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RESEARCH ARTICLE

Detection of Unrecognized Low-Level mtDNA Heteroplasmy May Explain the Variable Phenotypic Expressivity of Apparently Homoplasmic mtDNA Mutations

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Mitochondrial DNA (mtDNA) mutations are an important cause of human disease. Most mtDNA mutations are found in heteroplasmy, in which the proportion of mutant vs. wild-type species is believed to explain some of the observed high phenotypic heterogeneity. However, homoplasmic mutations also observe phenotypic heterogeneity, which may be in part due to undetected low levels of heteroplasmy. In the present report, we have developed two assays, using DHPLC and Pyrosequencing (Biotage AB, Uppsala, Sweden), for reliably and accurately detecting low-level mtDNA heteroplasmy. Using these assays we have identified a three-generation family segregating two mtDNA mutations in heteroplasmy: the deafness-related m.1555A>G mutation in the 12S rRNA gene (MTRNR1) and a new variant (m.15287T>C) in the cytochrome *b* gene (MTCYB). Both heteroplasmic mtDNA mutations are transmitted through generations in a random manner, thus showing differences in mutation load between siblings within the family. In addition, the developed assays were also used to screen a group of deaf subjects of unknown etiology for the presence of heteroplasmy for both mtDNA variants. Two additional heteroplasmic m.1555A>G samples, previously considered as homoplasmic, and two deaf subjects carrying m.15287T>C variant were identified, thus confirming the high specificity and reliability of the approach. The development of assays for reliably detecting low-level heteroplasmy, together with the study of heteroplasmic mtDNA transmission, are essential steps for a better knowledge and clinical management of mtDNA diseases. Hum Mutat 29(2), 248–257, 2008. **© 2007 Wiley-Liss, Inc.**

KEY WORDS: mitochondria; deafness; heteroplasmy; 12S rRNA; MTRNR1; MTCYB

INTRODUCTION

Mitochondria are essential organelles in a cell as they are responsible not only for the generation of cellular energy in the form of ATP by oxidative phosphorylation, but also for the control of apoptosis [Wallace, 1999]. The 16.5-kb human mitochondrial genome encodes 13 essential respiratory chain polypeptides as well as two rRNAs and 22 tRNAs that are required for mitochondrial protein synthesis [Anderson et al., 1981]. Mitochondrial genetics features several unique characteristics including high mutation rate of the mitochondrial DNA (mtDNA) and a high copy number of the mitochondrial genome, due to the presence of hundreds to thousands of mitochondria per cell, each carrying a large and variable number of mtDNA molecules. In addition, mtDNA is generally maternally inherited [Wallace, 1992].

mtDNA mutations are an important cause of human disease and have been associated with many clinical abnormalities, including various forms of hearing loss, neuropathies, myopathies, cardiomyopathies, diabetes, Alzheimer disease, and Parkinson disease. Phenotypic heterogeneity is a hallmark of mitochondrial disorders [DiMauro and Schon, 2001]. Heteroplasmy (the coexistence of both normal and mutant mtDNA in a single individual) is present in many mtDNA disorders, so that the proportion of mutant mtDNA in any cell or tissue may be

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extremely variable, giving rise to variable clinical manifestations due to organ-specific energetic requirements [Shoffner and Wallace, 1994]. In most mtDNA disorders there seems to be a threshold effect, such that tissues function normally unless the proportion of mutant mtDNA rises above a particular level. Therefore, heteroplasmy may underlie some of the variability in penetrance and severity observed in mitochondrial diseases.

Prenatal and postnatal genetic testing and interpretation for mitochondrial disorders is problematic, mainly due to the presence of heteroplasmy and the unpredictable nature of its inheritance. The reliable measurement of heteroplasmy of mtDNA mutations in different tissues and a better knowledge of mtDNA transmission through generations, may help identify individuals who are at risk of developing specific complications and allow improved prognostic advice for patients and relatives.

In the present study, we have developed two assays for the detection and quantification of mtDNA mutations, based on denaturing high performance liquid chromatography (DHPLC) and Pyrosequencing technologies (Biotage AB, Uppsala, Sweden). By using these approaches we have identified a three-generation heteroplasmic family harboring two mtDNA variants, the deafness-related m.1555A>G mtDNA mutation in the 12S rRNA gene (MTRNR1; MIM 561000) and the novel m.15287T>C variant in the mitochondrial *cytochrome b* (MTCYB; MIM 516020) gene. We also used the newly developed assays to screen for the presence of the two substitutions, either in homoplasmy or heteroplasmy, in additional familial cases of nonsyndromic hearing loss and control samples.

MATERIALS AND METHODS

Patients and Families

Familial cases of sensorineural hearing loss have been collected from different Spanish clinical centers with the aim of studying the molecular basis of hearing loss. The Spanish control samples were unrelated blood donors, all of Caucasian origin. Informed consent was obtained from all participants prior to their participation in the study, in accordance with Hospital Son Dureta and CRG Review Board and Ethics Committee.

