrial DNA are one of the most important causes of sensorineural hearing loss, especially in the 12S ribosomal RNA (rRNA) gene. We have analyzed the mtDNA 12S rRNA gene in a cohort of 443 families with hearing impairment, and have identified the A1555G mutation in 69 unrelated cases. A1555G is not a fully penetrant change, since only 63% of subjects with this change have developed hearing impairment. In addition, only 22% of the 183 A1555G deaf subjects were treated with aminoglycosides. Two novel nucleotide changes (T1291C and T1243C) were identified. T1243C was found in five deafness cases and one control sample. Mutation T1291C was detected in all maternally related individuals of a pedigree and in none of 95 control samples. Conservation analysis and comparison of the 12S rRNA structure with the 16S rRNA of Escherichia coli showed that the T at nucleotide 1243 and A at nucleotide 1555 are conserved positions. Prediction of RNA secondary structure showed changes in all 12S rRNA variants, the most severe being for T1291C. The reported data confirm the high prevalence of mutation A1555G in deafness cases and the major role of the 12S rRNA gene in hearing. The two novel changes reported here might have different contributions as deafness-related variants. T1291C fulfills the criteria of a disease-causing change. As in the case of mutation A1555G, the underlying phenotype of T1291C is not homogeneous for all family members, providing evidence for the implication of environmental and/or additional genetic factors.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Nonsyndromic hearing loss; Sensorineural hearing impairment; Mitochondrial DNA; 12S rRNA gene; RNA secondary structure

Hearing loss is a common sensory disorder affecting 1 in 1000 newborns and showing a genetic origin or predisposition in at least 50% of the cases [1]. When genetically determined, the inheritance pattern of hearing impairment can be autosomal dominant or recessive, X-linked, and mitochondrial [2].

A number of distinct mutations in the mitochondrial DNA (mtDNA) have been found to be associated with both syndromic and nonsyndromic forms of hearing impairment [3,4]. A recent study of two geographically distant European populations (Italy and UK) showed that at least 5% of cases of postlingual, nonsyndromic hearing impairment are attributable to known mtDNA mutations, representing one of the most frequent causes of hearing impairment [5]. The most commonly reported nonsyndromic deafness-causing mtDNA mutations are a C insertion or deletion at position 961 [6–8], C1494T [9] and A1555G in the 12S rRNA gene, and mutations A7445G [10–12], 7472insC [13,14], T7510C [15], and T7511C [16–18] in the rRNA Ser(U CN) gene. Recently, several other variants in the 12S rRNA gene have been identified in a cohort of Chinese pediatric subjects with aminoglycoside-induced and nonsyndromic hearing loss, suggesting that the
mitochondrial 12S rRNA gene is a hot-spot for deafness associated mutations [19].

In contrast with other deafness-associated mtDNA mutations, reported only in a few number of families, the A1555G mutation in the small ribosomal RNA gene (12S rRNA) has been associated with aminoglycoside-induced and adult onset nonsyndromic deafness in many families of different ethnic origins [20–24], with a prevalence of 0.5–2.4% in European sensorineural deafness patients and 3% in Japanese patients [11,25,26]. The resulting phenotype varies considerably among matrilineal relatives within families or among different families carrying the A1555G mutation, ranging from severe deafness, to moderate progressive hearing loss or even completely normal hearing. Incomplete penetrance and variable expressivity of hearing loss associated with mutation A1555G are thought to be due to the interaction between genetic factors, such as nuclear modifier genes or mitochondrial haplotype, and environmental factors, such as aminoglycoside antibiotics [27–31]. Although aminoglycosides are known to trigger deafness onset, their role in the development of hearing loss in subjects carrying the A1555G mutation has been estimated to be as low as 20% when a large number of patients and families have been studied [20–24]. On the other hand, nucleotide 1555 maps to a phylogenetically conserved and a functionally well-characterized domain of the small subunit rRNA, in the decoding site of the ribosome. The mutation is predicted to alter the secondary structure of the 12S rRNA molecule, in a way that it resembles more closely its bacterial counterpart, being the deafness-associated phenotype the consequence of this structural change [20–24].

