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**Mitochondrial nonsyndromic sensorineural hearing loss. A clinical, audiological
and pathological study from Italy and revision of the literature**

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Abstract

Over the last decade 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. Their real incidence as a cause of deafness is yet poorly understood and generally underestimated.

Among the known mtDNA mutations, the A1555G in the 12S gene has been identified to be one of the most common genetic cause of deafness, and it has been described both associated to nonsyndromic progressive sensoryneural hearing loss (SNHL) and to aminoglycoside induced SNHL.

In this study, we have investigated the presence of mtDNA alterations in patients affected by idiopathic nonsyndromic SNHL, both familiar and sporadic, in order to evaluate the frequency of mtDNA alterations as a cause of deafness and to describe the audiological manifestations of mitochondrial nonsyndromic SNHL.

Consistently with literature data, we found the A1555G mutation to be responsible of a relevant percentage (5,4%) of cases affected with isolated idiopathic sensorineural hearing impairment.

Key words: nonsyndromic sensorineural hearing loss, mtDNA mutation, A1555G mutation, mitochondrial deafness, cochlea

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Introduction

Over the past decade, knowledge on genetic cause of deafness has considerably increased. Among genetic factors, the mitochondrial DNA (mtDNA) mutations are clearly responsible of several forms of syndromic and nonsyndromic deafness, although the role of these mutations is still poorly understood (Fischel-Ghodsian 2003).

The cochlea is very sensible to mitochondrial dysfunctions, either because its function is highly energy dependent and because the hair cells do not replicate. The exact mechanism of cochlear damage in mtDNA-associated disorders is still unclear. Normal hearing is dependent upon the hair cells function and the stria vascularis, which maintain the ionic gradients necessary for sound signal transduction (Chinnery et al. 2000; Fischel-Ghodsian 2003). Both the stria vascularis and the hair cells are highly metabolically active and rich in mitochondria, so can be easily compromised by a dysfunction in mitochondrial ATP production, consequent to mtDNA mutations (Fischel-Ghodsian 2003). Moreover, the hair cells of the cochlea do not replicate and consequently tend to accumulate mutant mtDNA. For this reason progressive sensorineural hearing loss (SNHL), both in its syndromic and nonsyndromic forms has recently been associated to a large number of mtDNA alterations (Braverman et al. 1996; Casano et al. 1998; Chinnery et al. 2000; Del Castillo et al. 2003; Deschauer et al. 2001; El-Schahawi et al. 1997; Estivill et al. 1998; Hutchin et al. 2001a; Hutchin et al. 2000; Lehtonen et al. 2000; Prezant 1993; Santorelli et al. 1999; Seidman et al. 1996; Sevier et al. 1998). Similarly to other mitochondrial pathologies, also mitochondrial deafness is widely clinically variable. This variability is most likely related to the complex interaction between mtDNA, nuclear DNA and environmental factors, so that a mtDNA alteration probably causes deafness only in association to other genetic or environmental co-factors (Ballana et al. 2006; Bravo et al. 2006; Fischel-Ghodsian 2003).

Nonsyndromic progressive SNHL has been detected in association to mitochondrial genomic dysfunctions. This is demonstrated by the presence of mtDNA mutations in families with nonsyndromic progressive SNHL with a maternal inheritance as well as in patients affected with sporadic nonsyndromic progressive SNHL.

The most commonly reported nonsyndromic deafness-causing mtDNA mutations are the A1555G (Ballana et al. 2006; Braverman et al. 1996; Casano et al. 1998; Casano et al. 1999; Fischel-Ghodsian 2003), the C1494T (Zhao et al. 2004), and a C insertion or deletion at position 961 (Bacino et al. 1995; Yoshida et al. 2002; Li et al. 2004) in the 12S rRNA gene and the mutations A7445G (Hutchin et al. 2001a; Tekin et al. 2003; Hyslop et al. 1997), 7472insC (Hutchin et al. 2001b; Verhoeven et al. 1999), T7510C (Hutchin et al. 2000) and T7511C (Chapiro et al. 2002; Ishikawa et al. 2002; Li R. 2005) in the tRNAser (UCN) gene. Recently, several other variants in the 12S rRNA gene have been identified (Li Z et al. 2005). Among these mtDNA mutations, the A1555G is the most frequent and it has been described both associated to nonsyndromic progressive SNHL and to aminoglycoside induced SNHL. The mtDNA mutations associated to nonsyndromic SNHL are generally homoplasmic or almost homoplasmic (>85%.) (Fischel-Ghodsian 2003).

Also mtDNA deletions, such as the *common deletion* (10.4 Kb) have been described in relation to nonsyndromic SNHL (Ueda et al. 1998); to this regard we have reported a patient affected by sudden nonsyndromic bilateral SNHL, harbouring a 2,7 Kb mtDNA deletion (Berrettini et al. 2001).

In this study we have investigated the presence of the already known mtDNA mutations in patients affected by idiopathic nonsyndromic SNHL, both familiar and sporadic. The objectives were to evaluate the frequency of mtDNA alterations as a cause of deafness and to describe the audiological manifestations of mitochondrial nonsyndromic SNHL.

Materials and Methods

167 consecutive patients, affected with idiopathic nonsyndromic SNHL were studied at the ENT Unit of the University of Pisa, in collaboration with the Neurological Unit, with the aim to investigate the presence of mtDNA alterations as a cause of deafness.

The sample was composed by 81 males and 86 females, mean age 59 years (ranging from 15 to 76 years).

