Detection of deafness-causing mutations in the Greek mitochondrial genome

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Abstract. Mitochondrion harbors its own DNA, known as mtDNA, encoding certain essential components of the mitochondrial respiratory chain and protein synthesis apparatus. mtDNA mutations have an impact on cellular ATP production and many of them are undoubtedly a factor that contributes to sensorineural deafness, including both syndromic and non-syndromic forms. Hot spot regions for deafness mutations are the *MTRNR1* gene, encoding the 12S rRNA, the *MTTS1* gene, encoding the tRNA for Ser$^{(UCN)}$, and the *MTTL1* gene, encoding the tRNA for Leu$^{(UUR)}$. We investigated the impact of mtDNA mutations in the Greek hearing impaired population, by testing a cohort of 513 patients suffering from childhood onset prelingual or postlingual, bilateral, sensorineural, syndromic or non-syndromic hearing loss of any degree for six mitochondrial variants previously associated with deafness. Screening involved the *MTRNR1* 961delT/insC and A1555G mutations, the *MTTL1* A3243G mutation, and the *MTTS1* A7445G, 7472insC and T7510C mutations. Although two patients were tested positive for the A1555G mutation, we failed to identify any subject carrying the 961delT/insC, A3243G, A7445G, 7472insC, or T7510C mutations. Our findings strongly support our previously raised conclusion that mtDNA mutations are not a major risk factor for sensorineural deafness in the Greek population.

Keywords: 961delT/insC, A1555G, A3243G, A7445G, 7472insC, T7510C, Greece, mitochondrial DNA, mutation, sensorineural deafness

1. Introduction

Clinically significant hearing loss is present in at least 1.9 per 1,000 infants at birth and the prevalence rises to at least 2.7 per 1,000 by the age of four [1]. Genetic causes of hearing loss are estimated to account for 68% of cases expressed at birth and 55% of those expressed by the age of four. Genetic deafness is divided into syndromic forms, in which hearing loss is associated with a variety of other anomalies, and non-syndromic forms. The syndromic forms account for 30% of prelingual genetic deafness and include several hundred deafness syndromes, with the underlying genetic defect being

found in about 30 of them [2,3]. In non-syndromic genetic deafness of prelingual onset, autosomal recessive inheritance predominates (80%), but autosomal dominant (20%), X-linked (1%), and mitochondrial (< 1%) forms have also been described [4]. Hearing loss can also be non-genetic in origin, induced by factors such as ototoxic drugs, perinatal infections, traumas etc. However, many cases are multifactorial, involving a collaboration of exogenous factors and mutations in single genes or several genes [5,6]. The severity of hearing loss may range from mild to profound and the damage in hearing may include all frequencies [6].

mtDNA is transmitted only through the matrilineal lineage [7]. Mitochondrial mutations are collected in the human mitochondrial genome database MITO-MAP [8]. mtDNA mutations can be divided into large rearrangements and mutations limited to a few base-pairs, the majority being point mutations. Most mtD-

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NA deletions, duplications, insertions, inversions, or other complex rearrangements involve several genes, as mitochondrial genes are located close to each other. Large deletions generally remove at least one tRNA gene and are therefore likely to cause a translational defect and dysfunction of multiple components of oxidative phosphorylation, and consequently the whole energy process [9]. Remarkably, specific mutations in tRNA genes can also lead to non-syndromic deafness, whereas other organ systems remain unaffected. This illustrates the limited genotype-phenotype correlation in mitochondrial disease. In the Caucasian population, at least 5% of postlingual, non-syndromic hearing impairment is due to known mtDNA mutations, thus representing the most frequent cause of hearing loss after the 35delG mutation in the *GJB2* gene encoding connexin 26 [10]. In oriental populations the frequency might be even higher. Especially the mitochondrial genes *MTRNR1* and *MTTS1* encoding the 12S rRNA and tRNA$^{Ser(UCN)}$ respectively, have been found to be associated with non-syndromic hearing loss. However, it is unclear which percentage of postlingual hearing loss is due to mtDNA mutations, as these mutations might be both constitutional but also acquired (somatic).

In this study of mtDNA deafness-causing mutations, we have selected specific point mutations of the mitochondrial genome reported to be associated with sensorineural hearing loss [6]. We have previously reported preliminary data for the A1555G [11] and A7445G [12] mtDNA mutations. Here we present our data of screening for the 961delT/insC, A1555G, A3243G, A7445G, 7472insC, and T7510C mtDNA mutations in the Greek deafness population, and we summarize our findings with this report and the previously reported data [11,12].