Family S292 was ascertained through the Genetic Service at Hospital Son Dureta in Palma de Mallorca. A comprehensive clinical history and physical examination were performed, including information on severity and age of onset of hearing impairment, the exposure to some kind of ototoxic substances such as aminoglycosides, and any other medical diagnoses of relevance. Whenever possible, pure tone hearing thresholds were determined for 125, 250, 500, 1000, 2,000, 4,000, and 8,000 Hz, measured in dB. The degree of hearing loss was defined according to the mean hearing loss as follows: normal $\geq$20 dB; mild = 20–40 dB; moderate = 41–70 dB; severe = 71–95 dB; and profound >95 dB. Total DNA from peripheral blood was extracted from 13 of the family members using standard procedures. In six of the cases, DNA was obtained from a second blood sample and saliva, to assess the possibility of sample switching or contamination and to determine the degree of heteroplasmy at a different time and in another tissue. Saliva DNA was extracted following the manufacturer's protocol from 2 ml of saliva, obtained using the Oragene DNA self-collection kit (DNA Genotek, Ontario, Canada).

mtDNA Sequencing

To confirm the sample sources and assess the possibility of paternal mtDNA inheritance, a DNA fragment of 417 bp from the mitochondrial hypervariable region 2 (HV2) was amplified (forward primer 5′-tcacaggtctatcaccctattaacc-3′ and reverse primer 5′-tgcataccgccaaaagataa-3′). The resulting PCR product was sequenced using an ABI PRISM 3730*xl* DNA Analyzer and ABI PRISM BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA). The sequences obtained were compared with the revised Cambridge reference sequence (GenBank accession number NC_001807.4).

For two brothers from Family S292, the entire mitochondrial genome was sequenced. mtDNA was PCR-amplified in overlapping fragments and each fragment was analyzed by direct sequencing and the resultant sequence data was compared with the human mtDNA consensus Cambridge sequence (GenBank accession number NC_001807.4).

Detection and Quantification of mtDNA Mutations

PCR-RFLP analysis. Detection of m.1555A>G mutation was first performed by PCR-amplifying a 340-bp DNA fragment containing the mutation site (forward primer 5′-GCTCAGC CTATATACCGCCATCTTCAGCAA-3′ and reverse primer 5′-TTTCCAGTACACTTACCATGTTACGACTTG-3′), followed by digestion with restriction endonuclease *Hae*III. In the wild type allele, digestion results in two fragments of 216 and 123 bp. The mutation specifically creates a novel restriction site, resulting in three fragments (216 bp, 93 bp, and 30 bp) [Estivill et al., 1998b].

DHPLC analysis. DHPLC was performed using the 3500-HT WAVE nucleic acid fragment analysis system (Transgenomic, Crewe, UK). For m.1555A>G mutation, a 340-bp fragment was amplified using the same primers as for the PCR-RFLP analysis. The PCR products were denatured for 5 minutes at 95°C and then gradually reannealed by decreasing sample temperature from 95°C to 24°C at a rate of –0.04°C per second. That enables the efficient formation of homo- and heteroduplexes. Reannealed PCR products were then separated using a DNASep HT Cartridge (Transgenomic), a column that allows the separation of nucleic acids in a size- and conformation-dependent manner. The column is kept in the L-7300+ oven (Transgenomic) and separation takes place by means of ion-pair reversed-phase liquid chromatography under a given analysis temperature, over a period of time and through a linear acetonitrile (ACN) gradient (flow rate of 1.5 ml/minute). Both the solvent gradient and the analysis temperature were determined using the Transgenomic Navigator Software (version 1.6.1) according to the length, sequence, and melting behavior of the amplicon. The elution of PCR products was monitored with the L-7400 UV detector at 260 nm in millivolts and analyzed using the D-7000 program (Transgenomic) and the Transgenomic Navigator Software (Transgenomic).

To elute heteroduplexes peaks, a fragment collector was used. The fractions of interest were isolated using the FCW-200 in-line Fragment Collector (Transgenomic). Fragment collection took place in two steps. A first DHPLC run was performed under the analysis conditions in order to determine the retention time of the fragments of interest and this reference data (time window) were then entered into the Fragment Collector software. In a second run, the collection event takes place at the given retention time and the collected fraction elutes in a mixture of triethylammonium acetate (TEAA), ACN, and water.

Pyrosequencing assay. Pyrosequencing technology (PSQ96MA) (Biotage AB) is a real-time sequencing method for the analysis of short to medium length DNA sequences. To detect and quantify the mutation load for m.1555A>G and m.15287T

>C carriers, specific SNP assays using Pyrosequencing were developed (for m.1555A>G assay: forward 5′-CGACATTTAACTAAAACCCCTACGC-3′, reverse 5′-GTTGGGTGCTTTGTGTTAAGCT-3′, and sequencing 5′-CACTTACCATGTTACGACT-3′ primers; and for m.15287T>C assay: forward 5′-CAGTAGACAGTCCCACCCTCACA-3′, reverse 5′-TAAGCCGAGGGCGTCTTT-3′, and sequencing 5′-CCCTCACACGATTCTTTA-3′ primers). Sequence identification was performed by the PSQ SQA software (Biotage AB), and percentage of mutation load was determined using the quantification function of the software.

Threshold Detection of m.1555A>G and m.15287T>C Mutations

DNA from a wild-type individual and a carrier of each mutation were amplified using primers 5′-TGCTCGCCAGAACACTACGA-3′ and 5′-TGGACAACCAGCTATCACCA-3′ for the m.1555A>G mutation, and 5′-ACATCGGCATTATCCTCCTG-3′ and 5′-AGTAAGCCGAGGGCGTCT-3′ for m.15287T>C. The resulting PCR-amplified fragments were cloned into pGEM-T vector (Promega, Madison, WI) and colonies were sequenced to identify two clones, one with the wild-type genotype and another with the mutation. The wild-type and mutated DNA were mixed to generate samples with known mutation loads ranging from 5 to 100%. Each sample was analyzed in triplicate using the DHPLC and Pyrosequencing assays (as described above) to determine the detection threshold for each technique. As Pyrosequencing allows the quantitative determination of mutation load with high sensitivity and specificity [White et al., 2005], a standard curve was built for both mutations.