Any known cause of SNHL had previously been excluded by means of a complete blood and immunological evaluation, according to a previously published protocol (Berrettini et al. 1998) including sequence analysis of Connexin 26 and 30 genes.

A high resolution computed tomography (HRCT) of the petrous bone and a high resolution magnetic resonance (HRMR) of the labyrinth excluded the presence of inner ear malformations in all the patients.

The whole sample of patients was submitted to an accurate audiological evaluation as follows.

An accurate clinical history was performed to investigate the presence of clinical signs or symptoms suggestive for mitochondrial dysfunction and the presence of a familial history of hearing impairment or mitochondrial diseases. Otoscopy and otomicroscopy were performed as well as pure tone audiometry, speech audiometry, tympanometry and stapedial reflex study, brainstem evoked auditory potentials (BEAPs) and otoacoustic emissions.

All the patients included in the study were affected by bilateral SNHL. 19/167 patients (11,3%) were pre-lingually deafened and in 6/167 patients (3,6%) the hearing impairment had a sudden onset. 8/167 patients (4,7%) had been exposed to aminoglycosides during the neonatal period, or later before hearing loss onset.

In all the patients the research of homoplasmic and high rate heteroplasmic mtDNA mutations and deletions on peripheral lymphocytes was performed (note 1). The following mtDNA point mutations have been investigated:

- A1555G, 961T, T1095C of the 12S rRNA gene;
- A7445G, 7472 insC, T7510C and T7511C of the tRNA^{Ser}(UCN) gene;
- A3243G of the tRNA^{Leu}(UUR) gene;
- the point mutation A8926G has been investigated only in patients with diabetes in whom the A3243G point mutation had been excluded;
- mtDNA deletions have also been investigated.

All patients with a mtDNA mutation underwent a neurological examination as well as the dosage of lactic acid blood, both at rest and during exercise. Furthermore, after having obtained their consent, in those patients a muscle biopsy was performed. Routine histological and histochemical studies, as well as biochemical analysis of the respiratory chain enzymes activities was performed as described (Sciocco et al. 1996). On mtDNA extracted from muscle presence of the above mentioned point mutations and mtDNA deletions was also investigated (note 1).

Where possible, the relatives of the patients harbouring mtDNA mutations underwent an audiological evaluation and the research of the mutation on peripheral blood lymphocytes.

Note 1: molecular analysis technique.

Screening of mtDNA

Total DNA from patients' blood or, if available, muscle is extracted using standard protocols. The mtDNA point mutations are screened by PCR-RFLP analysis. tRNAs mutations are detected by single strand conformational polymorphism (SSCP) analysis. We use a set of primers that amplifies all the tRNAs coding regions. Reactions are performed in 25 μ l of 10 mM Tris-HCl (pH 8.9) containing 0.4 μ M each of the forward and reverse oligonucleotide, 1.5 mM MgCl₂, 0.2 mM each of dATP, dGTP, and dTTP, 0.02 mM dCTP, 1 μ Ci of α -³²[P] dCTP, and 1.25 units of Taq DNA polymerase (Roche, Indianapolis, IN). PCR conditions are: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 30 seconds, and a final extension step at 72°C for 7 min. Samples are denatured and separated on 6% MDE polyacrylamide gel (BME, Rockland, ME) with 5% glycerol, according to the manufacturer's protocol. Conformations of single-stranded DNA are visualized by autoradiography using

BIOMAX film (Kodak, Rochester, NY). Samples with abnormal patterns are directly sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and a 310 Automatic Sequencer (Applied Biosystem, Foster City, CA).

In all cases, the presence of the mutations are confirmed by PCR-RFLP analysis.

Southern blot and Long PCR analysis for the presence of mtDNA deletion(s) are performed as previously described (Zeviani et al.1988).

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Results

We found the mtDNA A1555G point mutation in 9 of the 167 patients (5,4%) affected by idiopathic nonsyndromic SNHL. We did not find any of the remaining investigated mutations in peripheral blood lymphocytes.

In all the patients the sequence analysis of Connexin 26 and 30 genes resulted negative, as well as the radiological study of the inner ear.

In table I the main features of the patients harbouring the mtDNA mutations are summarized (table I).

Below, a brief description of the clinical cases harbouring the mutations is reported.

1) A.M., 54 years old male. The patient was affected by isolated slowly progressive SNHL, started at age 27-yrs after streptomycin exposure. The audiometric pattern was a downsloping hearing loss (figure 1a). The homoplasmic A1555G point mutation was detected in blood. Family history was positive for hearing impairment, with a matrilinear pattern of transmission (figure 1b). Not all the hearing impaired relatives have been exposed to aminoglycosides. We have analysed the mtDNA from peripheral lymphocytes of the patient's brothers, sister and nephews and we detected the homoplasmic A1555G mutation in all of them (figure 1b).

The vestibular function examined in the proband was normal. The HRCT of the petrous bone and the HRMR of the labyrinth were both normal. Neurological examination as well as lactic acid levels were both normal. Signs of mitochondrial dysfunction were present at muscular level as demonstrated by the presence of ragged-red and COX negative fibers (figure 1c). The ultrastructural analysis showed mitochondria with focal loss of cristae and crystalline-like bodies.

