Heteroplasmy for the 1555A>G mutation in the mitochondrial 12S rRNA gene in six Spanish families with non-syndromic hearing loss

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Hearing impairment is the most prevalent sensory disorder and genetic causes are thought to be responsible for over 60% of the cases in developed countries.[1] Inherited hearing impairment is highly heterogeneous from both the clinical and genetic points of view.[1 2] It varies in age of onset, severity, and audiological characteristics, and it can be associated or not with other clinical features (syndromic or non-syndromic hearing impairment). Genetic transmission includes autosomal (dominant and recessive), X linked, and maternal inheritance patterns. This unparalleled heterogeneity is well illustrated by the fact that over 70 loci in the nuclear genome have been reported to be involved in non-syndromic hearing impairment, and about 30 genes have been isolated from their critical intervals.[3] Furthermore, a number of different mutations in several genes of the mitochondrial genome are responsible for syndromic and non-syndromic forms of hearing loss.[4 5]

Mutations responsible for maternally inherited non-syndromic hearing loss are so far confined to only two genes in the mitochondrial genome. These include mutations 7510T>C[6] and 7511T>C[7] in the $tRNA^{Ser(UCN)}$ gene, and 1095T>C[8] and 1555A>G[9] in the gene for the 12S rRNA. This last mutation is responsible for a dual phenotype, since it also confers increased susceptibility to the ototoxic action of aminoglycoside antibiotics.[9] Most of these mutations have been reported in a small number of families from several countries, with the exception of 1555A>G, which seems to be more frequent than the others,[10–13] although its real prevalence remains to be determined in most populations. Remarkably, in Spain it accounts for about 15-20% of all familial cases of non-syndromic hearing loss, irrespective of their mode of inheritance and age of onset[14 15] (our unpublished results). In a majority of these patients, the hearing loss is not attributable to aminoglycoside ototoxicity. A phylogenetic analysis of mitochondrial DNA (mtDNA) haplogroups, performed on 50 unrelated Spanish families, showed that the 1555A>G mutation could be caused by over 30 independent mutational events, occurring in mtDNA haplogroups which are common in all European populations.[16] These data indicate that the high detection rate of this mutation in Spain is not the result of a single major founder event, at least with regard to the mitochondrial genome. Given the high prevalence of the 1555A>G mutation in Spain, and the possibility of preventing aminoglycoside ototoxicity in mutation carriers, its detection has become a priority in routine genetic testing. In contrast to other mutations in the mtDNA, which are frequently heteroplasmic,[4 5] the 1555A>G mutation has been found in homoplasmy in all but one of the families reported so far.[17] In that study, the mutation was found in heteroplasmy in three subjects, with the proportion of mutant copies (the "mutation load") ranging from 85-94%. Here we report the genetic and clinical characterisation of six novel unrelated Spanish families segregating the 1555A>G mutation in heteroplasmy, with a wide range of percentages of mutant copies in a total of 19 subjects.

Key points

- Mutation 1555A>G in the 12S rRNA gene of the mitochondrial genome is responsible for non-syndromic hearing loss, as well as for increased susceptibility to the ototoxicity of aminoglycoside antibiotics.
- In almost all the cases reported so far the mutation was found in homoplasmy. Here we report the clinical and genetic characterisation of six Spanish families with sensorineural hearing loss, totalling 19 subjects with heteroplasmy for 1555A>G.
- The proportion of mutant copies ranged from 3.75-96.60%. Subjects carrying less than 20% of mutant copies were asymptomatic or had a mild hearing loss, whereas heteroplasmic subjects with over 52% of mutant copies suffered from moderate to severe hearing loss.
- Taking the six families together, there is a correlation of the mutation load with the severity of the hearing loss. However, when studying the families separately, this correlation is confirmed in three of them and excluded in another.
- Our study illustrates the difficulties in extracting general principles from the analysis of the genotype-phenotype correlation regarding the 1555A>G mutation.