2. Materials and methods

2.1. Materials

For the present study we recruited a large cohort of 656 unrelated individuals suffering from hearing impairment of childhood onset. These patients were referred to our Department for molecular analysis from all the major childhood deafness centers of Greece, from 1999 until 2010. We used an extensive questionnaire in order to exclude cases of non-Greek origin, unilateral or mixed hearing loss, otosclerosis, history of infections during pregnancy, bacterial meningitis, need of neona-

tal ventilation, and history of head trauma associated with skull fracture and loss of consciousness. Cases with a long-term hospital intensive care treatment, poor DNA quality, and complete absence of clinical history were also excluded from the present study. By clinical genetic evaluation we separated the cases of syndromic deafness from the non-syndromic ones. Although the A1555G mtDNA mutation has been so far reported only in non-syndromic cases, in the present study we applied this protocol also in 35 cases of syndromic hearing impairment. Finally, our cohort consisted of 513 unrelated Greek patients with sensorineural, bilateral, syndromic and non-syndromic hearing loss of any degree. All cases were categorized in respect of their inheritance pattern, age of onset, and degree of hearing loss.

2.2. Methods

By pure tone audiometry the level of hearing loss was classified as mild (20–39 dB), moderate (40–69 dB), severe (70–89 dB), or profound (> 90 dB), from an average at 500–2000 Hz in the better ear.

DNA was isolated from EDTA-anticoagulated blood samples by a standard salting out procedure [13]. All samples had been previously tested for the frequent *GJB2* 35delG mutation and the coding region of the *GJB2* gene was sequenced in 35delG heterozygotes in order to investigate the possible existence of a second mutation in compound heterozygosity with the 35delG mutation (unpublished data).

Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) protocols were recruited for the detection of the mtDNA deafness mutations. The methods used for the molecular analyses of this study are summarized in Table 1. For all mutations, the restriction fragments were visualized on 2% agarose gels under UV light. The PCR protocols used in this study are available upon request. Informed consent was obtained for all participating patients. The study was approved by the Ethics Committee of the Institute of Child Health, Athens, Greece (Reference approval No: ICH/G.15).

3. Results

3.1. Clinical evaluation results

A questionnaire evaluation of the patients revealed 24 cases of non-Greek origin, six cases of bacterial

Table 1
Methods used for the molecular analyses of six mtDNA mutations associated with sensorineural hearing loss

Mutation	Mitochondrial DNA gene (GenBank, GI:251831106)	Primers	Enzyme*	Amplified fragment (bp)	
				Normal sample (bp)	Mutant sample (bp)
961delT/insC	*MTRNR1*	F: 5'–AAGAGTGTTTTAGATCACCCGC–3' R: 5'–CGGTAAGCATTAGGAATGCC–3'	BsrBI	2437	
				1713, 703, 21	1713, 724
A1555G	*MTRNR1*	F: 5'–GCTCAGCCTATATACCGCCATCTTCAGCAA–3' R: 5'–TTTCCAGTACACTTACCATGTTACGACTGG–3'	HaeIII	339	
				216, 123	216, 93, 30
A3243G	*MTTL1*	F: 5'–CCTCCCTGTACGAAAGGAC–3' R: 5'–GCGATTAGAATGGGTACAATG–3'	HaeIII	238	
				169, 37, 32	97, 72, 37, 32
A7445G	*MTTS1*	F: 5'–GGATGCCCCCCACCCTACC–3' R: 5'–CCTACTTGCGCTGCATGTGCC–3'	XbaI	216	
				168, 48	216
7472insC	*MTTS1*	F: 5'–ACATAAAATCTAGACAAAAAAGGAAGGAAT–3' R: 5'–CTTCTATGATAGGGGAAGTAGCGT–3'	XcmI	208	
				208	168, 40
T7510C	*MTTS1*	F: 5'–GGATGCCCCCCACCCTACC–3' R: 5'–CCTACTTGCGCTGCATGTGCC–3'	HinfI	216	
				150, 35, 31	98, 52, 35, 31

*New England Biolabs, Inc, Ipswich, MA, USA.