In the present study, we have screened the 12S rRNA gene in our cohort of Spanish deaf patients for the presence of sequence changes. We have identified 69 cases carrying the A1555G mutation, confirming the high prevalence of this specific mutation in the population of study. In addition, we have identified two novel changes in the mitochondrial 12S rRNA gene, T1243C and T1291C. The T1243C was found in 5/443 unrelated patients and 1/160 controls tested. The T1291C change was detected in a Cuban family affected of nonsyndromic sensorineural deafness, segregating perfectly with hearing loss. Modelling of 12S rRNA secondary structures for all identified variants resulted in changes in their predicted secondary structure, supporting that T1291C variant is a deafness-causing mutation.

Materials and methods

Patients and families. We have collected 443 families or sporadic cases affected of nonsyndromic sensorineural hearing loss from different Spanish clinical centers. Three hundred and thirteen of the cases included in the study belong to families with at least two affected members, while 130 samples were sporadic cases. Families with deafness were classified as autosomal dominant, autosomal recessive or X-linked, in accordance with the patterns of transmission of deafness. Hundred and seven pedigrees out of the 313 familial cases showed a segregation pattern of deafness consistent with maternal transmission due to the presence of affected maternal relatives and the lack of father to son transmission. The control subjects for molecular analysis were 100 general population individuals from Spain.

Clinical information such as the severity and age of onset of hearing impairment, the exposure to some kind of ototoxic substances, specifically aminoglycosides, and any other medical diagnoses were evaluated from at least one member of each pedigree. Whenever possible, pure tone hearing thresholds were determined for 125, 250, 500, 1000, 2000, 4000, and 8000 Hz, measured in dB. The degree of hearing loss was defined according to the mean hearing loss as follows: normal ≤20 dB; mild = 20–40 dB; moderate = 41–70 dB; severe = 71–95 dB; and profound >95 dB.

Mutational analysis of DFNB1 locus and 12S rRNA gene. After obtaining written informed consent, total DNA was extracted from peripheral blood using standard procedures. The samples were tested for the presence of mutations in the coding region of GJB6, the two deletions affecting GJB6 and the A1555G mutation in the 12S rRNA gene, prior to the analysis of the mtDNA 12S rRNA.

Mutation detection for GJB2 was performed by direct sequencing of the entire coding region. To detect GJB6 deletions, a specific PCR assay was used, as described by del Castillo et al. [32].

The analysis of the 12S rRNA gene was performed by direct sequencing. Once the new variants were identified, the genotyping of other deaf patients and control samples was performed using different methods. The detection of the A1555G and T1291C mutations was performed by PCR amplification of a 340-bp fragment (forward 5'-GCTAGACTGA TATTACGCATCTCAGAAA-3' and reverse 5'-TTTCCAGTACACT TACCATGTTACGACTG-3'), followed by the digestion with restriction endonuclease HaeIII, as both changes introduce a cleavage site for this enzyme. Screening of the T1243C variant was performed using the Pyrosequencing technology (PSQ96MA) (Biotage AB, Sweden). Specific SNP assays were designed by Pyrosequencing (forward 5'-TAAACCCC CGATCAACCTCAC-3', reverse 5'-TCCACCTCTGACCCCTTGA-3', and sequencing 5'-GATCAACCTACGCAC3'). Sequence identification was performed automatically by the SQA software.

Secondary structure prediction. Structures for the wild type and mutated human mitochondrial 12S rRNAs were generated using the RNAfold software from the Vienna RNA package [33]. RNAfold predicts RNA secondary structure based on minimum energy requirements and pair probabilities.

Results

A1555G mutation is a common cause of deafness in Spanish patients

Among the 313 families from our cohort, 215 (69%) of the pedigrees showed an autosomal recessive segregation pattern, 97 (31%) were considered autosomal dominant and one (0.3%) was classified as X-linked. Among them, 107 pedigrees (34%) showed a segregation pattern likely to correspond to maternal transmission. These pedigrees had information for at least three generations and the inheritance pattern was characterized by the presence of affected maternal relatives and the lack of father to son transmission. The affected subjects of the 107 families compatible with maternally inherited deafness showed bilateral and sensorineural hearing loss as the sole clinical symptom. There was a wide variability in the age at onset of deafness within and between families, although most of the patients presented late-onset, progressive deafness.