METHODS

Subjects

Familial cases of non-syndromic hearing loss were collected with the only criterion of having at least two affected subjects. Our collection procedure did not cause any other bias, such as preferential selection of large pedigrees or compatibility with maternal inheritance. A total of 649 unrelated Spanish families were enrolled in the study. After getting informed consent, peripheral blood samples were obtained from all participating family members, and DNA extraction was performed by standard procedures.

Mutation detection

Screening for the 1555A>G mutation was carried out by PCR amplification of a 339 bp DNA fragment containing the mutation site, followed by digestion with restriction endonuclease *Hae*III, as described previously.[14] In the wild type allele, digestion results in two fragments of 216 bp and 123 bp. The mutation specifically creates a novel restriction site, and so digestion results in three fragments (216 bp, 93 bp, and 30 bp).

Quantification of the mutation load

The proportion of mutant copies was quantified by detection of fluorescently labelled PCR products separated by capillary electrophoresis. A 359 bp DNA fragment was amplified with

hot start FastStart *Taq* DNA polymerase (Roche) using 75 ng of DNA from the subject as template. Primer sequences were: upper primer 5′-AGACGTTAGGTCAAGGTG-3′; lower primer, 5′-GTTTAGCTCAGAGCGGTC-3′. The upper primer was fluorescently labelled at 5′ with TET. PCR was carried out in a final volume of 15 µl with the following conditions: an enzyme activation step, at 95°C for six minutes; 10 cycles of denaturation at 94°C for 15 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 30 seconds; 13 cycles of denaturation at 89°C for 15 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 30 seconds; plus a final extension step at 72°C for 10 minutes. Subsequently, 8 µl of the PCR product were digested at 37°C for two hours with a large excess (10 units) of restriction endonuclease *Alw*26I (Fermentas), in a final volume of 10 µl. Three microlitre aliquots of digestion were subjected to capillary electrophoresis in an ABI Prism 310 Genetic Analyzer (Applied Biosystems) according to the recommendations of the manufacturer. By using this technique, two fluorescent products can be detected in a given sample, wild type (127 bp) and/or mutant (359 bp, since the 1555A>G mutation destroys the restriction site for *Alw*26I). In heteroplasmic subjects, the proportion of mutant copies was estimated from the peak areas of the fragments, in quantification experiments from three independent PCR amplifications. The quantification results were consistent with those previously observed in agarose gels after *Hae*III digestion.

RESULTS AND DISCUSSION

At least one subject from each of the 649 collected families was tested for the presence of the mitochondrial 1555A>G mutation, the result being positive in 105 families (16%). In the positive cases, a search for mutation carriers was performed on all the remaining participating relatives. This screening showed heteroplasmy for the mutation in 19 subjects from six unrelated families, which also included 12 subjects with the mutation in homoplasmy (fig 1).

All of the mutation carriers in the families with heteroplasmy were studied clinically. There were records of treatment with aminoglycoside antibiotics in only two subjects (S138 I.2 and S160 I.2, streptomycin). Other environmental factors were excluded as causes of hearing loss in all the subjects. No syndromic features were found. 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 conduction, confirmed that the hearing loss was bilateral and sensorineural in all affected subjects. Audiograms for air conduction are shown in fig 1. There were no vestibular symptoms except in patient S141 I.2, who reported episodes of positional vertigo. Patients S141 I.2, S141 II.3, and S338 II.1 reported bilateral tinnitus.