RESULTS

Identification of a Heteroplasmic Family With Abnormal Inheritance of m.1555A>G

A three-generation family with various members affected of sensorineural hearing loss was ascertained through the Genetic Service of Hospital Son Dureta (Fig. 1A). A total of 13 subjects (seven deaf and six hearing) were analyzed both genetically and clinically. All of them were interviewed at length to identify either personal or family histories of hearing loss, use of aminoglycosides, and other clinical abnormalities. Most of the affected individuals (6/7) exhibited bilateral, sensorineural hearing impairment with clinical manifestations varying from mild to profound hearing loss and characterized by a more severe loss of hearing at high frequencies (Table 1; Fig. 2). In one case (Patient III6), hearing loss was unilateral. The age at onset of deafness was found to range from 5 to 30 years and four of the patients reported previous exposure to aminoglycoside antibiotics with the age at onset correlating with the period of drug administration. Although the pedigree did not clearly show a typical pattern of inheritance, it was compatible either with an autosomal recessive or with a maternal mode of transmission.

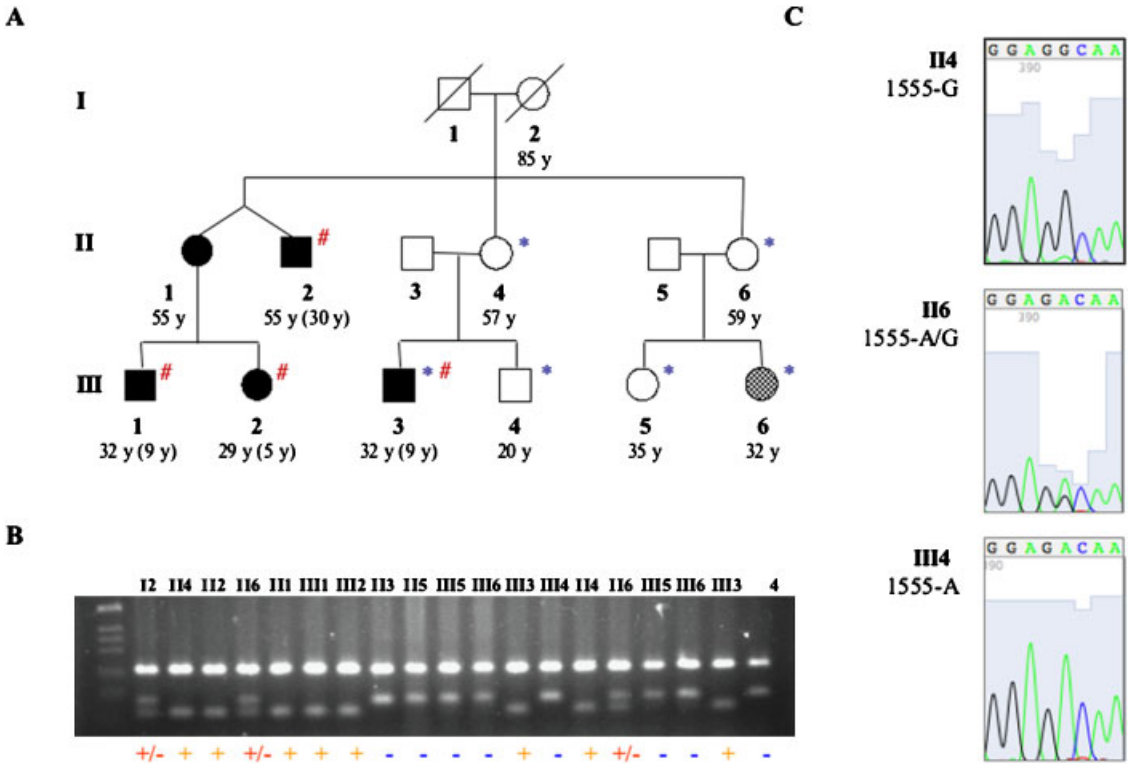

FIGURE 1. **Identification of mtDNA mutation m.1555A>G in a family with hearing impairment. A: Pedigree segregating mtDNA mutations in heteroplasmy. Solid symbols indicate clinically deaf individuals, checkered symbol a unilateral affected individual, and open symbols unaffected individuals. Age in years and age of onset (in parentheses) is shown below of subject symbols. ♯Denotes individuals who had a history of exposure to aminoglycosides. *Indicates subjects from which two independent blood samples and saliva were obtained. B: PCR-RFLP analysis of m.1555A>G mutation. In wild-type allele, digestion results in two fragments; whereas the mutant DNA shows a different restriction pattern, resulting in three fragments. C: Sequence of a mutant (Patient II4), a heteroplasmic (Patient II6) and a wild-type subject (Patient III4). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

TABLE 1. Clinical Evaluation and Heteroplasmic mtDNA Quantification in a Three-Generation Pedigree With Hearing Impairment Using Pyrosequencing Specific Assays*

	Quantification						
	m.1555A>G (%A)		m.15287T>C (%T)		Phenotype		
Patient sample	Blood	Saliva	Blood	Saliva	HL degree	Age of onset (years)	Use of aminoglycosides
I2	76.9		65.8		Normal	na	–
II1	10.1		4.7		Mild	un	–
II2	18.9		12.9		Mild	30	+
II3	98.7		99.7		Normal	na	–
II4	9.8	10.9	9.3	9.8	Normal	na	–
II5	98.7		98.7		Normal	na	–
II6	66.7	75.3	63.2	74.3	Normal	na	–
III1	10.6		7.3		Severe	9	+
III2	8.9		4.7		Profound	5	+
III3	3.9	4.3	4.7	4.7	Severe	9	+
III4	98.6	97.7	97.4	96.9	Normal	na	–
III5	94.9	89.4	94.1	93.8	Normal	na	–
III6	93.9	88.2	92.2	87.1	Moderate[a]	un	–

*Values are the mean of two independent assays with three replicate measurements each.
[a]Unilateral hearing loss.
na, not applicable; un, unknown.