Table 2
Distribution of the deafness patients according to the inheritance pattern, age of onset, and degree of hearing loss

Cases		Non-syndromic (%)	Syndromic (%)	Total (%)
Inheritance	Sporadic	301 (58.7)	15 (2.9)	316 (61.6)
	Familial	138 (26.9)	2 (0.4)	140 (27.3)
	Unknown	39 (7.6)	18 (3.5)	57 (11.1)
Age of onset	Prelingual	378 (73.7)	15 (2.9)	393 (76.6)
	Postlingual	79 (15.4)	1 (0.2)	80 (15.6)
	Unknown	21 (4.1)	19 (3.7)	40 (7.8)
Degree of hearing loss	Mild	27 (5.3)	1 (0.2)	28 (5.5)
	Moderate	67 (13.1)	3 (0.6)	70 (13.7)
	Severe	115 (22.4)	5 (1.0)	120 (23.4)
	Severe/Profound	3 (0.6)	– (–)	3 (0.6)
	Profound	209 (40.7)	6 (1.2)	215 (41.9)
	Unknown	57 (11.1)	20 (3.9)	77 (15.0)

meningitis, four cases of rubella in pregnancy, two with toxoplasmosis in pregnancy, five cases with a long-term hospitalization, and four cases of cytomegalovirus infection during pregnancy. Forty nine cases had a complete lack of clinical history. Audiological examination revealed 33 cases of unilateral hearing loss, one case with mixed hearing loss, and two cases with otosclerosis. Laboratory testing showed that 13 cases failed to provide DNA of sufficient quality. Therefore, a total of 143, were excluded from this study. Among the 513 subjects included in the study, nineteen patients had been previously subjected to aminoglycosides. Nineteen patients had been previously subjected to aminoglycosides.

Clinical genetic evaluation of patients with additional symptoms to hearing impairment indicated that, of the 513 subjects that were included in this study, 478 were non-syndromic and 35 were syndromic cases. The results from the categorization according to the inheritance pattern, age of onset, and degree of hearing loss are shown in Table 2.

3.2. Molecular analyses results

Previously reported screening for the A1555G mt-

Table 3
Results from screening Greek patients for mtDNA deafness-causing mutations

Mutation	Available samples	Excluded	Tested	Result	Reference
961delT/insC	656	147	509	0/509	This study
A1555G	656	143	513	2/513 (0.4%)	[11] and this study
A3243G	656	150	506	0/506	This study
A7445G	656	143	513	0/513	[12]
7472insC	656	144	512	0/512	This study
T7510C	656	149	507	0/507	This study

DNA mutation included 478 unrelated Greek patients with sensorineural, bilateral, non-syndromic hearing loss of any degree [11]. Two patients were found with the A1555G mtDNA mutation. Both were previously subjected to aminoglycosides, and one of them was heterozygous for the *GJB2* 35delG mutation [11]. Screening for the A7445G mtDNA mutation included 513 unrelated Greek individuals suffering either from prelingual or postlingual bilateral, sensorineural, syndromic or non-syndromic, hearing impairment. None of the patients was found to harbor the mutation [12]. For the detection of the 961delT/insC, A3243G, 7472insC, and T7510C mtDNA mutations, PCR amplification was not possible in four, seven, one and six samples respectively, due to poor DNA quality. None of the 35 cases of syndromic hearing impairment was found to harbor the A1555G mutation in the present study. All samples were tested negative for the 961delT/insC, A3243G, 7472insC, and T7510C mtDNA mutations. The results from the mutation screening are summarized in Table 3.

4. Discussion

mtDNA mutations occur spontaneously at a high rate and most changes are neutral polymorphisms without clinical significance [15]. Furthermore, there appears to be a class of slightly deleterious mutations that modify the risks of mitochondrial disease, as in a multifactorial model, and distinct mtDNA mutations may act synergistically to modulate disease expression [16]. Consequently, it might be difficult to determine the pathogenicity of a novel mtDNA variation. Mitochondrial disease is characterized by an impressive degree of variation, and both inter- and even intrafamilial variation is the rule rather than the exception [6]. It is proposed that the accumulation of mtDNA mutations and the subsequent cytoplasmic segregation of these mutations during life is an important contributor both to the ageing process and to several human degenerative diseases [17]. Environmental factors (diet, toxic factors, etc) might further contribute to the progressive break-

down of mitochondrial function with age, resulting in a late-onset disease. Specific mtDNA mutations usually lead to progressive hearing loss with an age of onset varying from childhood to early adulthood.