As expected, the pattern of transmission of the 1555A>G mutation was consistent with maternal inheritance in all the families (fig 1). The proportion of mutant copies was determined in the 19 heteroplasmic subjects and ranged from 3.75-96.60% (fig 1). The mutation load in the offspring of a heteroplasmic mother was highly variable. For instance, subject S338 I.2 (52.14% of mutant copies) has two sons with the mutant allele in homoplasmy within our detection limits, and another son with values close to homoplasmy for the wild type allele (3.84% of mutant copies). A wide variation is also observed in the offspring of subject S138 I.2 (fig 1). In addition, pedigrees S160 and S068 have some relevant characteristics. Subject S160 I.2, homoplasmic for the mutant allele within our detection limits, has two heteroplasmic daughters (94.74% and 96.15% of mutant copies, respectively). This result indicates that subject S160 I.2 keeps some wild type copies at least in the germline. In peripheral blood, the wild type allele would have been lost or would be in a proportion small enough to go undetected. As regards pedigree S068, heteroplasmic subject II.5 has two sibs (a brother and sister) who are apparently homoplasmic for the wild type allele. However, both her brother (II.1) and a son of her sister (III.1) are affected by bilateral sensorineural hearing loss, more severe in the high frequencies. Two hypotheses may explain these data. First, I.2, the mother of subjects II.1, II.3, and II.5, would have carried the 1555A>G mutation in heteroplasmy. The mutation would remain in II.5, but would have been lost in II.1 and II.3. If this were the case, the hearing loss in subjects II.1 and III.1 would have a cause different from the 1555A>G mutation, which is a plausible explanation given the genetic heterogeneity of non-syndromic hearing loss. It should be taken into account, however, that the characteristics of the hearing loss in subjects II.1 and III.1 closely resemble those of other members of the family, but it is also true that high frequency hearing loss is the most common type. The second hypothesis concerns the possibility that peripheral blood from subjects II.1, II.3, and III.1 contained a very small, undetected, proportion of mutant copies (apparent homoplasmy) or none at all (real homoplasmy). However, the mutation load in the inner ear would be large enough to be pathogenic in subjects II.1 and III.1. Were this the case, its implications would be relevant for genetic diagnosis (see below).

We investigated the effect of heteroplasmy on the severity of the hearing loss. Subjects carrying less than 20% of mutant copies were asymptomatic (S138 II.1 and II.2, S297 III.2, S338 II.3 and II.4), or had a mild hearing loss (subject S297 IV.1, with a U shaped audiogram). Conversely, the remaining 13 subjects, with percentages of mutant copies between 52.14% and 96.60%, suffered from hearing loss. Two of them, monozygotic twins from family S141 (II.1 and II.2) with 70.93% and 64.48% of mutant copies, respectively, had a mild hearing loss for high frequencies. Five others (S068 II.5 and S138 II.3, II.4, II.5, and II.6), with percentages of mutant copies between 69.78% and 96.60%, had normal hearing for low and middle frequencies, but suffered from moderate or severe hearing loss for high frequencies. In the remaining six cases, the audiogram shape was sloping, affecting both middle and high frequencies (S141 II.3, S160 II.1, and S338 I.2; mutant copies ranging from 52.14% to 94.74%) or all the frequencies (S138 I.2, S141 I.2, and S160 II.3; mutant copies ranging from 61.03% to 96.15%). In this last group, subject S138 I.2 had a history of treatment with streptomycin. The study of 10 subjects from families S068, S160, and S338, carrying the mutation in homoplasmy, showed that eight of them had hearing losses which were more severe than those of their heteroplasmic relatives (only one homoplasmic subject, S160 I.2, had a history of treatment with streptomycin). The two remaining cases (S160 II.5 and II.6) were asymptomatic, but it should be considered that they are younger than their four affected sibs, and may be below the age of onset.

A statistical analysis of our data for all the six families showed significant correlation of the mutation load with the hearing thresholds, for all the frequencies (125-8000 Hz range) (fig 2A), and for only the high frequencies (2000-8000 Hz range) (fig 2B). However, these results must be interpreted cautiously, as indicated by intrafamilial analysis. In three families (S068, S160, S338), the severity of the hearing loss clearly correlates with the mutation load. Regarding family S141, the proportion of mutant copies influences the severity, but this seems to be modulated also by age. Conversely, no apparent correlation is observed between the severity of the hearing loss and the mutation load in four sibs from family S138 with mutant copies ranging from 69.78% to 96.60% (II.3-II.6).