All the samples from our cohort, independently of their inheritance pattern, were analyzed for mutations in the DFNB1 locus and for the A1555G mutation in the 12S rRNA gene. The A1555G mutation was found in 65
families (61%) from the 107 deafness pedigrees compatible with maternal transmission and in four of the sporadic cases, resulting in a total of 69 unrelated samples with the mtDNA A1555G mutation, indicating that 15% of our cohort of Spanish deaf patients is a carrier of the A1555G mutation. The 69 families or sporadic cases positive for the A1555G mutation included a total of 290 individuals, of which 183 were deaf A1555G carriers (63%) and 107 were asymptomatic carriers of the mutation (37%).

Twenty-four out of the 69 families or sporadic cases positive for the A1555G mutation reported previous exposure to aminoglycoside antibiotics, either for the index case or for some of the family members. This represented only 35% of the families. All the patients who received aminoglycosides \( (n = 40) \) became deaf, but these patients represent only 22% of the total of deaf carriers of the A1555G mutation. The role of aminoglycosides in the group of pedigrees here studied is limited, indicating that other environmental or nuclear factors would determine the onset, and severity of hearing loss.

Two novel nucleotide changes in the mtDNA 12S rRNA gene

The entire coding region of the mtDNA 12S rRNA gene has been analyzed in the index cases of deafness families with a mode of inheritance compatible with maternal transmission, and negative for mutations in the \( DFNB1 \) locus and the 12S rRNA A1555G mutation. Direct sequencing of the entire 12S rRNA gene resulted in the identification of two novel mtDNA variants in homoplasmy, T1243C and T1291C.

The T1243C variant was first identified in a patient affected of sensorineural hearing loss from one Italian family. The screening of this specific variant in the whole cohort of 443 sporadic and familial Spanish deafness families with an unknown genetic cause of hearing loss resulted in the identification of four additional cases carrying T1243C (Fig. 1). All of them were affected of sensorineural hearing loss and reported no previous aminoglycoside exposure. Audiometric evaluations of affected carriers revealed a more severe hearing impairment at high frequencies, but with differences in the severity and age at onset of hearing impairment between individuals (Table 1), which, although not exclusive, are common features of hearing loss associated to mtDNA mutations, particularly of mutation A1555G. However, the T1243C variant was also detected in 1 out of 160 control samples tested and none of the pedigrees studied was informative enough to clearly conclude that the T1243C change is the cause of hearing loss.

Fig. 1. mtDNA variant T1243C in the 12S rRNA in available nuclear pedigrees and its audiometric alterations. (A) Pedigrees of families with the T1243C variant and sequence chromatograms from an affected individual and a control, showing the T to C nucleotide change. (B) Available audiometries of affected patients with the T1243C variant.
The T1291C variant was identified in a three-generation family of Cuban origin, with members affected of sensorineural hearing impairment. DNA was obtained from six individuals, four affected and two nonaffected (Fig. 2). Clinical characterization included family history of hearing loss, which was consistent with maternal inheritance, use of aminoglycosides, age of onset and pure tone audiometry. All affected individuals exhibited bilateral, sensorineural progressive hearing impairment as the sole clinical symptom. None of them reported a previous history of aminoglycoside exposure. Audiometric studies showed a more severe loss of hearing at high frequencies and a wide range in the age at onset of hearing impairment, varying from 7 years (III-4 and IV-1) to 40 years (II-1 and II-3). The offspring of the deaf father II-1 does not report any hearing problems. Although mutation T1291C was present in homoplasmy in all affected individuals, they differ in the severity and the age of onset, suggesting the involvement of environmental and/or genetic factors in the phenotype. The T1291C has been tested in the rest of our cohort of deaf patients and 100 control samples with negative results in all cases, suggesting that it may be a deafness-related variant.