2) D.S., 34 years old female. The patient was affected by isolated slowly progressive SNHL started at age 15-yrs. The hearing loss was mild to moderate and the audiogram

shape was flat (figure 2a). The patient has never been exposed to aminoglycosides. The homoplasmic A1555G point mutation was detected in blood. Family history was negative for hearing impairment (figure 2b). The A1555G mutation was also detected in patient's mother, sister and daughter, although none of them had any hearing problem (figure 2b).

The vestibular function examined in the proband was normal. Both the HRCT of the petrous bone and the HRMR of the labyrinth were normal. Neurological examination as well as lactic acid levels were both normal. Ragged-red and COX negative fibers were also observed in muscle biopsy, and ultrastructural studies evidenced focal loss of cristae and the presence of crystalline bodies within mitochondria (figure 2c).

3) M.L., 66 years old female. The patient was affected by profound bilateral SNHL, started at age 24-yrs, after streptomycin assumption and progressively worsened. She used bilateral hearing aids, without substantial benefits and was a candidate to cochlear implantation. The patient harboured the homoplasmic A1555G mutation. The proband had three daughters, all positive for the A1555G mutation. Two of them were affected by SNHL. One of the two hearing impaired daughters developed the hearing loss after aminoglycoside exposure. A vestibular function examination showed bilateral labyrinth areflexia. The HRCT of the petrous bone and the HRMR of the labyrinth were both normal. Neurological examination was normal, while lactic acid kinetic study showed slightly abnormal lactate levels after exercise. The patient refused a muscular biopsy.

4) M.G., 36 years old female. The patient was affected by severe bilateral SNHL, started at age 18-yrs and progressively worsened. Since her 20's, she used bilateral hearing aids. No exposure to aminoglycosides was known. The patient had two severely hearing impaired twins, aged 3-yrs, who had been exposed to aminoglycosides in neonatal intensive care unit. The mother and both sons carried the homoplasmic

A1555G mutation. The vestibular function in the proband was normal. The HRCT of the petrous bone and the HRMR of the labyrinth were both normal. Neurological examination was normal as well as lactic acid levels. The patient refused a muscular biopsy.

5) L.E., 40 years old female. The patient was affected by severe bilateral SNHL, started at age 19-yrs and progressively worsened. She used a unilateral hearing aid, since she was 26-yrs. The patient had never been exposed to aminoglycosides. We detected the homoplasmic A1555G mutation in peripheral lymphocytes mtDNA. Family history was negative for hearing impairment, and she has a normal hearing daughter. Both mother and daughter harbour A1555G change.

The vestibular function in the mother was normal. The HRCT of the petrous bone and the HRMR of the labyrinth were both normal. Neurological examination as well as lactic acid levels were both normal. Muscle biopsy was normal.

6) G.A.M., 50 years old female. The patient was affected by bilateral downsloping SNHL, started during childhood and progressively worsened (figure 3a). The patient had never been exposed to aminoglycosides. The family history was positive for hearing impairment. The patient had two hearing impaired brothers and one normal hearing sister. One of the brothers, affected with severe bilateral hearing loss, had been exposed to streptomycin before the hearing loss onset. The patient, her brothers and sister harboured the homoplasmic A1555G mutation. The mutation was also detected in her normal hearing sons (figure 3b).

The vestibular function explored in the proband was normal. The HRCT of the petrous bone and the HRMR of the labyrinth were both normal. Neurological examination as well as lactic acid levels were also normal. Muscle biopsy was normal.

7) C.D., 49 years old female. The patient was affected by bilateral severe downslipping SNHL, started at age 6-yrs and progressively worsened. The patient suffered also of type II diabetes and hypertension. The patient had never been exposed to aminoglycosides. The patient's mother was hearing impaired. The patient had two sons, both with normal hearing. We have detected the homoplasmic A1555G mutation the proband. The other relatives were not analysed.

The vestibular function was normal. The HRCT of the petrous bone and the HRMR of the labyrinth were both normal. Neurological examination as well as lactic acid levels were both normal. Muscle biopsy was normal

8) F. S., 66 years old male. The patient was affected by bilateral profound SNHL, started during childhood and progressively worsened. At age 25-yrs, since the hearing loss became severe bilaterally, the patient started using hearing aids, without substantial benefits. The patient had never been exposed to aminoglycosides. The patient suffered also of type II diabetes. The patient had four brothers, all of them hearing impaired; in particular three of them were affected by severe bilateral hearing loss and were hearing aids users. The patient's daughter was normal hearing. We have detected the homoplasmic A1555G mutation in the patient and in his brothers.

The vestibular function in the proband was normal. The HRCT of the petrous bone and the HRMR of the labyrinth were both normal. Neurological examination as well as lactic acid levels were both normal. Muscle biopsy was normal.

9) M. A. 71 years old male. The patient was affected by bilateral profound SNHL, started during childhood and progressively worsened after streptomycin assumption at age 28-yrs. At age 28-yrs, since the hearing loss became severe bilaterally, the patient started using hearing aids, without substantial benefits. The patient was also affected by a mild form of type II diabetes. The patient had two brothers and one of them was

hearing impaired and hearing aids user. The patient's daughter was also hearing impaired. We have detected the homoplasmic A1555G mutation in the father but not in the daughter.

The vestibular function in the proband was normal. The HRCT of the petrous bone and the HRMR of the labyrinth were both normal. Neurological examination, lactic acid levels and muscle biopsy were.