In subjects with only high frequency hearing loss, it was difficult to ascertain their age of onset, since frequently they were not aware of their hearing loss. However, a majority of cases with the mutation in heteroplasmy reported that the hearing loss first manifested in adulthood (between 17 and 50 years of age). In contrast, in eight out of 10 subjects carrying

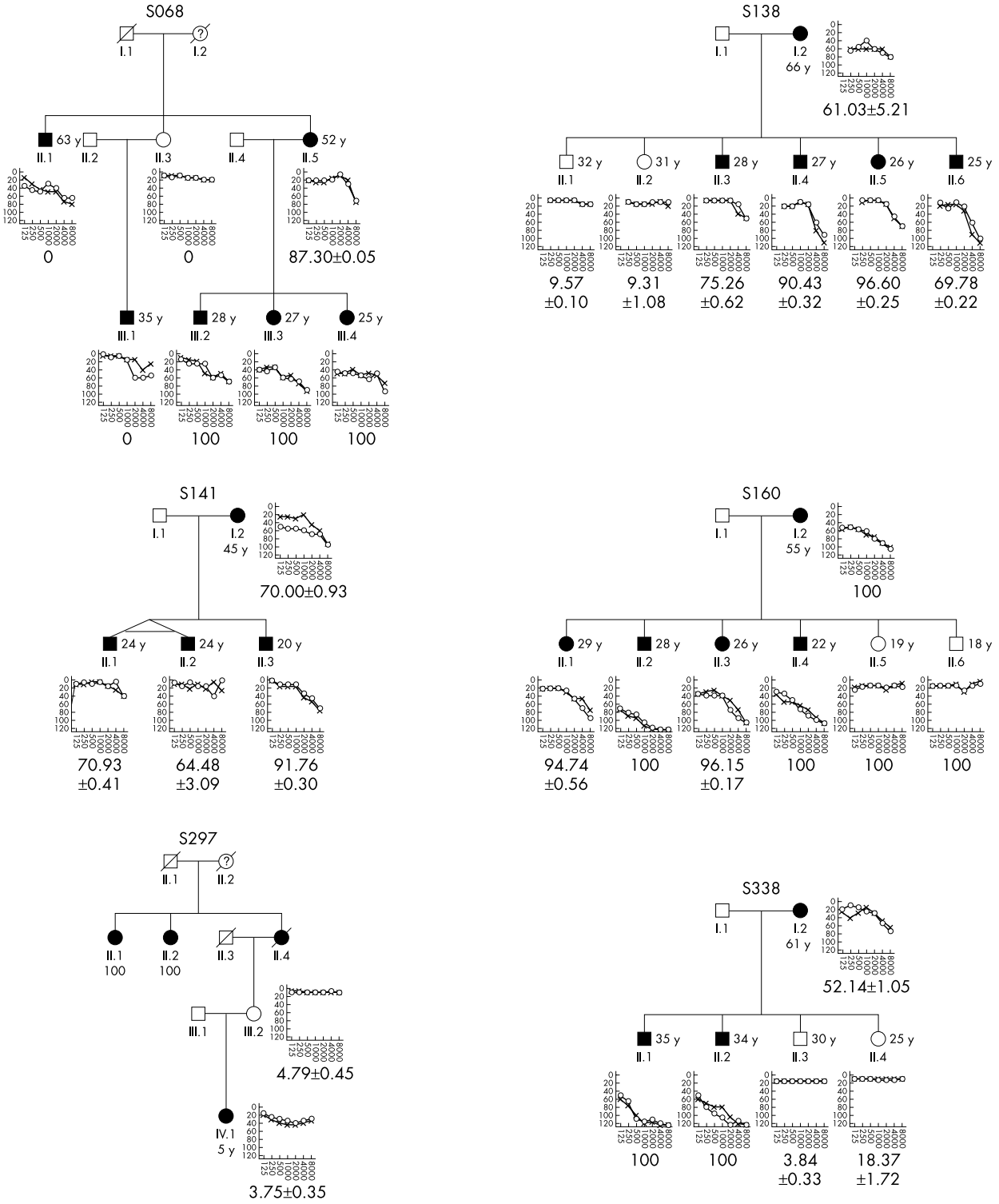

Figure 1 Pedigrees of the six Spanish families segregating the 1555A>G mutation in heteroplasmy. A question mark inside a symbol is used to represent subjects whose clinical status could not be ascertained. Age (in years) and audiograms are shown below or to the right of subject symbols. Hearing level (in dB) is plotted versus sound frequency (in Hz). Since the hearing loss was sensorineural in all cases, only results for air conduction are depicted. Circles, right ear; crosses, left ear. For each subject, the proportion (%) of mutant copies (mean of three independent experiments (standard deviation)), estimated from DNA from peripheral blood, is indicated below the audiogram.

