Molecular basis of deafness linked to mitochondrial DNA mutations

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Molecular basis of deafness linked to mitochondrial DNA mutations

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Xavier Estivill Pallejà **Ester Ballana Guix**

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Contents

Preface

La seqüenciació del genoma humà ha marcat una fita important en la història de la biologia. Com a conseqüència, la genètica i la genòmica han experimentat un progrés enorme. Això ha permès un millor coneixement tant de les causes genètiques de malalties humanes, com del per què de les diferències comunes entre individus.

Com a sistemes complexos que som tots els éssers vius, hem de considerar el paper que tenen les interaccions entre les diferents parts del genoma a l'hora d'especificar el resultat final, és a dir, el fenotip. Igualment, podem dir que el genoma conté un conjunt d'instruccions, però que la forma en què aquestes es porten a terme depèn, també de contingències ambientals i històriques. Per tant, la naturalesa de les instruccions genètiques no és completament determinista en tots els casos, si bé hi ha una sèrie de processos en què sí que es compleix aquesta perfecta relació entre herència i expressió final. Aquesta mateixa situació es presenta amb certes alteracions genètiques i amb el desenvolupament de patologies, la qual cosa facilita enormement el diagnòstic precoç i obre les possibilitats per a la teràpia genètica. Però la gran majoria de fenotips, incloent-hi moltes condicions d'interès per a la medicina, tenen una base complexa, és a dir, no existeix "el gen" que determina el caràcter de forma unívoca, sinó que aquest és el resultat de l'acció simultània de molts gens, no tots amb la mateixa participació, juntament amb l'efecte de l'ambient.

Aquesta tesi doctoral va arrencar en aquest punt, tenint com a objectiu l'aprofundiment en les bases genètiques d'un tipus de sordesa lligada a mutacions al

DNA mitocondrial i de la qual se'n tenien evidències de la implicació tant de factors ambientals com diversos factors genètics.

D'altra banda, els tests basats en l'ADN són un dels primers usos comercials i d'aplicació mèdica d'aquests nous descobriments de la genètica. Aquests tests poden ser utilitzats per al diagnòstic de malalties, confirmació diagnòstica, informació del pronòstic, així com del curs de la malaltia, confirmar la presència de malaltia en pacients assimptomàtics i amb diferents graus de certesa, predir el risc de futures malalties en persones sanes i en la seva descendència. Aquest és l'objectiu final, i sovint encara utòpic, de la recerca en biomedicina: una millor comprensió del procés biològic, que derivi en un millor tractament i prevenció de la malaltia.

Aquesta tesi també ha volgut contribuir humilment en aquest aspecte. Durant aquests anys s'han recollit centenars de mostres de famílies sordes, amb la finalitat de donar un "diagnòstic" de la causa genètica. Poques vegades ho hem aconseguit, però en qualsevol cas, si això alguna vegada ha ajudat a algú d'alguna manera, ja em dono per satisfeta.

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Introduction

1. The auditory system

As a finely developed system, evolved to detect small rapid changes in pressure, the auditory system is a masterpiece. Through a combination of mechanical tricks and physiological processing, the process of hearing allows humans and animals to interact with a complex environment, and to communicate with one-another.

1.1 ANATOMY OF THE AUDITORY SYSTEM

The mammalian ear contains sense organs that serve two different functions: hearing and equilibrium. It is composed of three parts, which are anatomically and functionally different: the outer ear, the middle ear and the inner ear (Figure 1.1A).

The **outer ear** consists of the visible portion called auricle, or pinna, which projects from the side of the head to the tympanic membrane. The function of the outer ear is to collect sound waves and guide them to the tympanic membrane. The **middle ear** is a narrow, air-filled cavity in the temporal bone, where a chain of three tiny bones is located: the malleus, the incus, and the stapes, collectively called the auditory ossicles. This ossicular chain conducts sound from the tympanic membrane to the inner ear. The **inner ear** is a system of fluid-filled passages and cavities located within the temporal bone. The inner ear consists of two functional units: the vestibular apparatus, consisting of the vestibule and semicircular canals, which contains the sensory organs of postural equilibrium; and the snail-like cochlea, which contains the sensory organ of hearing. These sensory organs are highly specialized endings of the eighth cranial nerve, also called the vestibulocochlear nerve.