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Discussion

Genetic deafness amounts for about 60% of SNHL; among genetic causes of deafness, mtDNA alterations play an important role. To this regard over the last decade a number of distinct mutations in the mtDNA have been found to be associated with both syndromic and nonsyndromic forms of hearing impairment (Ballana et al. 2006, Van Camp et al. 2000; Fischel-Ghodsian 2003), although their real incidence is yet poorly understood and generally underestimated. The reported frequency of mitochondrial deafness is quite variable, ranging from 3 to 27%, depending on the variability of the studies of the literature (Gardner et al. 1997; Oshima et al. 1999; OStergaard et al. 2002; Tamagawa et al. 1996; Tekin et al. 2003; Usami et al. 2000; Usami et al. 1997): in fact most of the papers are difficult to interpret, both for the differences in the patients selection criteria (sporadic or familial cases) and for the variability in the mutations investigated and this lack of uniformity leads to quite variable and not completely comparable results (Gardner et al. 1997; Oshima et al. 1999; OStergaard et al. 2002; Tamagawa et al. 1996; Tekin et al. 2003; Usami et al. 2000; Usami et al. 1997). To this regard the reported frequency of the most frequent deafness-related mtDNA mutations, the A1555G and the A3243G, ranges from 3 to 6,9% in sporadic cases (Oshima et al. 1999; Lehtonen et al. 2000; Usami et al. 2000; Jacobs et al. 2005), while a higher rate is generally reported in familial cases, ranging from 16 to 27% (Estivill et al. 1998; Del Castillo et al. 2003; Bravo et al. 2006).

Consistently with literature data, our study results demonstrate that mitochondrial dysfunctions are a relevant cause of sensorineural deafness: 5,4% (9/167) of our patients affected with idiopathic SNHL harboured the A1555G mutation, thus confirming that the A1555G point mutation is the most frequent among the known mtDNA mutations related to sensorineural deafness and is globally one of the most relevant causes of genetic deafness. In our patients, we did not observed any other mtDNA mutations. In a previously described patient affected with profound sensorineural hearing loss and

submitted to cochlear implant we detected the A3243G point mutation at a very low level (3%) in muscle and urine, but not in peripheral blood (Mancuso et al. 2004). The A3243G mutation is one of the most common mtDNA mutations related to deafness and it is considered to cause mainly syndromic deafness and rarely nonsyndromic hearing loss (Fischel-Ghodsian 2003; Chinnery et al. 2000). Oshima et al. (1999) described three patients harbouring the A3243G mutation affected with nonsyndromic SNHL, reporting a wide variability in the audiological features as well as in the evidence of a clear matrilineal kind of transmission. Chinnery et al. reported two A3243G cases with nonsyndromic SNHL (2000) our knowledge, this is the first case observed in Italy of nonsyndromic deafness with a very low level of A3243G mutation. These data reinforce the finding that this mutation can be responsible for both syndromic and nonsyndromic deafness, similarly to other mtDNA mutations such as the A7445G (Fischel-Ghodsian 2003). In our patient, the A3243G mutation was present at very low level in muscle (3%) and in urine (1%), while was not present at peripheral blood level and it is difficult to explain how such low levels of mutant mtDNA cause mitochondrial dysfunction and clinical symptoms. We could speculate that a mutation with high pathogenic potential as A3243G may be clinically limited to deafness if present at very low levels. Furthermore, this case confirms that mitochondrial deafness is suitable of treatment by CI, as already reported (Hill et al. 2001; Raut et al. 2002; Rosenthal et al. 1999; Tono et al. 1998).

As evidenced by the over mentioned case, the heteroplasmic mtDNA mutations can be missed if researched only in peripheral blood lymphocytes and not in muscle. Therefore, we could speculate that the incidence of mtDNA mutations as a cause of deafness could be more frequent than reported in literature.

Besides being one of the most common causes of nonsyndromic SNHL, the homoplasmic A1555G point mutation in the mitochondrial 12S rRNA gene has been described in association to aminoglycoside induced deafness. This mutation was first

discovered by Prezant et al. (1993) in a large Arab-Israeli family, with a striking pattern of maternal inheritance and a quite variable clinical expression. Later it was detected in various ethnic groups from Europe (Casano et al. 1998; Casano et al. 1999; Del Castillo et al. 2003; Estivill et al. 1998; OStergaard et al. 2002), Asia (Reid et al. 1997; Tamagawa et al. 1996; Tekin et al. 2003; Usami S. 2000; Usami et al. 1997) and Africa (Gardner et al. 1997), both in familial and in sporadic cases (Bykhovskaya et al. 2004a; Casano et al. 1998; Casano et al. 1999; Li R. 2005; Kupka et al. 2002; Tekin et al. 2003; Malik et al. 2003). The reported prevalence varies from 0,5 to about 5% in Europe and 3,45% in Japan (Casano et al. 1998; Scrimshaw et al. 1999; Kupka et al. 2002), although higher percentages have been found in Spain (Estivill et al. 1998; Del Castillo 2003, Bravo et al. 2006). This reported variability is probably due to the heterogeneity of the samples of the various studies and to the different use of aminoglycoside antibiotics among the different countries.