As mutations in the *DFNB1* locus are the most prevalent genetic cause of hearing loss [Estivill et al., 1998a], samples were first analyzed for mutations in *GJB2* and the described deletions in *GJB6* [del Castillo et al., 2005, 2002], with negative results (data not shown). The m.1555A>G mutation was also tested by PCR amplification of a 340-bp fragment followed by the digestion with restriction endonuclease *Hae*III. Eight out of the 11 maternally-related family members were positive for mutation m.1555A>G, with two of them being heteroplasmic. Surprisingly, in three of the third-generation maternally-related subjects as well as in the two nonrelated fathers, digestion with *Hae*III failed to identify the mutation m.1555A>G (Fig. 1B). This mutation was also undetectable by direct sequencing (Fig. 1C). Remarkably, the two sons of the mother (Patient II4), who was detected as a homoplasmic carrier of mutation m.1555A>G showed contradictory results: one of them is not affected and presumably homoplasmic for the A allele and his brother is deaf and presumably homoplasmic for the G allele.

To assess the possibility of sample switching or contamination, different microsatellites were genotyped without identifying any abnormal segregation pattern and, therefore, confirming the pedigree structure (data not shown). mtDNA is assumed to be exclusively maternally inherited. There is, however, a case report of a patient with mitochondrial disease, in which a mtDNA mutation was paternally transmitted [Schwartz and Vissing, 2002]. To discard the possibility of paternal mtDNA inheritance in the present family, a fragment of mtDNA hypervariable region 2 (HSV2) was sequenced in all available family members. The probability of two random individuals showing identical mtDNA haplotypes in this region is <5% [Stoneking et al., 1991]. On the basis of the identity of DNA sequences, it was confirmed in all cases that mtDNA was maternally inherited (data not shown). These results suggested the presence of low-level heteroplasmic mtDNA in some of the members of this family, which was not detected with the traditional screening method.

DHPLC and Pyrosequencing Are Accurate and Efficient Techniques for the Detection and Quantification of Low-Level Heteroplasmic mtDNA Mutations

It has been described that PCR and sequencing alone require heteroplasmy to be present at a minimum of 20% to be detected. Thus, we developed two additional assays using more sensitive techniques to detect heteroplasmy: DHPLC and Pyrosequencing [van Den Bosch et al., 2000; White et al., 2005].

DHPLC allows a rapid and accurate detection of heterozygous and heteroplasmic mutations and has been extensively used for the diagnosis of cystic fibrosis [Le Marechal et al., 2001], breast cancer [Eng et al., 2001], and acute lymphoblastic leukemia [zur Stadt et al., 2001], among other disorders. DHPLC has also been used for mutation scanning of the whole human mitochondrial genome for both homoplasmic and heteroplasmic mutations with low proportion of mutant mtDNA [Wulfert et al., 2006].

To assess the sensibility of DHPLC for detecting heteroplasmy for the m.1555A>G mutation it was necessary to produce heteroplasmic mtDNA samples with known mutation load. This was achieved by mixing cloned wild-type and mutant fragments of mtDNA at known concentrations to produce a series of samples with mutation loads between 5% and 100%. These samples, as well as the patients' samples, were subjected to DHPLC analysis. By analyzing the DHPLC patterns obtained from the known mutation load mixtures, we tested the sensitivity and specificity of the assay. From these results, we could identify heteroplasmy from 10% of wild-type mtDNA (1555A), but it was easier to detect low levels of mutant mtDNA (1555G), as low as 5% (Fig. 3A).

When the family samples were analyzed, clear heteroduplex patterns were observed for Patients I2, II6, III5, and III6. This confirms the presence of mutation m.1555A>G in heteroplasmy in two cases (Patients I2 and II6) and allows the identification of two additional heteroplasmic samples (Patients III5 and III6) (Fig. 3B). In addition, the comparison of DHPLC patterns from the family members with those obtained from the standard mixtures, indicated that some other samples could harbor low-level heteroplasmic mtDNA, especially in the case of Patients II1, II2, II4, and III3 (Fig. 3B). To circumvent this problem and clearly identify heteroplasmic samples, we used a fraction collector to elute the heteroduplex fraction, which represents an equimolar mixture of mutant and wild-type DNA strands. These fractions were PCR amplified, then they were suitable for mutation identification through DNA sequencing, as fragment collection enriched them with the low-level mtDNA genome (Supplementary Fig. S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). Using this approach, the presence of heteroplasmy, even at very low levels, was confirmed in