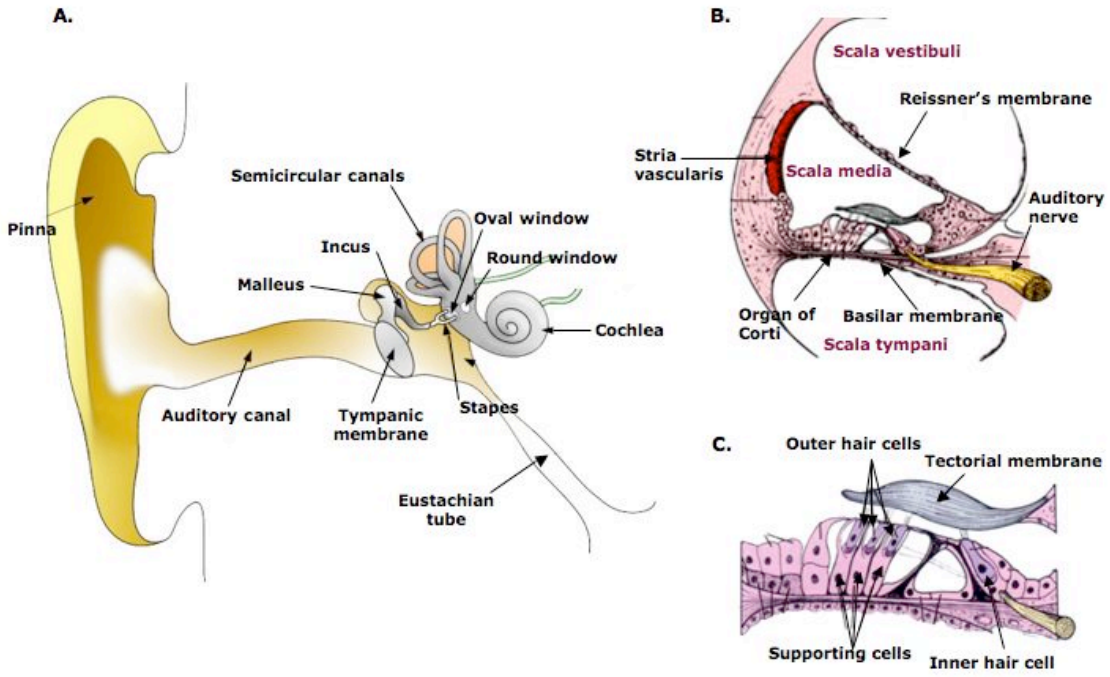

Figure 1.1. The human ear. **A.** Schematic drawing of the human ear. **B.** Transversal section of the cochlea. **C.** Organ of Corti.

The **cochlea** is a coiled, tapered tube containing the auditory branch of the mammalian inner ear. Its main component is the Organ of Corti, the sensory organ of the auditory system, which converts the sound waves into electrical impulses (Figure 1.1B).

The cochlea is divided into three fluid-filled compartments (scala timpani, scala vestibuli and scala media), separed by two membranes (Reissner's membrane and basilar membrane). The scala vestibuli and scala timpani are bathed with perilimph and the scala media is filled with endolimph. Perilimph and endolimph have different ionic concentrations, which create the so-called endocochlear potential and make possible the transduction of sound signals. This direct current potential difference is about +80 millivolts and results from the difference in potassium content between the two fluids: the perilimph concentrarion is similar to other corporal fluids, with a high sodium concentration and low potassium, whereas the endolimph is an extracellular fluid with unusually high potassium concentration. The endocochlear potential is thought to be maintained by the continual transport of potassium ions from the perilymph into the cochlear duct by the stria vascularis, an epithelia located in the lateral wall of the scala media.

The **Organ of Corti** is seated on the basilar membrane. Different cell types can be distinguished in the Organ of Corti: sensory cells, known as hair cells; supporting cells and neurons (Figure 1.1C). There are two types of hair cells, a row of inner hair cells and three rows of outer hair cells. The inner hair cells are pure

receptor cells that transmit signals to the acoustic nerve and the auditory cortex. The outer hair cells have both sensory and motor elements that contribute to hearing sensitivity and frequency selectivity by amplifying sound reception. Each hair cell has in its apical pole a bundle of stereocilia, packed with actin filaments and linked to each other at their tips. Stereocilia are the mechanoreceptive structure of the hair cells as they deflect in response to sound vibration. The stereocilia are bathed by endolimph, whereas the basolateral cell surfaces of the hair cells are bathed by perilimph. The tectorial membrane is an extracellular gel-like matrix positioned above the hair cells that provides a mass against which stereocilia can bend.

Other cell types present in the Organ of Corti are supporting cells and neurons. There are different types of supporting cells surrounding the hair cells. Its main function is the recycling of ions to maintain the endocochlear potential. The spiral ganglion contains the cell bodies of the neurons innervating the Organ of Corti. It contains around 35000 neurons, of two different types: type I, which innervate inner hair cells; and type II, which contact with outer hair cells. The axons of these neurons form the auditory nerve that projects to the brainstem, forming the cranial nerve VIII.

1.2 PHYSIOLOGY OF HEARING

The outer ear directs sound waves from the external environment to the tympanic membrane. Sound waves impinging on the head are captured by the auricle, the visible portion of the outer ear, and conveyed through the auditory canal, to the tympanic membrane. When the sound waves reach the tympanic membrane, it vibrates: the greater the force of the sound waves, the greater the deflection of the membrane and the louder the sound; the higher the frequency of a sound, the faster the membrane vibrates and the higher the pitch of the sound is. The motion of the membrane is transmitted through the middle ear by the ossicular chain: the malleus is connected to the tympanic membrane; the stapes is attached to the oval window in the inner ear; and the incus articulates with both ossicles. The action of the stapes transmits the sound waves to the perilymph of the scala vestibuli.