The phenotype varies considerably among matrilineal relatives within families or among different families, ranging from severe deafness to moderate progressive hearing loss or even completely normal hearing (Ballana et al. 2006, Braverman et al. 1996; Casano et al. 1998; Casano et al. 1999; Fischel-Ghodsian 2003). Ballana et al. (2006) found that 63% of the subjects harbouring the A1555G mutation presented SNHL, while the remaining 37% were asymptomatic. In several cases a bilateral down sloping SNHL has been described, while flat or rising audiograms have been reported in a minority of cases. In our patients the hearing threshold shape was downsloping in all, with the exclusion of one patient who showed a flat curve. The age of onset is also very variable. It is more common in adult or young adult age, although congenital or childhood onset cases have been reported (Braverman et al. 1996; Casano et al. 1998; Estivill et al. 1998). In our patients, the hearing loss onset was dated in childhood or adulthood. The site of lesion is generally reported to be the cochlea, as demonstrated by otoacoustic emissions, stapedius reflex study and BEAPs results (Braverman et al.

1996; Casano et al. 1998; El-Schahawi et al. 1997; Estivill et al. 1998; Lehtonen et al. 2000; Lopez-Bigas et al. 2000; Shohat et al. 1999). Usually, the vestibular function is normal also in cases with aminoglycoside exposure. (Braverman et al. 1996; Fischel-Ghodsian 2003; Tono et al. 2001). The relationship between A1555G and aminoglycoside exposure is also quite variable, and aminoglycosides seem to play a role only in about 20% of the cases. Today in developed countries aminoglycosides are used rarely and this could affect the data (Estivill et al. 1998; Prezant et al. 1993; Fischel-Ghodsian 2003; Casano et al. 1998; Hutchin et al. 1993). Aminoglycosides seem to enhance the susceptibility to develop hearing loss in patients harbouring this mutation. However, even in the absence of aminoglycoside exposure, the mutation could be *per se* responsible of hearing impairment (Prezant et al. 1993; Estivill et al. 1998, Fischel-Ghodsian 2003; Casano et al. 1998; Hutchin et al. 1993; Lopez-Bigas et al. 2000). Some authors reported that patients carrying the A1555G mutation exposed to aminoglycosides develop a more severe deafness with an earlier onset (Bacino et al. 1995; Braverman et al. 1996; Casano et al. 1998; Casano et al. 1999; El-Schahawi et al. 1997; Estivill et al. 1998; Noguchi et al. 2004). However other studies did not confirm that hypothesis (Matsunaga et al. 2004). Two out of the three patients of our sample with profound SNHL had been exposed to aminoglycosides.

The expression of the deafness phenotype in carriers of the A1555G mutation probably requires the contribution of additional environmental and/or genetic factors. The factors that could modulate the expressivity of this mutation in hearing impairment have not yet been resolved and some hypotheses have been proposed. Aminoglycosides are probably only one of the factors interacting with the mutation in determining the deafness phenotype. Mitochondrial haplotypes may explain some of the differences between families and ethnic groups (Achilli et al. 2004; Torroni et al. 1999) and also the nuclear background may modulate the phenotypic expression of the mutation (Guan et al. 2000; Guan et al. 2001). Recent studies by Bykhovskaya et al. revealed that nuclear modifying

factors are likely to be numerous and, although a region in chromosome 8p23 has been proposed as a putative localization for a modifier locus, the gene has not been identified (2001; 2004a; 2004b). Connexin 26 gene has also been hypothesized to be a modifier gene, but according to recent studies by Lopez-Bigas and coworkers (2000) mutations in the Connexin 26 gene does not seem to modify the deafness phenotype due to the A1555G mutation. In the present study Connexin 26 and 30 genes mutation were not present in any of the subjects harbouring the A1555G mutation.

In 2003, Del Castillo and coworkers found some individuals (5,7% of the subjects) with a variable degree of heteroplasmy (from 3,75% to 96,6%). The Author attempted to correlate the rate of heteroplasmy to the severity of the phenotype and found that most of the patients with a low rate heteroplasmy (below 20%) presented a normal hearing or a mild hearing impairment, while subjects with a high rate heteroplasmy (above 52%) were affected by moderate to severe sensorineural hearing loss (Del Castillo et al. 2003).

The variability of the phenotype related to mtDNA mutations is confirmed also by our study. The audiological features of our patients harbouring the A1555G mutation are quite variable. The degree of hearing impairment was mild-moderate in 1 case, moderate in 2 cases, severe in 3 patients and profound in the remaining 3. The audiometric threshold shape was also variable, downsloping in 8 cases and flat in 1 patient. The hearing loss was progressive in all the patients, started in childhood in 4 patients and in young adulthood in the remaining 5. As far as the aminoglycoside exposure, only 2 among the 9 patients carrying the A1555G mutation developed hearing loss after exposure to those antibiotics, while in one patient there was a progression of the hearing impairment after streptomycin exposure. A matrilinear transmission was clearly evident in 6/9 patients, while in one patient the family history was positive for hearing loss, but not suggestive of a matrilinear inheritance. The site of lesion was the cochlea in all the patients, while the vestibular function was normal in every A1555G

patient, but one who presented a bilateral labyrinth areflexia and who had been exposed to aminoglycosides (see table I).

Some variability was also present between members of the same family, regarding both the degree of hearing loss and the association between hearing loss onset and aminoglycoside exposure.

In 6 of the 9 patients with the A1555G mutation we have executed a muscle biopsy and interestingly in two of them we found muscular abnormalities consistent with mitochondrial dysfunction, such as red-ragged and COX negative fibres, and mitochondrial ultrastructural abnormalities. To our knowledge only one study has been published in which a muscular study was performed in patients with nonsyndromic deafness, harbouring the A1555G point mutation (Yamasoba et al. 2002). These authors found muscular alterations in the two patients subjected to muscular biopsy. These findings, demonstrating muscular involvement in some patients affected by isolated deafness and harbouring the A1555G mutation, are extremely interesting and future researches could explain if muscular involvement in these cases plays a role in the variability of the phenotypic expression of the mutation.