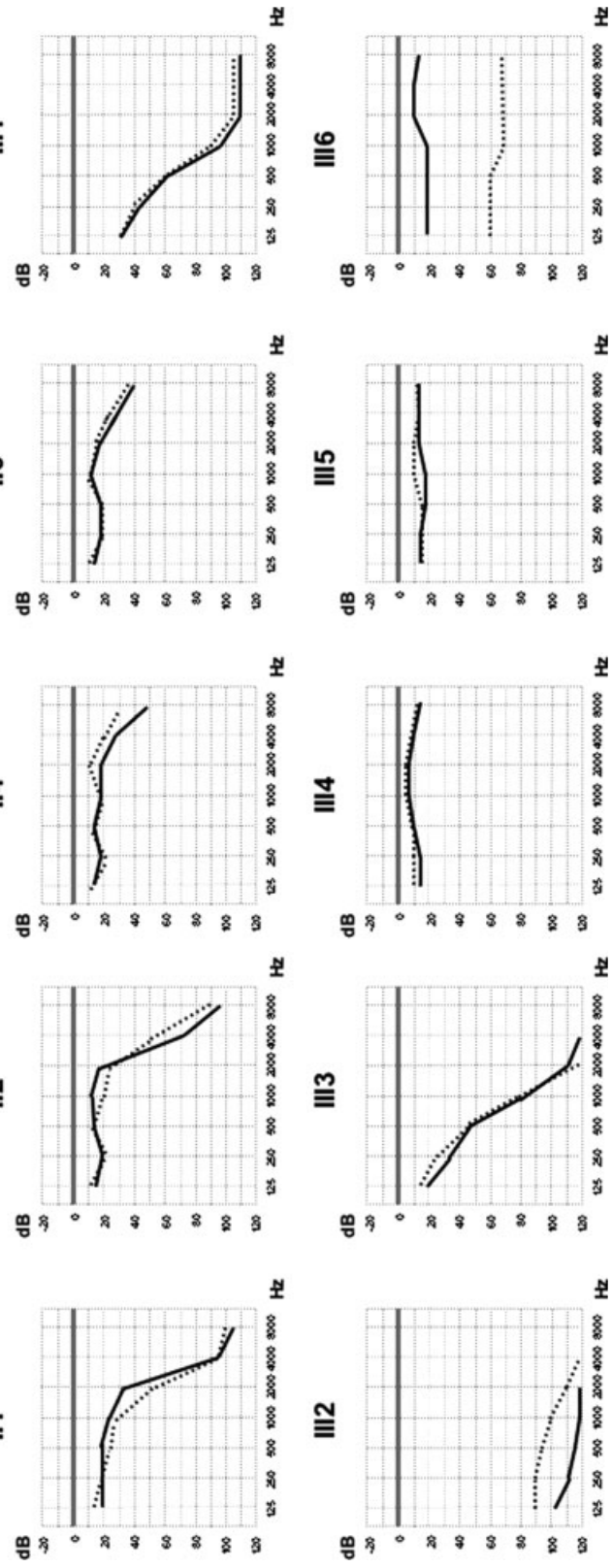

FIGURE 2. Pure tone audiometries of the three-generation pedigree subjects segregating mtDNA mutations in heteroplasmy. Hearing level (in dB) is plotted vs. sound frequency (in Hz). Pure tone hearing thresholds were determined for 250, 500, 1,000, 2,000, 4,000, and 8,000 Hz, measured in dB nHL (based on normal hearing subjects). The PTA was calculated from the sum of audiometric thresholds at 500, 1,000, 2,000, 4,000, and 8,000 Hz. Solid lines, right ear; dashed lines, left ear.