The mechanical vibrations of the stapes at the oval window create pressure waves in the perilymph of the scala vestibuli, which is then transmitted to the endolymph inside the cochlear duct (Figure 1.2A). As a result, the basilar membrane vibrates, causing the organ of Corti to move against the tectorial membrane. These leads to bending of the stereocilia, thereby opening potassium

channels and allowing potassium entrance into hair cells from the potassium-rich endolimph (Figure 1.2B). The influx of potassium ions results in a change in membrane potential proportional to the intensity of the acoustic stimulus. This depolarization of hair cells then activates calcium channels on the basolateral side of the cells, leading to calcium influx into the hair cells. This influx triggers the release of the neurotransmitter glutamate at the hair cell base. Glutamate binds to the afferent nerve terminals that surround the base of the hair cells, resulting in an action potential being propagated down the afferent nerve fibers. The hair cells are repolarized when potassium ions leave these cells and enter the epithelial supporting cells. The potassium ions then diffuse to the stria vascularis through gap junctions and are secreted back into the endolimph, resetting the mechanoelectrical transduction system.

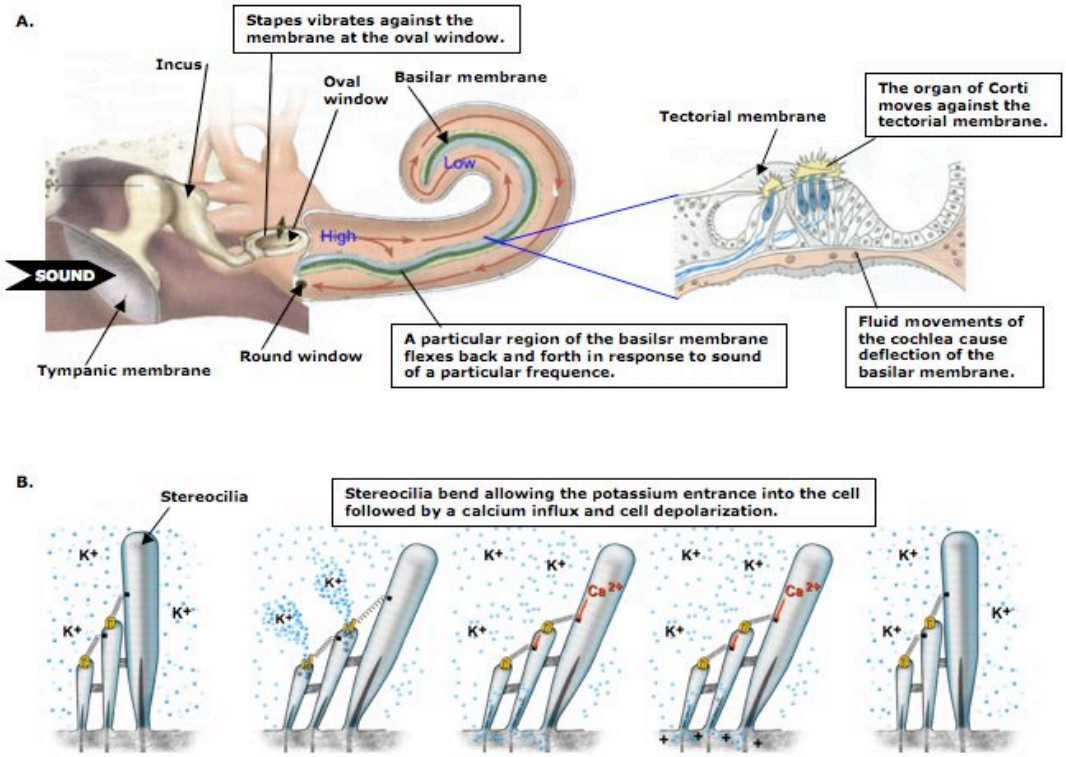

Figure 1.2. Physiology of hearing. **A.** Sound transmission in the inner ear. **B.** Sequential representation of the events leading to the depolarization of hair cells.

Within the cochlea, the different frequencies of complex sounds are sorted out. The cochlea analyzes sound frequencies by means of the basilar membrane, which exhibits different degrees of stiffness, or resonance, along its length. Loudness also is determined at this level by the amplitude, or height, of the vibration of the basilar membrane. As a sound increases, so does the amplitude of the vibration. This increases both the number of hair cells stimulated and the rate at which they generate nerve impulses.

Afferent nerves from the spiral ganglion terminate in the cochlear nucleus in the brainstem. Axons from neurons in the cochlear nucleus project to the superior olive, inferior colliculus, medial geniculate nucleus (in the thalamus) and, finally, the auditory cortex.

1.3 HEARING LOSS

Hearing loss is the most common sensory disorder worldwide, affecting 6-8% of the population in developed countries[1]. Hearing loss is common at all ages, but it is also a common birth defect. A hearing defect beginning in early childhood has dramatic effects on speech acquisition and psychosocial development. Later onset of a hearing defect seriously compromises the subject's quality of life, as the affected individual becomes increasingly socially isolated.

1.3.1 Classification of hearing loss

The traditional classification of hearing loss into two categories, **conductive** and **sensorineural** is based on the defective anatomical structures involved. Conductive deafness is associated with outer-ear or middle-ear abnormalities, whereas sensorineural deafness is caused by a defect located anywhere along the auditory pathway, from the cochlea to the auditory cerebral cortex. Most cases of sensorineural hearing loss are due to inner ear defects.