It is worth noting that 3/9 patients carrying the A1555G mutation were affected by type II diabetes. To our knowledge this is the first time that diabetes is reported in patients with A1555G mutation and deafness, and further studies are necessary to establish if this association is incidental or related to the mutation. In literature there are only sporadic cases reported of A1555G patients affected with hearing impairment associated to other clinical manifestations, such as Parkinson, spinal and pigmentary disturbances (Nye et al. 2000) and restrictive cardiomyopathy (Santorelli et al. 1999; Fischel-Ghodsian 2003).

Conclusions

Mitochondrial related hearing loss can present in a variety of clinical forms, both syndromic and nonsyndromic, with variable degrees of severity. MtDNA mutations are a relevant cause of nonsyndromic hearing loss and the A1555G point mutation is one of the most frequent causes of genetic deafness.

Consistently with literature data, we found the A1555G mtDNA mutation to be responsible of a relevant percentage (5,4%) of cases affected with isolated idiopathic sensorineural hearing impairment. The age of onset and the audiological features (severity of hearing loss, the hearing threshold shape, relation with aminoglycosides exposure) were quite variable, between different families and within the same families.

Interestingly, in two patients harbouring the A1555G mutation, we found muscular abnormalities. Further researches could explain if this mutation, rather than being an organ specific mutation, might cause a systemic disorder or if the muscular involvement in these cases plays a role in the variability of the phenotypic expression of the mutation. Additionally, in three out of the nine A1555G patients, diabetes type II was associated to deafness. Further studies are needed to establish if the association is incidental or related to the A1555G mutation phenotype.

Finally, we want to highlight that as mtDNA mutations represent one of the most frequent causes of deafness, they should be routinely screened for, especially because their identification has quite different genetic counselling implications from nuclear genes mutations. Moreover, because of the susceptibility of individuals carrying the A1555G mutation and their maternal relatives to aminoglycosides exposure, at least genetic screening for this mutation among deaf patients should be considered. The early detection of the mutation would enable prevention of the onset and/or progression of hearing loss (such as avoiding exposure to aminoglycosides).

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Table I: general and audiological features of the sample of patients harbouring mtDNA mutations.

Patients	mtDNA mutation	SNHL degree	Audiometric pattern	Age at onset	HL progression	Site of lesion	Vestibular function	Aminoglycoside exposure	Family history positive for HL	Lactate kinetic	Muscular biopsy
1) A.M., m. 54 yrs	A1555G	Moderate	Bilateral, downsloping	27 yrs	Yes	Cochlear	Normal	Yes*	Yes	Normal	Pre-ragged-red fibres and COX negative and SDH hyperreactive fibres. Ultrastructural mitochondrial damage.
2) D.S., f. 34 yrs	A1555G	Mild-moderate	Bilateral, flat	15 yrs	Yes	Cochlear	Normal	No	No	Normal	Pre ragged-red fibres and COX negative and SDH hyperreactive fibres. Ultrastructural mitochondrial damage.
3) M.L., f. 66 yrs	A1555G	Profound	Bilateral, downsloping	24 yrs	Yes	Cochlear	Bilateral labyrinth areflexia	Yes*	Yes	Abnormal	Not executed
4) M.G., f. 36 yrs	A1555G	Severe	Bilateral, downsloping	18 yrs	Yes	Cochlear	Normal	No	Yes	Normal	Not executed

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5) L.E., f. 40 yrs	A1555G	Severe	Bilateral, downsloping	19 yrs	Yes	Cochlear	Normal	No	No	Normal	Negative
6) G.A.M., f. 50 yrs	A1555G	Moderate	Bilateral, downsloping	Childhood	Yes	Cochlear	Normal	No	Yes	Normal	Negative
7) C.D., f. 49 yrs	A1555G	Severe	Bilateral, downsloping	6 yrs	Yes	Cochlear	Normal	No	Yes	Normal	Negative
8) F.S., m. 66 yrs	A1555G	Profound	Bilateral, downsloping	Childhood	Yes	Cochlear	Normal	No	Yes	Normal	Negative
9) M.A., m. 71 yrs	A1555G	Profound	Bilateral downsloping	Childhood	Yes	Cochlear	Normal	Yes**	Yes ***	Normal	Not executed

*: the hearing loss started after aminoglycoside exposure.