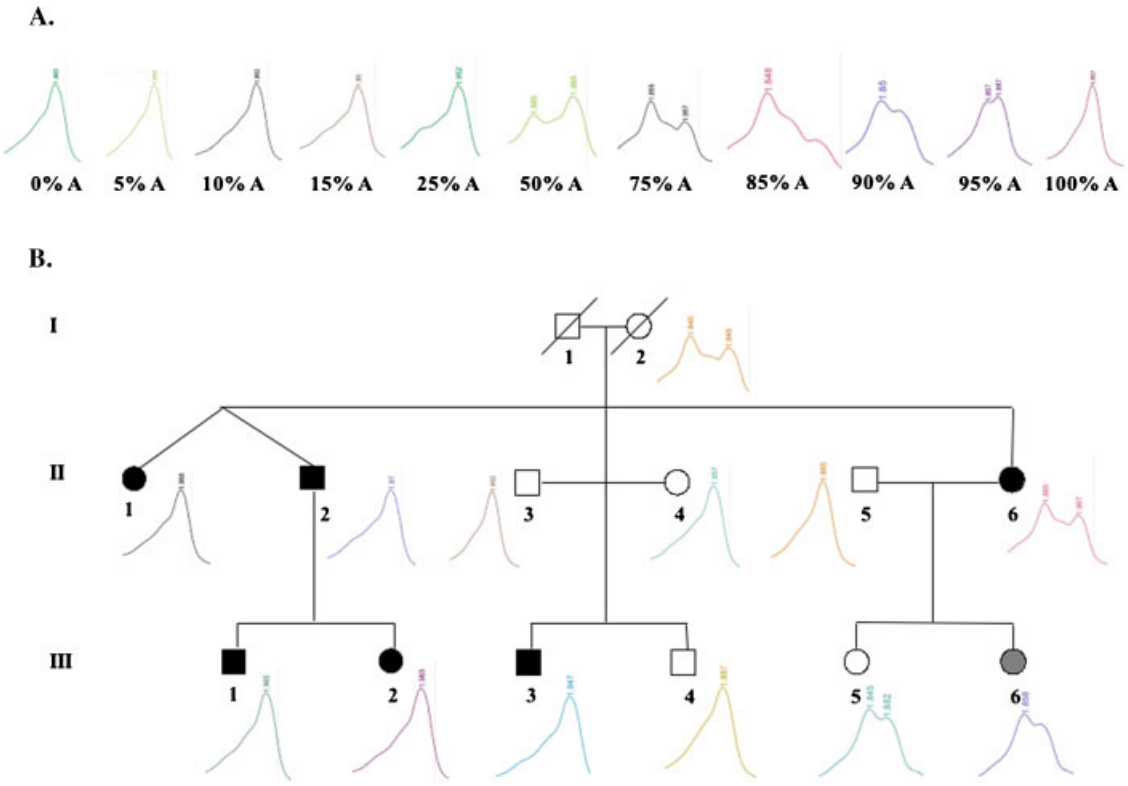

FIGURE 3. **DHPLC profiles for mutation m.1555A>G. A: DHPLC patterns of standard samples with known mutation load. B: DHPLC patterns of the three-generation pedigree segregating m.1555A>G mutation in heteroplasmy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

all maternally-related subjects, except for Patient III3, one of the discordant brothers, in which only mutant mtDNA could be detected.

Pyrosequencing technology has emerged as a new and accurate method for detection of single nucleotide polymorphisms (SNPs) [Fakhrai-Rad et al., 2002] and has allowed the detection and quantification of specific mtDNA mutations [Andreasson et al., 2002; White et al., 2005]. A specific pyrosequencing assay was developed for detecting and quantifying the m.1555A>G mutation. To assess specificity, sensitivity, and accuracy of the assay, we used the known mutation load mixtures standards previously used for testing DHPLC performance (Supplementary Fig. S2). A level of 5% heteroplasmy was reliably detected (standard deviation [SD] 1.7). Quantification of three replicate experiments showed a linear regression with a regression coefficient of 0.991x, an intercept of 0.465, and an R^2 of 0.999. This demonstrates the specificity of the pyrosequencing assay and its high sensitivity and accuracy of quantification.

The quantification of the family samples allowed us the identification of both wild-type and mutant mtDNA in all maternally-related individuals with highly variable proportions of wild-type DNA ranging from 8% to almost 100%, except for Patient III3, where again only mutant mtDNA could be identified (Table 1). This could be due to a real absence of wild-type mtDNA or, alternatively, the levels of wild-type mtDNA would be under the detection threshold of the pyrosequencing assay (less than 5%). Two different blood samples were obtained from six family members, to discard manipulation errors and to study mutation load variations over time. Linear regression was performed for testing whether the slope is equal to 1 (perfect correlation), indicating no differences between two blood samples (p-value = 0.8303). These results indicate no time-dependent variation in mutation load and give further evidences of the reliability of the techniques.

The effect of heteroplasmy in the severity of hearing loss was also investigated. All normal-hearing family members had more than 75% wild-type mtDNA, except for Patient II4. However, this is a common situation in carriers of mutation m.1555A>G. Deaf family subjects had less than 20% wild-type mtDNA, with the exception of Patient II6. Thus, no clear genotype–phenotype correlation could be drawn from these data.

mtDNA Heteroplasmy Quantification in Other Tissues and Samples

To investigate the distribution of heteroplasmy between tissues in a single individual, DNA from saliva was also extracted in six of the subjects. The proportion of A:G heteroplasmy at position 1555 in the saliva-obtained mtDNA was examined by PCR-RFLP, sequencing, DHPLC, and Pyrosequencing. No significant differences in mutation load between blood and saliva were found in the six subjects tested, when linear regression was performed to test whether perfect correlation (slope is equal to 1) (p-value = 0.99). These results suggest that the mutation is in the germline rather than somatic and that heteroplasmy is established early in development.

Both assays were used to screen the presence of m.1555A>G mutation in 190 deaf subjects of unknown cause, with negative results. A total of 69 additional pedigrees known to carry the m.1555A>G mutation [Ballana et al., 2006] were also analyzed, with the aim of detecting low-level heteroplasmy not previously identified with less sensitive techniques. We detected two new heteroplasmic samples, which carry low-level wild-type mtDNA

and were previously misclassified as homoplasmic. Thus, both DHPLC analysis and Pyrosequencing are robust, effective, and efficient for detecting and quantifying mtDNA mutations, and much more sensitive and accurate than the PCR-RFLP technique.

Heteroplasmic m.1555A>G Mutation Is Inherited With m.15287T>C in *cytochrome b*

Hearing loss associated with mutation m.1555A>G is characterized by incomplete penetrance and variable expressivity of hearing impairment. This phenotypic variability is thought to be due to the contribution of additional genetic factors, such as nuclear modifier genes or mitochondrial haplotype [Bykhovskaya et al., 2000, 1998, 2001; Guan et al., 2000, 2001].

To further investigate the role of mtDNA genome in the phenotypic variability and transmission pattern of heteroplasmic mtDNA in the family reported here, we sequenced the entire mtDNA of the two discordant brothers (Patients III3 and III4). Surprisingly, together with m.1555A>G, another nucleotide difference was identified between them: m.15287T>C, a novel mtDNA variant in the *MTCYB* gene, present also in the m.1555A>G carrier (Table 2). Variant m.15287T>C results in an amino acid change; phenylalanine at position 181 is replaced by leucine (p.MT-CYB:Phe181Leu; GenBank NP_536855.1). This residue is evolutionary conserved at both the DNA and protein level, thus giving indirect evidence of a putative pathogenic role of m.15287T>C (Fig. 4).