Hearing loss can be further classified by several other criteria including the severity, age of onset, the cause and the association with other clinical manifestations. Considering the degree of severity, hearing loss can be divided into **mild**, **moderate**, **severe** or **profound**. The hearing deficit may begin before the development of speech (**prelingual**) or thereafter (**postlingual**). Most prelingual forms are present at birth (**congenital**), but some start in early infancy before the acquisition of language. In most cases, prelingual hearing loss is severe but stable, whereas postlingual hearing loss is usually moderate but progressive. Impaired auditory function can be the only clinical manifestation (**non-syndromic** or isolated forms of deafness) or be associated with other symptoms or anomalies (**syndromic** forms of deafness). There are several hundred syndromes involving hearing loss, which present a wide range of additional clinical manifestations.

1.3.2 Epidemiology and frequency of hearing loss

Hearing loss is a major public health concern due to its high incidence worldwide. It is the most common birth defect, with approximately 1/800 individuals affected by severe or profound deafness at birth, 1/300 children affected with congenital hearing loss of a lesser degree and another 1/1000 becoming profoundly hearing impaired before adulthood[2]. Postlingual hearing loss is much more frequent than prelingual hearing loss, affecting 10% of the population by the age of 60 years and 50% by the age of 80 years[2,3].

Data from the Spanish National Disability, Impairment, and Handicap Survey conducted in 1999 by the National Statistics Institute (Instituto Nacional de Estadística), illustrates the increase of hearing loss incidence with age, especially from 60 years onwards (Figure 1.3)[4].

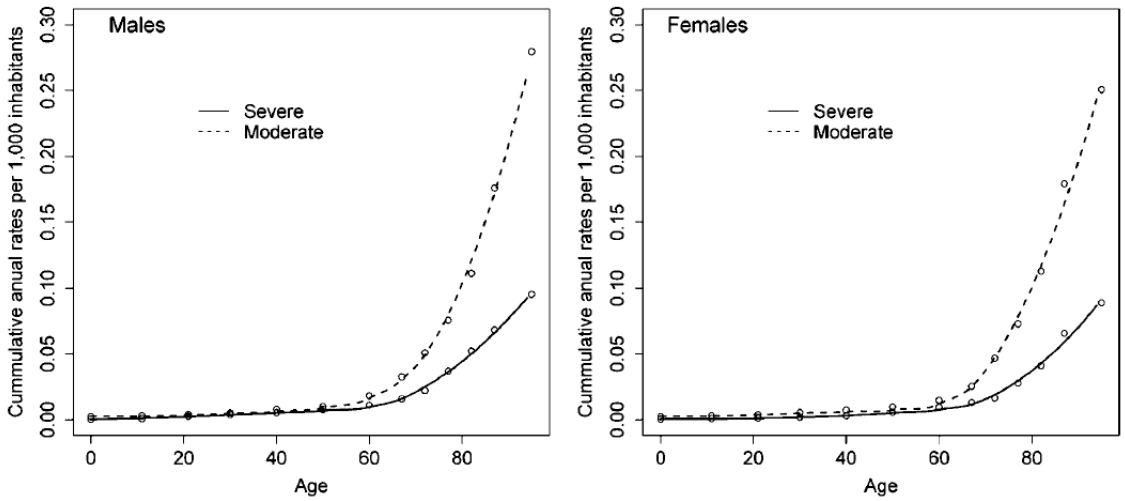

Figure 1.3. Cumulative net penetrance of hearing loss in Spain per 1,000 inhabitants by grade of severity and sex. The sample used in the study comprised residents in 80,000 dwellings. Information on the existence, type, and severity of disability for each member of the dwelling was recorded.

1.3.3 Clinical evaluation of hearing loss

In evaluating hearing impairments, audiologists rely on a wide range of tests designed to determine the type and extent of hearing loss. Some hearing tests require the person to co-operate, known as **subjective** tests, and others can measure hearing without co-operation, known as **objective** tests. The basic test is the audiometry, and when hearing loss is present, other tests help determine how much the hearing loss affects the person's ability to understand speech and whether the hearing loss is sensorineural, conductive, or mixed. Accurately diagnosing the cause, characteristics, and extent of hearing loss is critical to treating the problem and, if necessary, fitting with the most effective assistive listening device.

Audiometry

Audiometries are used to determine a person's ability to detect pure tones of various frequencies and to understand speech. Pure-tone audiometry measure how well someone can hear sounds of a different pitch and volume. Pitch or frequency is measured in cycles per second or Hertz (Hz). Most speech sounds are in the 500- to 4000-Hz range. Volume or intensity is measured in decibels (dB). Usual conversation ranges between 45 and 60 dB. Results of the test are recorded and printed on a graph called audiogram (Figure 1.4).