To investigate the impact of known deafness-causing mtDNA mutations in a Greek deafness population, we applied rapid PCR-RFLP protocols in a large cohort of syndromic and non-syndromic cases. Our research included mtDNA mutations that have been associated with non-syndromic (A1555G, A3243G, A7445G, 7472insC, T7510C) and syndromic (A3243G, A7445G, 7472insC) hearing impairment. We also included the 961delT/insC mtDNA variant which has been reported to be pathogenic in some studies and polymorphic in others.

The 961delT/insC mutation is a deletion of a single T with an insertion of a varying number of Cs in the *MTRNR1* gene. This mutation has been found in Chinese sporadic patients [18] and two Italian families with aminoglycoside ototoxicity [19,20]. In a recent study [21], a frequency of 2% among sensorineural hearing loss Japanese patients was reported, raising the possibility of a relatively high frequency of this mutation among hearing impaired patients. However, a similar frequency was found in control subjects and the hearing loss phenotype did not segregate with the mutation in families suggesting that the 961delT/insC mutation is non-pathogenic [21]. More recent studies have provided support that 961delT/insC is a polymorphism, and might be common in Asian [22] and African populations [23].

The A1555G mutation affecting the *MTRNR1* gene, which encodes the small subunit ribosomal RNA (12S rRNA), was the first mitochondrial mutation to be associated with non-syndromic hearing loss [24]. It is probably the most common mtDNA mutation causing hearing loss as it is present in 0.5 to 1% of hearing-impaired Caucasians [25,26], although a much higher prevalence has been reported among Spanish [27] and Asian [28] patients. The A1555G mutation has been found in many families with maternally inherited, non-syndromic hearing loss, and also in sporadic patients

with hearing loss after the use of aminoglycosides. The fact that none of our 35 syndromic cases was found to harbor the A1555G mtDNA mutation further supports the notion that the mutation is only associated with non-syndromic hearing impairment. Our frequency of 0.4% (2/513) is similar to that reported in other European populations [25].

The A3243G mutation of the *MTTL1* gene causes deafness and/or diabetes [29–31]. Neurosensory hearing loss is present in almost all patients. Many patients have retinopathy, myopathy, cardiomyopathy, encephalopathy, and kidney disease [31], providing a phenotypic continuum between mitochondrially inherited deafness and diabetes (MIDD) syndrome and myoclonic epilepsy, lactic acidosis, and stroke-like episodes (MELAS) syndrome. However, cases of non-syndromic cases carrying the A3243G mtDNA mutation have been reported [32,33].

The A7445G mutation in the *MTTS1* gene (called T7445C in some earlier papers) has been reported in seven families to date [34–40]. The hearing loss in some of these families was non-syndromic, but in some families combined with palmoplantar keratoderma. The mutation was present in homoplasmic, heteroplasmic or combined homo- and heteroplasmic genotypes with large differences in the level of heteroplasmy even within families.

The 7472insC mutation was first reported in a Sicilian family [41] with sensorineural hearing impairment in some family members and a combination of deafness with ataxia, dysarthria and myoclonus in others. In a large Dutch family the 7472insC mutation was found to be responsible for non-syndromic hearing loss in all family members but one, who additionally had ataxia and myoclonus, suggesting that modifying secondary factors must account for the intrafamilial difference in penetrance of the neurologic abnormalities [42]. Additional four families with hearing loss combined with myoclonic epilepsy, ataxia, and cognitive impairment have been reported [43,44]. A recent study identified a family in which, all affected members presented with non-syndromic deafness [33].

The T7510C mtDNA mutation in the *MTTS1* mitochondrial gene appears to be extremely rare, and has been so far reported in only three families from the United Kingdom [45], Spain [46], and North America [47] presenting non-syndromic hearing loss.

In this and in previous studies [11,12] we have reported our results from screening a Greek deafness population for six mtDNA mutations associated with sensorineural hearing loss. Mitochondrial mutations lead-

ing to syndromic and non-syndromic deafness have been previously reviewed [6]. Some of these mutations, which were not investigated in this study, including the A827G, T1095C, C1494T, and G7444A mtDNA mutations could potentially be involved in sensorineural deafness in the Greek population, and their impact remains to be elucidated in future studies.

Taking into consideration that mtDNA mutations accumulate with age and have been associated with postlingual hearing loss and presbyacusis [6], there is a chance that mtDNA mutation carriers will never get tested for mtDNA mutations. Our findings indicate that mtDNA deafness-causing mutations are not major risk factors, and that other genetic or environmental factors may be more important contributors to sensorineural deafness in this population.

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