All the family members were analyzed for the presence of the m.15287T>C variant by direct sequencing and pyrosequencing. The pyrosequencing assay was used to quantify the m.15287T>C mtDNA variant with the same strategy as for mutation m.1555A>G. Variant m.15287T>C was identified in heteroplasmy in all family members that also carried the m.1555A>G mutation, but not in the noncarriers. The specificity, sensitivity, and accuracy of the m.15287T>C assay were also assessed using pyrosequencing known mutation load mixtures standards (Supplementary Fig. S2). Quantification of three replicate experiments showed a linear regression with a regression coefficient of 0.9483x, an intercept of 4.4651, and a R^2 of 0.972. Although this assay performs a little worse than the m.1555A>G assay, sensitivity and accuracy for mutation load quantification are still very high.

The segregation pattern and quantification results obtained for variant m.15287T>C are very similar to that observed for mutation m.1555A>G (Table 1). These results indicate that both changes may have arisen in the same maternal ancestor and were then cosegregated. The analysis of 181 Spanish unrelated control samples resulted in the identification of one sample carrying m.15287T>C in homoplasmy. This sample did not carry the m.1555A>G mutation as well as neither did any of the other controls tested. In addition, 190 additional index cases affected of hearing loss of unknown etiology, were analyzed for the presence of variant m.15287T>C, identifying two additional nuclear families in homoplasmy (Supplementary Fig. S3). One of them was previously reported to carry the 1243T>C variant in the 12S rRNA gene, which also has an unknown role in the pathogenesis of deafness [Ballana et al., 2006].

TABLE 2. **mtDNA Sequence Variants and Amino Acid Changes Identified in the Two Brothers (Patients III3 and III4) Compared to the Human mtDNA Consensus Cambridge Sequence***

	Sample	
Gene	**Patient III3**	**Patient III4**
MT-RNR1	m.750A>G	m.750A>G
	m.1438A>G	m.1438A>G
	m.1555A>G	
MT-ND1	m.3915G>A (Gly203Gly)	m.3915G>A (Gly203Gly)
MT-TI	m.4314T>C	m.4314T>C
MT-ND2	m.4727A>G (Met86Met)	m.4727A>G (Met86Met)
	m.4769A>G (Met100Met)	m.4769A>G (Met100Met)
MT-ATP6	m.8860A>G (Thr112Ala)	m.8860A>G (Thr112Ala)
MT-CO3	m.9380G>A (Trp58Trp)	m.9380G>A (Trp58Trp)
MT-ND4	m.11253T>C (Ile165Thr)	m.11253T>C (Ile165Thr)
MT-ND6	m.14356C>T (Val106Val)	m.14356C>T (Val106Val)
MT-CYB	m.15287T>C (Phe181Leu)	
	m.15326A>G (Thr194Ala)	m.15326A>G (Thr194Ala)
MT-DLOOP	m.239T>C	m.239T>C
	m.263A>G	m.263A>G
	m.302.303insCC/CCC[a]	m.302.303insCC/CCC[a]
	m.310.311insC	m.310.311insC
	m.16362T>C	m.16362T>C
	m.16482A>G	m.16482A>G

*The only sequence differences between the patients are mutations m.1555A>G and m.15287T>C; Cambridge sequence (GenBank accession number NC.001807.4).
[a]A variable number of C were found to be inserted in between positions 302 and 303.

DISCUSSION

Mutations in mtDNA are recognized as an important cause of disease, with over 200 pathogenic defects identified in the mitochondrial genome [Brandon et al., 2005; Chinnery and Turnbull, 2000]. The presence of heteroplasmy, the threshold effect, and differential tissue distribution present a diagnostic challenge for clinicians dealing with patients harboring mtDNA mutations [Chinnery and Turnbull, 2000]. In the present study we have used two technologies for the reliable measurement of heteroplasmy in different tissues: DHPLC and pyrosequencing, to more easily overcome the problems derived from mtDNA heteroplasmy. The performance of both approaches was examined with two different mtDNA variants: the deafness-associated m.1555A>G mutation and a novel variant in the *MTCYB* gene,

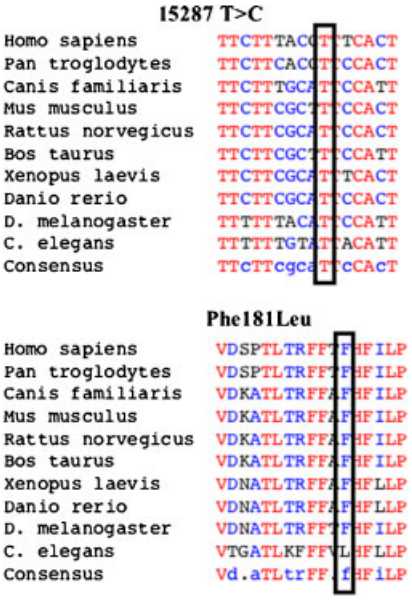

FIGURE 4. **Conservation of variant m.15287T>C across species at the DNA and protein levels. At both levels, T at position 15287 and Phe at position 181 are conserved throughout evolution; with the exception of *C. elegans* in which Phe is replaced by Leu. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

m.15287T>C, both identified in a three-generation heteroplasmic pedigree affected with nonsyndromic hearing loss.