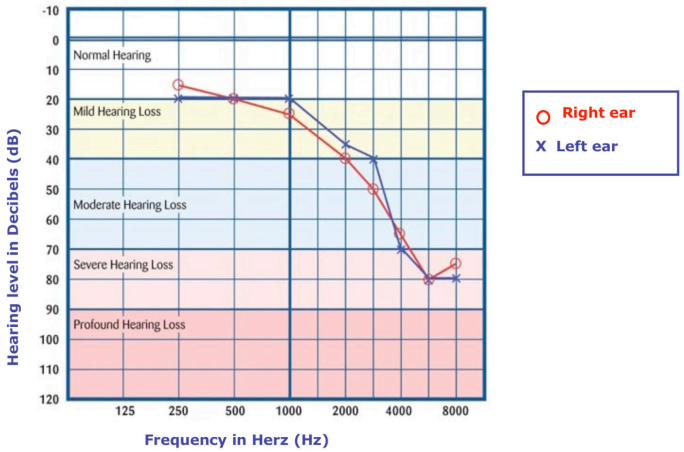

Figure 1.4. An audiogram illustrates a range of hearing across different frequencies and volumes. The right and the left ear are plotted separately on the graph. Hearing is considered normal if sounds from 250 to 8000 Hz can be heard at volumes of 20 dB or less.

Otoacoustic emissions

An otoacoustic emission (OAE) is a sound generated within the inner ear. These cochlear emissions are recorded using sophisticated electronics and are used routinely in many newborn nurseries to screen newborns for congenital hearing loss. Numerous studies have shown that OAEs disappear after the inner ear has been damaged, so OAEs are often used as a measure of inner ear health.

Auditory brainstem responses

The auditory brainstem responses test measures nerve impulses in the brain stem resulting from sound signals in the ears. Brain waves are stimulated by a clicking sound to evaluate the central auditory pathways of the brainstem. Test results are abnormal in people with some sensorineural types of hearing loss and in people with many types of brain tumors. Auditory brain stem response is used to test infants and also can be used to monitor certain brain functions in people who are comatose or undergoing brain surgery.

1.3.4 Causes of hearing loss

Hearing is a complex process, thus, it is not unexpected that the causes of hearing loss are also complex. Hearing loss can be due to environmental factors, genetic defects or a combination of both.

Most syndromic forms of deafness have a genetic origin, except for embryopathies due to rubella, toxoplasmosis or cytomegalovirus infection that can lead to polymalformations including hearing loss. Hundreds of syndromes associating hearing loss and disorders of the musculoskeletal, cardiovascular, urogenital, nervous, endocrine, digestive or tegumentary systems have been reported to have a genetic basis[1].

Nonsyndromic forms of deafness can be caused by environmental and/or genetic factors. The hereditary nature of nonsyndromic deafness was first reported in the sixteenth century by Johannes Schenck[1]. In 1621, the papal physician Paolus Zacchias recommended deaf people not to marry because of the risk of having deaf children[5]. Nowadays, it is believed that in developed countries, where prelingual nonsyndromic deafness mostly presents as sporadic cases, at least 50-60% of all nonsyndromic hearing loss cases are of genetic cause[6]. Approximately 25% of nonsyndromic forms of deafness are caused by environmental factors such as prematurity, neonatal hypoxia, pre or postnatal infections, exposure to ototoxic drugs, trauma or tumours. Hearing loss aetiology remains unknown in 15-25% of cases, but increasing evidences show that genetic predisposition or susceptibility may also play an important role in this group[7,8].

Late-onset forms of hearing loss used to be thought to result from a combination of genetic and environmental causes. Recent evidences indicate that the genetic contribution could have been underestimated, being genetic factors either the direct cause or susceptibility factors that may determine the onset of hearing impairment together with other genetic or environmental factors[1].

In summary, genetic causes account for the largest proportion of all hearing loss cases. Combined data of syndromic and nonsyndromic forms of hearing loss, suggests that over 80% of all cases of congenital deafness is of genetic origin in developed countries. In addition, the proportion of cases with a clear environmental aetiology is likely to decline as better therapies for bacterial and viral infections are implemented, acoustic trauma in the workplace is recognized and prevented, and ototoxic drugs are avoided.

1.4 GENETICS OF HEARING LOSS

The study of hereditary hearing loss deals with two main objectives: (i) identification of causative genes and the underlying pathogenic process in each form of deafness; and (ii) elucidation of the molecular and cellular mechanisms of hearing.

Given the remarkable sensitivity and specificity of the auditory function, it is not surprising the estimation that approximately 1% of all human genes (i. e. about 300 genes) are involved in the hearing process[9] and therefore, they are potential candidates to give rise to hearing impairment when mutated.

Hundreds of syndromic forms of deafness have been described and the underlying genetic defect identified for many of the most common forms. However, the vast majority of genetic deafness is nonsyndromic[10].

Hearing loss can follow different inheritance patterns. Early interest in the mode of inheritance of hearing loss was stimulated by the French physician Pierre Ménière in 1846[11]. In his lecture, "Upon marriage between relatives considered as the cause of congenital deaf-mutism", he was the first to recognize the autosomal recessive origin of "deaf-mutism", now well established as the principal mode of transmission[12]. According to its transmission pattern, hereditary forms of nonsyndromic hearing impairment are categorized in: (i) **autosomal dominant**, involving 15-20% of cases; (ii) **autosomal recessive**, which accounts for around 80% of cases; (iii) **X-linked** in 1-2% of cases and (iv) **mitochondrial** in at least 1% of cases but the frequency increases up to 20% in some populations.