the mutation in homoplasmy, onset was in early childhood (between 1 and 5 years of age).

The study of the genotype-phenotype correlation in subjects carrying the 1555A>G mutation in homoplasmy, which are the vast majority of the cases reported so far, has shown considerable heterogeneity in age of onset, evolution, severity, and other audiological features of the hearing loss resulting from this mutation.[4 5] This variability has been attributed to the influence of both environmental and genetic factors. Undoubtedly, aminoglycoside antibiotics induce a severe worsening of the hearing loss in mutation carriers. In addition, there is in vitro evidence of the influence of the nuclear background

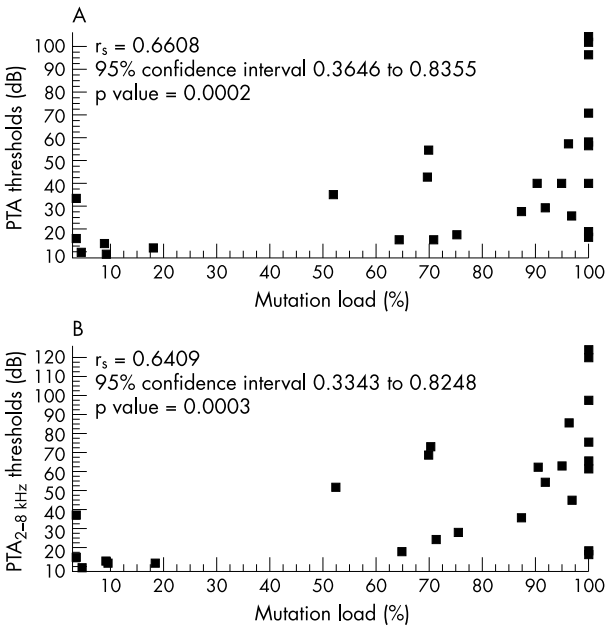

Figure 2 Statistical correlation analysis of mutation load (proportion of mutant allele) and hearing thresholds. Data from a total of 27 subjects from the six families were included in the analysis (the two subjects with a record of treatment with aminoglycoside antibiotics were excluded). Spearman rank correlation tests were performed using GraphPad InStat version 3.05 for Windows 95 (GraphPad Software, San Diego, California, USA). r_s, Spearman correlation coefficient. (A) Pure tone average (PTA) of hearing thresholds for all the frequencies (125-8000 Hz range) versus mutation load. (B) Pure tone average (PTA) of hearing thresholds for the high frequencies (2-8 kHz range) versus mutation load.

in modulating the phenotype caused by the 1555A>G mutation.[18 19] Also, the hypothesis of the existence of nuclear genes acting as modifiers of mitochondrial hearing loss has recently received strong support.[20–23] The existence of a not negligible percentage of cases with heteroplasmy (5.7% in our sample of 105 families with the 1555A>G mutation) adds more complexity to the picture. Our study of a set of 19 heteroplasmic subjects illustrates the difficulties in extracting general principles from the analysis of the genotype-phenotype correlation regarding this mutation. First, among our heteroplasmic cases, most of the subjects carrying less than 20% of mutant copies were asymptomatic, whereas all of the subjects with mutation loads higher than 52% suffered from hearing loss. This suggests that there is a threshold in mutation load for manifestation of clinical symptoms. However, it is also known that there exist subjects homoplasmic for 1555A>G, who are asymptomatic (for example, subjects S160 II.5 and II.6). Second, statistical analysis of our data indicates a significant correlation of the the severity of the hearing loss with the mutation load when considering the six families altogether. However, when studying the families separately, this correlation is confirmed in three of them and excluded in another (family S138). This situation may be because of intrafamilial differences in the nuclear background modulating the phenotype, and/or individual variability in mutation load in peripheral blood and inner ear. In fact, it has been reported that the level of heteroplasmy for a given mutation can vary among different tissues within the same person.[24]