The m.1555A>G mutation is located in the mitochondrial small ribosomal RNA gene (12S rRNA) and has been associated with aminoglycoside-induced, nonsyndromic hearing loss in many families worldwide [Ballana et al., 2006; Casano et al., 1998; Estivill et al., 1998b; Fischel-Ghodsian et al., 1993; Hutchin et al., 1993; Prezant et al., 1993]. In contrast with most mtDNA mutations, which are almost always found in heteroplasmy, m.1555A>G is usually homoplasmic. Heteroplasmy for mutation m.1555A>G has only been described in seven families, with variable mutation loads reported [el-Schahawi et al., 1997; del Castillo et al., 2003]. Using the newly-developed assays, we have detected low-level mtDNA heteroplasmy in a three-generation pedigree, solving a first-sight discordance in mtDNA inheritance. In addition, two subjects previously considered homoplasmic for the m.1555A>G mutation were also recognized as heteroplasmic, suggesting that the use of traditional screening methods, which have a lower sensitivity, such as PCR-RFLP or direct sequencing, could have prevented the detection of low-level m.1555A>G heteroplasmic subjects [Sekiguchi et al., 2003].

It has also been reported that mtDNA mutations can segregate and accumulate in certain tissues, because the level of heteroplasmy is frequently lower in blood leukocytes compared to affected tissues [Meierhofer et al., 2005]. We obtained mtDNA from saliva, which is a noninvasive technique that has demonstrated a good performance in the assays and allowed us to draw inferences regarding the origin of mtDNA heteroplasmy. The fact that no differences in mutation load were identified between tissues suggests that heteroplasmy is established early in development, at least in the first generation and is then inherited to the next generations.

Similar to our results, only in a few of the previously described m.1555A>G heteroplasmic cases was the estimated mutation load correlated with the severity of hearing loss [del Castillo et al., 2003]. Thus, the contribution of heteroplasmy in the phenotypic differences associated with m.1555A>G mutation seems to be small in most of the cases. However, it is important to note that although no differences in mutation load were identified between the two distinct tissues analyzed, the exact situation in the cochlea cannot be inferred with certainty. Taking this into account together with the difficulties in the establishment of genotype–phenotype correlations when hearing status is involved, the slight correlation found on this family is of relevance.

On the other hand, additional genetic factors may determine the differences in the phenotypic expression of mutation m.1555A>G. The search of additional mtDNA factors that could influence the phenotype associated with m.1555A>G mutation in this three-generation heteroplasmic pedigree resulted in the identification of a novel mtDNA variant in the mitochondria-encoded *MTCYB* gene, m.15287T>C, cosegregating with the m.1555A>G mutation. The most likely explanation for this cosegregation is that the two sequence changes occurred simultaneously, or nearly so, within the same mtDNA molecule in a recent maternal ancestor and were then cosegregated in the matrilineal progeny.

Although position 15287 is conserved both, at DNA and protein levels, the pathogenic role of variant m.15287T>C is unclear. Cytochrome *b* plays a central role as a catalytic subunit in complex III of the respiratory chain [Fisher and Meunier, 2001]. Several point mutations in human *MTCYB* have been associated to several diseases, such as Leber hereditary optic neuropathy (LHON), mitochondrial myopathy, isolated complex III deficiency, and mitochondrial encephalopathy, all characterized by impaired complex III activity [Andreu et al., 1999; Legros et al., 2001]. Hearing impairment is the sole clinical symptom of m.15287T>C carriers; none of them presenting evidence of respiratory complex III deficiency. Thus, m.15287T>C variant might be a polymorphism with no pathogenic effect. Alternatively, it could have a specific role in the inner ear, which would determine the onset and severity of hearing impairment when found together with 12S rRNA mutations, as suggested by the fact that m.15287T>C in deaf cases has been found associated with m.1555A>G or 1243T>C. Further studies are needed to determine the role of this m.15287T>C variant, specially in conjunction with 12S rRNA mutations.

The study of pedigrees segregating mtDNA heteroplasmic mutations provides insight into the complex process of mtDNA transmission. In the family reported here, third generation individuals are effectively fixed either for wild-type or mutant mtDNA. Studies at cellular level of pathogenic mtDNA point mutations suggest that only those that allow survival of the oocyte, either because they are present at low-levels or lead to little biochemical defects, will be passed on [Blok et al., 1997; Howell et al., 1992, 1996; Parsons et al., 1997]. Mutation m.1555A>G has been shown not to have severe functional effects, compromising cell survival only in cochlear hair cells [Guan, 2004]. This could explain the apparently random fixation of m.1555A>G nearly in homoplasmy in one-half of the third-generation subjects on the pedigree studied, in comparison with other mutations showing more severe functional effects in which mtDNA heteroplasmy levels are maintained with less significant fluctuations [Jenuth et al., 1996; Poulton et al., 1998].

In summary, the newly developed assays have demonstrated to be useful to reliably detect heteroplasmy, which is an important issue for a better management of mtDNA diseases. Issues of sensitivity, specificity, and labor-intensive methodologies inherent to classical molecular genetic techniques have been overcome by the use of DHPLC and pyrosequencing. Moreover, the two techniques are complementary rather than excluding. DHPLC can be used as a screening strategy to identify novel mtDNA variants, as it is a rapid, cost-effective, and sensitive method for detecting mtDNA mutations and polymorphisms. Once the variant is identified, the mitochondrial mutation load can be quickly and accurately detected and quantified by pyrosequencing. The use of different DNA sources, together with the implementation of these technologies, can help the identification, better treatment, and risk assessment of individuals with disease-associated mtDNA mutations, as well as the evolutionary and forensic studies based on mtDNA polymorphisms.

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