Generally, patients with autosomal recessive hearing impairment have prelingual and profound deafness, and patients with autosomal dominant hearing impairment have postlingual and progressive hearing impairment. This observation may be explained by the complete absence of functional protein in patients with recessive disorders, whereas dominant cases may be consistent with initial function and subsequent hearing loss due to a cumulative, degenerative process[10].

1.4.1 Identification of deafness genes

Despite the fact that genetic basis of hearing loss have been studied for decades, identification of deafness causative genes began only in the 1990s. The main obstacles to successful chromosomal mapping of genetic defects leading to hearing loss included: (i) inaccessibility of the cochlea, which lies within the temporal bone; (ii) high genetic heterogeneity; (iii) absence of clinically distinctive signs for the various gene defects; and (iv) assortative mating.

Initially, only genes implicated in syndromic hearing loss were localized and identified. Syndromic forms could be classified into homogeneous groups in which linkage analysis and positional cloning are feasible[13]. In contrast, traditional methods of genetic linkage analysis are not optimal for gene identification in nonsyndromic hearing loss cases. On one hand, isolated deafness is characterized by high genetic heterogeneity. On the other hand, more than one cause of hearing loss can segregate in a family as a consequence of assortative mating, which is relatively common among deaf individuals. Therefore, successful use of genetic linkage for mapping hearing disorders has been restricted to large consanguineous families or geographically isolated populations[10].

A complementary method to genetic linkage analysis for gene identification is the utilization of tissue or organ-specific cDNA libraries to provide candidate genes[10]. Cochlear cDNA libraries from human[14,15] and mouse[16] have provided valuable biological tools for gene discovery in the mammalian cochlea. Moreover, the identification of mouse models of specific forms of deafness has allowed both the discovery of new deafness causative genes and a better understanding of the pathobiology and underlying molecular mechanisms of genetic mutations from fetal to adult ages.

1.4.2 Hearing loss genes

Progress in identifying genes involved in deafness has been remarkable over the past few years. To date, more than 130 loci have been identified involved in syndromic or nonsyndromic hearing loss[17].

Syndromic forms of hearing loss are usually monogenic, although rare cases exist with a digenic mode of inheritance[16]. The genes underlying around 100 different syndromes have been identified. Some of these syndromes are genetically heterogeneous (i. e. mutations in different genes lead to the same clinical outcome)[17].

In the case of nonsyndromic hearing loss, 105 loci have been located all along the genome. The different gene loci for nonsyndromic forms of deafness have been called DFN (for deafness) and are numbered in chronologic order of discovery. Autosomal dominant loci are referred to as DFNA, autosomal recessive loci as DFNB and X-linked loci as DFN. Of the different loci reported for nonsyndromic hearing loss, 46 are DFNA, 53 for DFNB and 5 for DFN. In addition, a Y chromosome-linked locus and two genes on the mitochondrial genome have to be added to the list. The gene involved has been characterized in 44 of the cases (Table 1.1).

Table 1.1. Loci and genes responsible of nonsyndromic hearing loss[17].

AUTOSOMAL DOMINANT LOCI		
Locus name	**Location**	**Gene**
DFNA1	5q31	DIAPH1
DFNA2	1p34	GJB3 / KCNQ4
DFNA3	13q12	GJB2 / GJB6
DFNA4	19q13	MYH14
DFNA5	7p15	DFNA5
DFNA6/DFNA14	4p16.3	WFS1
DFNA7	1q21-q23	ni
DFNA8/DFNA12	11q22-24	TECTA
DFNA9	14q12-q13	COCH
DFNA10	6q22-q23	EYA4
DFNA11	11q12.3-q21	MYO7A
DFNA13	6p21	COL11A2
DFNA15	5q31	POU4F3
DFNA16	2q24	ni
DFNA17	22q	MYH9
DFNA18	3q22	ni
DFNA19	10 (pericentr.)	ni
DFNA20/DFNA26	17q25	ACTG1
DFNA21	6p21	ni
DFNA22	6q13	MYO6
DFNA23	14q21-q22	ni
DFNA24	4q	ni
DFNA25	12q21-24	ni
DFNA27	4q12	ni
DFNA28	8q22	TFCP2L3
DFNA30	15q25-26	ni
DFNA31	6p21.3	ni
DFNA32	11p15	ni
DFNA34	1q44	ni
DFNA36	9q13-q21	TMC1
DFNA37	1p21	ni
DFNA38	3q22	ni
DFNA39	4q21.3	DSPP
DFNA40	16p12	ni
DFNA41	12q24-qter	ni
DFNA42	5q31.1-q32	ni
DFNA43	2p12	ni
DFNA44	3q28-29	ni
DFNA47	9p21-22	ni
DFNA48	12q13-q14	MYO1A
DFNA49	1q21-q23	ni
DFNA50	7q32	ni
DFNA51	9q21	ni
DFNA52	4q28	ni
DFNA53	14q11-q12	ni
DFNA54	5q31	ni