Conservation and secondary structure of 12S rRNA nucleotide variants

Table 1
Available clinical data of samples carrying the T1243C mtDNA variant

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Sex</th>
<th>Hearing loss</th>
<th>Age of onset</th>
<th>T1243C</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>E19</td>
<td>10</td>
<td>F</td>
<td>+</td>
<td>Congenital</td>
<td>+</td>
<td>No pedigree information</td>
</tr>
<tr>
<td>S25.1</td>
<td>7</td>
<td>M</td>
<td>+</td>
<td>Congenital</td>
<td>+</td>
<td>No response in ABR</td>
</tr>
<tr>
<td>S25.2</td>
<td>37</td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S25.3</td>
<td>32</td>
<td>F</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S41.1</td>
<td>22</td>
<td>M</td>
<td>+</td>
<td>19 years</td>
<td>+</td>
<td>Tinnitus</td>
</tr>
<tr>
<td>S41.2</td>
<td>55</td>
<td>F</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S100.1</td>
<td>11</td>
<td>M</td>
<td>+</td>
<td>2 years</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>S100.2</td>
<td>40</td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S100.3</td>
<td>39</td>
<td>F</td>
<td>+</td>
<td>Unknown</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>S303</td>
<td>1</td>
<td>F</td>
<td>+</td>
<td>Congenital</td>
<td>+</td>
<td>No affected relatives</td>
</tr>
</tbody>
</table>

Fig. 2. mtDNA variant T1291C in the 12S rRNA in a Cuban genealogy with hearing impairment and its audiometric alterations. (A) Pedigree of the Cuban family and ethidium bromide-stained gel showing the PCR-RFLP analysis used for the detection of the T1291C variant. This change specifically creates a novel site for the restriction enzyme HaeIII, resulting in three fragments. (B) Audiometric evaluation of a mildly affected (III-4) and a severely affected (II-3) family members. * indicates individuals from which a DNA sample has been obtained.

E. Ballana et al. / Biochemical and Biophysical Research Communications 341 (2006) 950–957
antibiotics. The A1555G mutation is predicted to alter the secondary structure of the 12S rRNA molecule [20–24]. Under this assumption, we analyzed the newly identified sequence changes T1243C and T1291C, as well as the known pathogenic mutation A1555G, for their possible effects on secondary structure of the rRNA.

The comparison between human mitochondrial 12S rRNA and Escherichia coli 16S rRNA showed that the T at position 1243 and A at 1555 are highly conserved nucleotides. In the case of T at 1291, it is localized in an helix not present in the E. coli 16S rRNA (Fig. 3A) [34]. The sequence concordance for T1243 and A1555, and the discordance for T1291 between the human mitochondrial 12S rRNA and E. coli 16S rRNA was also confirmed by comparison in different species (Fig. 3B).

To further study the possible functional effect of the three changes, prediction of their secondary structures was performed using the RNAfold software [33]. In this model, the three changes resulted in a structural change compared to the wild-type prediction, being T1291C the most dramatic change affecting the secondary structure (Fig. 4).

Discussion

Mutations in the 12S rRNA gene, especially the A1555G mutation, have been shown to be a common cause of hearing impairment in different populations [6–9,20–24]. We have identified 69 unrelated patients, affected of non-syndromic hearing loss, positive for mutation A1555G. The 69 families correspond to 290 samples carrying the A1555G mutation, 183 affected (63%) and 107 asymptomatic individuals. The relationship between mutation A1555G and deafness after treatment with aminoglycosides is absolute; however, only 22% of deaf patients that carry this mutation received these antibiotics. These data suggest, as previously reported, that A1555G is a major factor in the onset of deafness, but other factors must contribute to the development of hearing loss in the affected subjects [6–9,20–24]. The high frequency of the A1555G mutation in the Spanish sensorineural deafness patients indeed suggests that this mutation is an important contributor to sensorineural deafness. In addition, our results confirm the importance of determining the prevalence of the mtDNA A1555G mutation in different populations and stress the need for mutation detection before the administration of aminoglycoside antibiotics.