The conclusions of our study are relevant for genetic diagnosis of mitochondrial mutations that are responsible for non-syndromic hearing loss. The estimations of mutation load obtained from mitochondrial DNA from peripheral blood may not always reflect accurately the real situation in the inner ear. In extreme cases, the mutation load may be pathogenic in the inner ear and remain undetectable in blood. Therefore, in large families with several affected subjects and a clear maternal inheritance of the disorder, several probands from different branches in the pedigree should be tested before excluding the presence of the mutation. This issue may be critical for prevention of aminoglycoside ototoxicity in subjects whose carrier status would go unnoticed.

Although the last few years have witnessed great advances in the understanding of mitochondrial pathogenesis, there are many important issues that remain unsolved, such as the basis of tissue specificity and the mechanisms by which a heteroplasmic mutation segregates and is fixed. Further investigation of these matters is needed to improve genetic counselling regarding the 1555A>G mutation.

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Uniparental disomy of chromosome 13q causing homozygosity for the 35delG mutation in the gene encoding connexin26 (*GJB2*) results in prelingual hearing impairment in two unrelated Spanish patients

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Inherited hearing impairment is a highly heterogeneous group of disorders with an overall incidence of about 1 in 2000 newborns.[1] In approximately 70% of cases, the auditory impairment is not associated with other clinical features, that is, it is non-syndromic. The most frequent condition is a severe or profound hearing loss of prelingual onset, which is inherited mainly as an autosomal recessive trait.[1] To date, 31 different DFNB loci for autosomal recessive non-syndromic hearing loss have been reported, and 16 genes have been identified.[2] Among these loci, DFNB1 in 13q12 stands out because of its complexity and clinical relevance. It contains the gene *GJB2*, which encodes connexin26, a component of intercellular gap junctions. Mutations in the *GJB2* gene are responsible for up to 50% of all cases of autosomal recessive hearing impairment in most of the populations tested so far, with a frequent mutation (35delG) accounting for up to 86% of the *GJB2* mutant alleles in white populations.[3 4] However, not all the DFNB1 mutations affect the *GJB2* gene. Recently, several research teams found a deletion in the 13q12 region which is frequently inherited in double heterozygosity with mutant *GJB2* alleles in affected subjects,[5–7] but it was also found in homozygosity.[6 7] Molecular characterisation of this deletion, termed del(*GJB6*-D13S1830), showed that it encompasses 342 kb and it does not affect the *GJB2* gene, but it truncates the gene encoding connexin30 (*GJB6*), another gap junction protein expressed in the inner ear.[6] The existence of this deletion was first suspected by the finding of inconsistencies in the segregation of genetic markers distal to *GJB2*.

Key points

- Mutations in the gene encoding the gap junction protein connexin26 (DFNB1 locus on 13q12) are responsible for up to 50% of all cases of autosomal recessive hearing impairment in most populations, the 35delG mutation being the most frequent in white populations.
- Here we report two unrelated cases of homozygotes for 35delG whose biological fathers were not carriers of the mutation. The study of the segregation of polymorphic genetic markers showed uniparental (maternal) disomy of chromosome 13, causing homozygosity for the mutation. In both cases, the disomic maternal gamete may have resulted from non-disjunction of chromosome 13 in meiosis II.
- These two cases represent the first description of UPD(13) with an abnormal phenotype, and they are also the first cases of UPD resulting in non-syndromic hearing impairment.

Here we report another inconsistency in the segregation of markers in the 13q12 region in two unrelated cases of subjects with prelingual hearing impairment. In these two cases, uniparental disomy of chromosome 13 caused homozygosity for