X-LINKED LOCI		
Locus name	**Location**	**Gene**
DFN1	Xq22	TIMM8A
DFN2	Xq22	ni
DFN3	Xq21.1	POU3F4
DFN4	Xp21.2	ni
DFN6	Xp22	ni

AUTOSOMAL RECESSIVE LOCI		
Locus name	**Location**	**Gene**
DFNB1	13q12	GJB2
DFNB2	11q13.5	MYO7A
DFNB3	17p11.2	MYO15A
DFNB4	7q31	SLC26A4
DFNB5	14q12	ni
DFNB6	3p14-p21	TMIE
DFNB7/DFNB11	9q13-q21	TMC1
DFNB8/DFNB10	21q22	TMPRSS3
DFNB9	2p22-p23	OTOF
DFNB12	10q21-q22	CDH23
DFNB13	7q34-36	ni
DFNB14	7q31	ni
DFNB16	15q21-q22	STRC
DFNB17	7q31	ni
DFNB18	11p14-15.1	USH1C
DFNB19	18p11	ni
DFNB20	11q25-qter	ni
DFNB21	11q	TECTA
DFNB22	16p12.2	OTOA
DFNB23	10p11.2-q21	PCDH15
DFNB24	11q23	ni
DFNB25	4p15.3-q12	ni
DFNB26	4q31	ni
DFNB27	2q23-q31	ni
DFNB28	22q13	TRIOBP
DFNB29	21q22	CLDN14
DFNB30	10p12.1	MYO3A
DFNB31	9q32-q34	WHRN
DFNB32	1p13.3-22.1	ni
DFNB33	9q34.3	ni
DFNB35	14q24.1-24.3	ni
DFNB36	1p36.3	ESPN
DFNB37	6q13	MYO6
DFNB38	6q26-q27	ni
DFNB39	7q11.22-q21.12	ni
DFNB40	22q	ni
DFNB42	3q13.31-q22.3	ni
DFNB44	7p14.1-q11.22	ni
DFNB46	18p11.32-p11.31	ni
DFNB47	2p25.1-p24.3	ni
DFNB48	15q23-q25.1	ni
DFNB49	5q12.3-q14.1.	ni
DFNB50	12q23	ni
DFNB51	11p13-p12	ni
DFNB53	6p21.3	COL11A2
DFNB55	4q12-q13.2	ni
DFNB57	10q23.1-q26.11	ni
DFNB58	2q14.1-q21.2	ni
DFNB59	2q31.1-q31.3	PJVK
DFNB60	5q22-q31	ni
DFNB62	12p13.2-p11.23	ni
DFNB65	20q13.2-q13.32	ni
DFNB66	6p21.2-22.3	LHFPL5

ni; non identified

The genetic basis of hearing loss is complex. Mutations in some genes can cause both recessive and dominant hearing loss; alternatively, different mutations

in the same gene may cause syndromic and nonsyndromic deafness[8]; and even within a single gene, the same mutation can give rise to quite variable phenotypes[18]. In some recessive hearing loss cases, the combination of two mutations in different genes from the same functional group may be the cause of the hearing impairment[18]. These examples of phenotypic heterogeneity demonstrate how the type of mutation within the gene and its allelic combinations can affect the overall clinical presentation, complicating therefore the establishment of genotype-phenotype correlations. Moreover, recent observations indicate that the phenotypic heterogeneity observed for a given mutation may also be due to the contribution of modifier genes.

Certainly, the great degree of genetic heterogeneity is indicative of a large number of genes orchestrating the hearing process. The identification and study of deafness causative genes has been a way to reveal the molecular mechanisms underlying the development and function of the auditory system. Another important consequence of gene discovery is the possibility to develop diagnostic tests and accurate genetic counselling.

As the vast majority of hereditary hearing impairments are nonsyndromic and the work performed in this doctoral thesis has dealed with nonsyndromic cases of hearing loss, the description of deafness causative genes will focus on nonsyndromic ones.

1.4.3 Genes involved in nonsyndromic hearing loss

The genetic approach has been useful for identifying the key components of the auditory process. Genes involved in the pathogenesis of hearing loss include ion channels, transcription factors, structural proteins, mitochondrial proteins and many others. Thus, the deafness causative genes here mentioned have been grouped into functional categories with the aim to have a more clear vision of the molecular components of the auditory function.

Ion homeostasis

A surprising aspect resulting from studying the auditory function has been the number of deafness genes affecting ionic homeostasis in the cochlea. Mutations have been reported in various genes affecting ion recycling, allowing the definition of recycling routes within the cochlea and the identification of the molecules involved.

◆ Connexins: *GJB2* and *GJB6*

In 1994, the first locus for autosomal recessive hearing loss, DFNB1, was located on chromosome 13q12[19]. DFNB1 locus contains two connexin genes, *GJB2* (or connexin 26) and *GJB6* (or connexin 30). Connexin 26 was the first gene to be identified as responsible of hearing loss[20]. Connexins are a family of genes that code for gap junction proteins. The gap junction or connexon forms a half-channel that docks with its counterpart from an adjacent cell to form an intercellular channel, enabling communication between cells. Gap junctions are believed to play a critical role in hearing, as they are responsible of the recycling of potassium ions from the Organ of Corti back into the endolymph[21].