Since mutations in mtDNA account for a high number of familial and sporadic sensorineural hearing loss cases [5], we considered the possibility that other mutations in the 12S rRNA gene could also be causing deafness. The search for additional mutations in the 12S rRNA gene led to the identification of two changes with possibly different roles as deafness-related variants.
T1243C was present in homoplasmy in five cases and one control, thus likely representing a polymorphism. However, a wide variety of symptoms have been reported in deaf patients with mitochondrial mutations, including cases of normal hearing [20–24]. Moreover, the A1555G mutation is thought to be a predisposing mutation, which needs the combined action of environmental factors and/or nuclear modifying genes to cause hearing impairment [27–31]. Taking this into account, we could not exclude a similar role for the T1243C variant, which in conjunction with other factors may lead to hearing loss.

The T1291C change was identified in homoplasmy in a single family affected of sensorineural deafness, but with different degrees of severity and ages at onset. T1291C is likely to be the disease-causing nucleotide variant, as it is located in the mtDNA 12S rRNA gene, it segregates with the disease in maternal relatives, the phenotype was similar to that associated with other mtDNA deafness causing mutations [35], and it was not found in any of the controls tested. Phenotypic heterogeneity is a hallmark of mitochondrial disorders and, this clinical heterogeneity presumably results from different nuclear backgrounds [35]. Therefore, the phenotypic variability in the members of this pedigree suggests again the involvement of either environmental factors or nuclear modifier genes [35].

Comparison of the 12S rRNA structure with the 16S rRNA of *E. coli* and prediction of their secondary structures shed light on the possible functional effects of the two new variants described. The T at nucleotide 1243 is a conserved position, suggesting an important role in its structure and function [34]. No comparison could be performed for the T at 1291, as it is located in a helix without a bacterial homologue. Evaluation of conservation of all three changes in different species showed again that the T at nucleotide 1243 is conserved throughout evolution, as well as the A at nucleotide 1555, while T at position 1291 is only present in human mtDNA. On the other hand, the prediction using RNAfold software showed changes in the secondary structure of the 12S rRNA in all three cases, being the one predicted for the T1291C variant the more severe for the T1291C. This suggests a more important functional effect of mutation T1291C, which would eventually lead to a more severe phenotype.

mtDNA mutations usually affect tissues with high energy requirements, such as muscle and brain, but also the cochlea. The exact mechanism of cochlear damage in mtDNA-associated disorders is unclear. Normal hearing is dependent upon the hair cells and the stria vascularis, which maintain the ionic gradients necessary for sound signal transduction. Both stria vascularis and hair cells are highly metabolically active and would be compromised.
by a dysfunction of intracellular mitochondrial ATP as a consequence of a mtDNA mutation [4].

For the A1555G and C1494T mutations, in which aminoglycoside-induced deafness is believed to be genetically-determined, it has been hypothesized that the mutations make the human mitochondrial small rRNA more similar to the bacterial rRNA, the target of aminoglycoside action [35]. Accumulation of aminoglycosides in cochlear mitochondria would lead to an inhibition of protein synthesis by interacting with the 12S rRNA carrying these mutations. As a result of this mitochondrial translation defect, the ATP production declines and the generation of reactive oxygen species increases, consequently damaging hear cells and giving rise to hearing impairment [35]. In the absence of aminoglycoside exposure, an analogue mechanism is expected, but the factors leading to a dysfunction of mitochondrial protein synthesis remain unknown. A similar scenario is also possible for the new changes we describe here, in which the phenotypic variability may be explained by the involvement of environmental or genetic factors, contributing to the penetrance of mtDNA mutations. To completely understand the pathogenic mechanism of mtDNA variants, it should be necessary to perform functional studies of cell lines derived from patients, but from the data presented here it seems clear that T1291C is a mtDNA disease-causing mutation.

Acknowledgments

We thank the patients for participation in the study. This work was financially supported by “Fundació La Marató de TV3” (993610) and “Instituto de Salud Carlos III,” FIS-ISCCII (G03/203). E.B. is the recipient of a FI fellowship from “Departament d’Universitats i Societat de la Informació,” Generalitat de Catalunya (2003FI00066).

References