** : the hearing loss worsened after aminoglycoside exposure.

***: the family history was not clearly suggestive of a matrilinear transmission.

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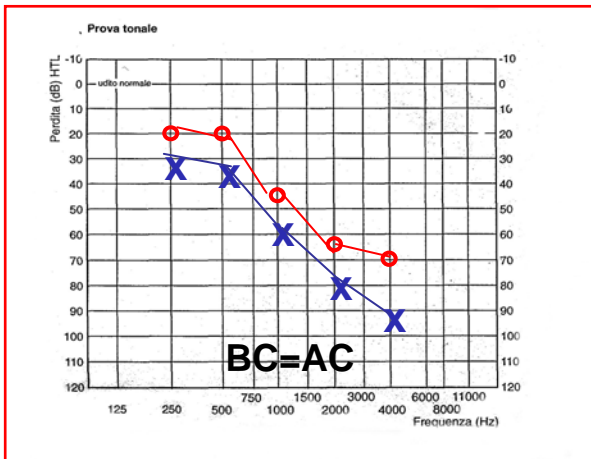
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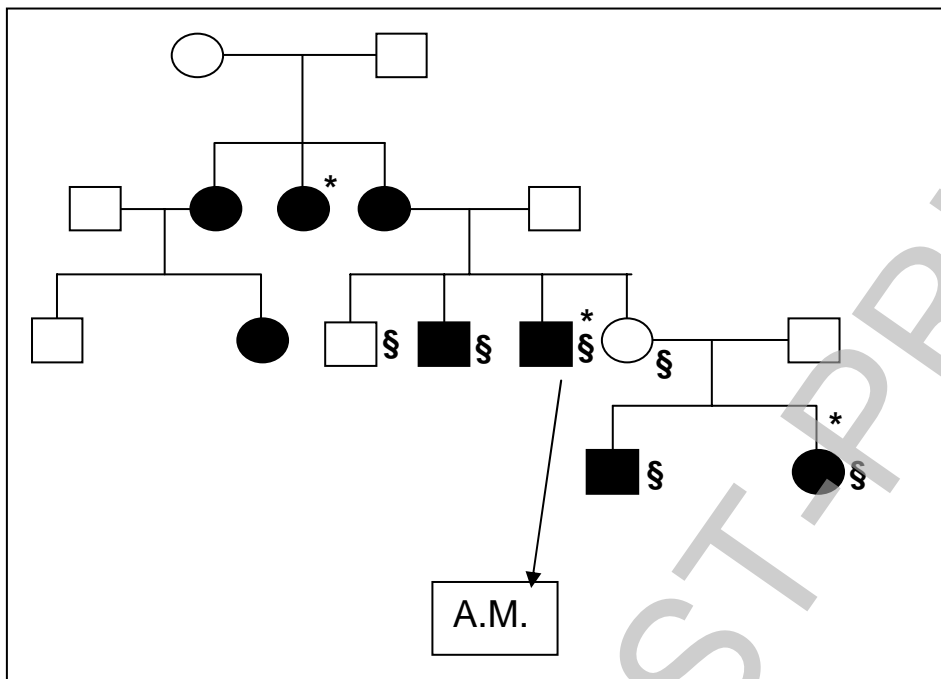
Figure 1a



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Figure 1b



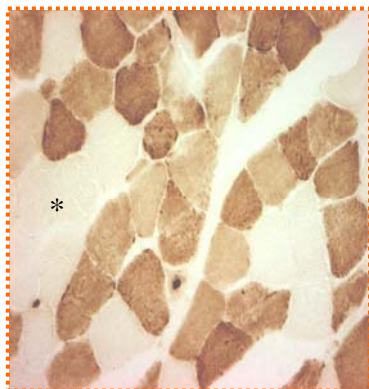
○ § subjects harbouring the A1555G point mutation

○* exposure to aminoglycosydes

○ not exposure to aminoglycosydes

- Woman normal hearing
- Woman with hearing loss
- Man normal hearing
- Man with hearing loss

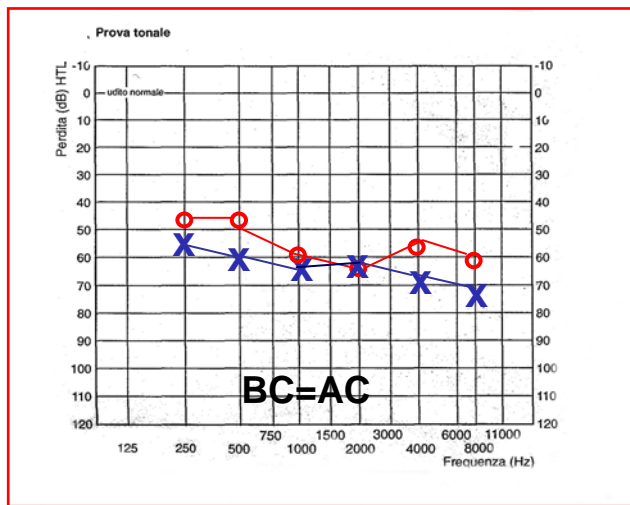
Figure 1c



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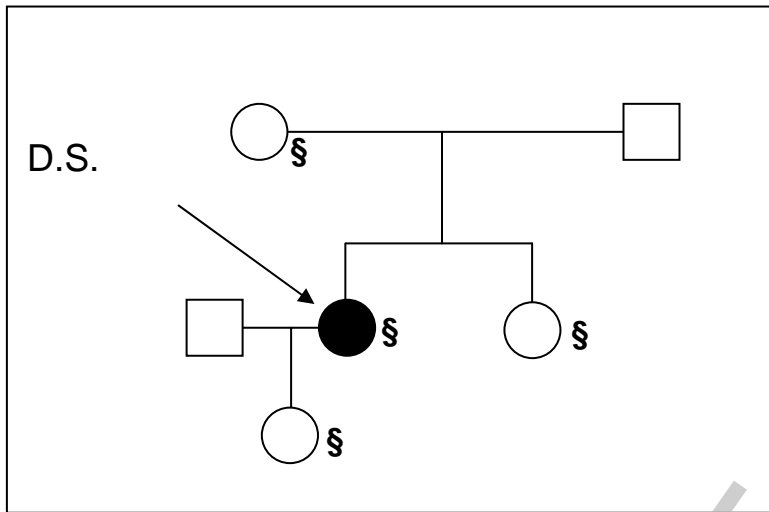
Figure 2a



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Figure 2b



○§ subjects harbouring the A1555G point mutation

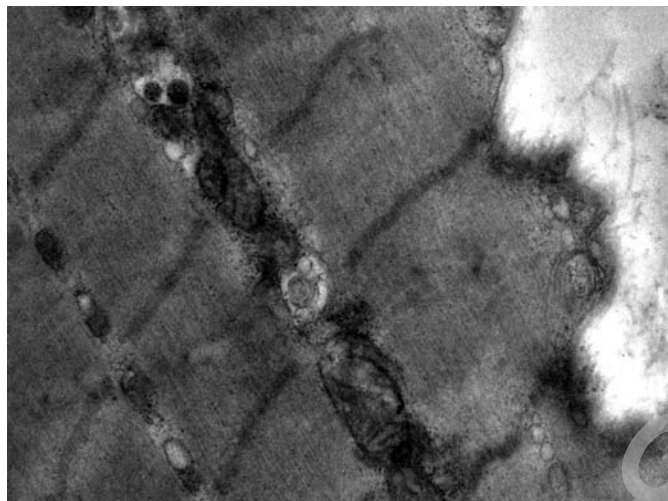
○* exposure to aminoglycosydes

○ not exposure to aminoglycosydes

- Woman normal hearing
- Woman with hearing loss
- Man normal hearing
- Man with hearing loss

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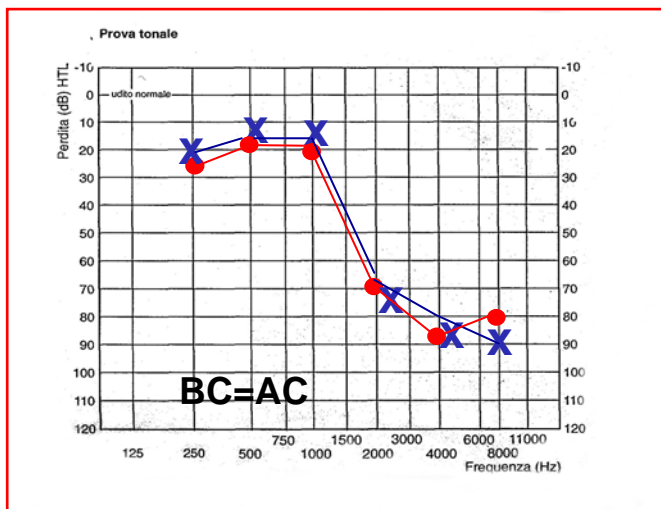
Figure 2c



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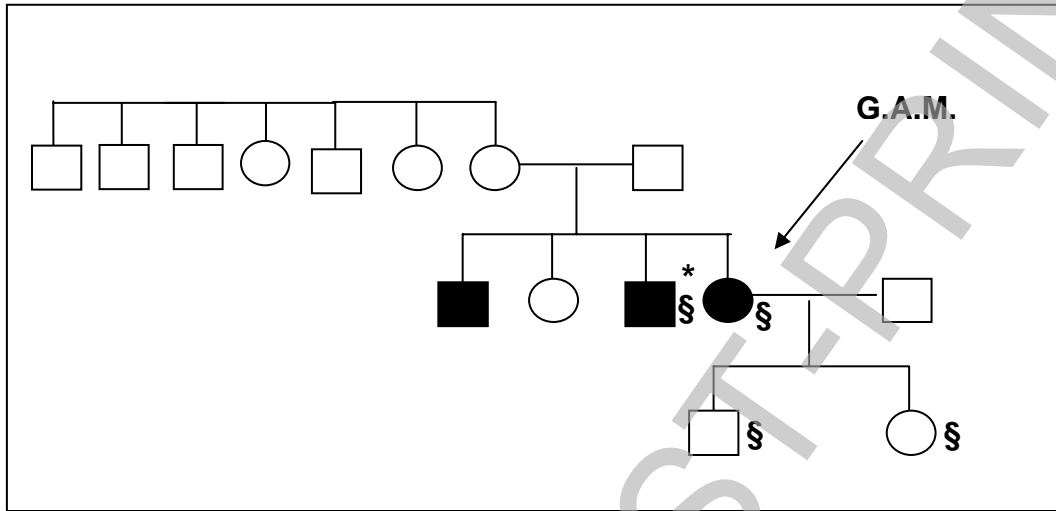
Figure 3a



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Figure 3b



○ § subjects harbouring the A1555G point mutation

○ * exposure to aminoglycosydes

○ not exposure to aminoglycosydes

- Woman normal hearing
- Woman with hearing loss
- Man normal hearing
- Man with hearing loss

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Figures' legend

Figure 1a: audiometric pattern (downsloping hearing loss) of patient A.M.

Figure 1b: Matrilinear pattern of transmission of hearing impairment in A.M.'s family.

Figure 1c: Muscle biopsy of patient A.M. Immunohistochemical assay for COX. The asterisk shows a COX negative fibres

Figure 2a: audiogram (flat shape) of patient D.S.

Figure 2b: pedigree of patient D.S.

Figure 2c: Muscular fibres with accumulation of mitochondria having variable shape and dimensions, within intermyofibrillar and subsarcolemmal sites. Mitochondria present focal loss of cristae and crystalline-like bodies. (Patient D.S.) X25,000

Figure 3a: Bilateral downsloping SNHL of patient G.A.M.

Figure 3b: Pedigree of patient G.A.M.

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