A somewhat surprising finding has been the high prevalence of mutations in *GJB2* gene accounting for up to 60% of autosomal recessive forms of hearing loss[22]; thus, representing the most common cause of sporadic and autosomal recessive nonsyndromic sensorineural hearing loss[22]. More than 100 different mutations have been reported in connexin 26 gene[23]. However, several recurrent population-specific mutations have been found. Mutation 35delG accounts for up to 85% of mutant alleles in European-Mediterranean populations, and its prevalence in normal hearing individuals has been estimated of 2%-4%[24]. Other mutations are particularly prevalent in other populations, such as 167delT among Ashkenazi Jews or 235delC in the Japanese population[22].

When testing for mutations in *GJB2* gene, a variable proportion of deaf individuals are diagnosed with only one *GJB2* mutation. A role for *GJB6*, the adjacent gene to *GJB2*, was first suggested in 1999, when a dominant mutation (T5M) in *GJB6* gene was described[25]. This was confirmed with the identification of two large deletions in the *GJB6* gene, which were responsible of nonsyndromic hearing loss when homozygous or in conjunction with a *GJB2* mutation[26,27]. As both genes are expressed in the cochlea where they can combine to form multi-unit hemichannels in the cell membrane[21], a digenic mechanism of inheritance was proposed. However, it seems now more likely that the large deletions in *GJB6* gene could affect an undiscovered upstream regulatory element of *GJB2*[28].

Identification of *GJB2* gene was a landmark in the genetics of hearing loss, because it pointed out the pivotal role of cochlear gap junction ion channels. Later on, other connexins have also been involved in deafness: mutations in connexin 31 (*GJB3*) have been found in families with nonsyndromic hearing loss and connexin 32 (*GJB1*) has been associated with X-linked Charcot-Marie-Tooth syndrome.

◆ Claudin 14 (*CLDN14*)

Claudins are essential components of tight-junctions. In the inner ear, tight junctions are involved mainly in the compartmentalization of endolymph. Mutations in *CLDN14* gene are responsible of profound congenital recessive deafness[29].

◆ Potassium channels: *KCNQ4*, *KCNQ1* and *KCNE1*

Mutations in three potassium channels genes from the same superfamily have been identified as a cause of hearing loss. The potassium channels encoded by these genes contribute to potassium homeostasis in the cochlea by mediating repolarization and resetting the electrical potential of hair cells[30].

KCNQ4 is mutated in dominant, progressive hearing loss cases[31]. Within the cochlea, *KCNQ4* gene product is found predominantly in outer hair cells, and may serve as the first step in ion recycling by allowing potassium to flow out of the hair cells through their basolateral membranes into the supporting cells. The potassium ions then recirculate through connexin channels to the stria vascularis, where they are secreted into the endolymph through potassium channels formed by the *KCNQ1* and *KCNE1* gene products[30]. Defects in both *KCNQ1* and *KCNE1* genes cause Jervell and Lange-Nielsen syndrome.

Hair-cell function

The intricate nature of the sensory epithelium and its highly organized stereocilia needs the maintenance of its precise structure to ensure proper function. Several molecules have been identified as having a vital role in hair-cell transduction because of their localization in or around the stereocilia. On the other hand, hair cells are also responsible of the synapses, which will allow the sound perception.

◆ Unconventional myosins: *MYO6, MYO7A*, *MYO15*

Myosins are a superfamily of proteins that exert mechanical forces through the binding to actin filaments. In the inner ear, myosins are present in the stereocilia of the hair cells and in the cuticular plate. Therefore, they might provide an anchor for actin in the hair cells and their stereocilia, being responsible not only for the tension on the tip links of the stereocilia and their movements, but also for the overall cytoeskeletal organization of the cells.

In mouse, mutant *MYO6* leads to a progressive fusion of hair cell stereocilia, mutant *MYO15* results in short stereocilia and mutant *MYO7A* leads to progressive disorganization of the stereocilia bundle[32-34]. The human mutations presumably have similar effects on hair cell development. *MYO6* mutations lead to autosomal dominant hearing loss[35], *MYO15* mutations are associated with profound congenital autosomal recessive hearing loss[36] and *MYO7A* mutations account for both autosomal dominant and recessive cases of hearing loss[37,38]. *MYO7A* mutations

have also been involved in Usher syndrome type 1B, a syndromic form of deafness combined with retinitis pigmentosa and vestibular abnormalities[37]. This demonstrates that similar macromolecular interactions are required for proper function in both the ear and eye.

◆ Other stereocilia components: *CDH23*, Espin, Vezatin

The identification of *MYO7A* led to the identification of other components of the transduction apparatus that may be candidates as deafness-causing genes. A yeast two-hybrid screen identified vezatin, a transmembrane protein that binds to Myosin VIIA tail[39]. A mutational screening of the gene has identified some possible pathological mutations in deaf patients from Italy and Spain[40]. It has also been suggested that myosin VIIA might interact with harmonin, known to underlie Usher syndrome type 1C[41,42].

Another essential cytoskeletal component of stereocilia is espin, an actin-binding protein. Two frameshift mutations in espin gene are responsible for nonsyndromic recessive hearing loss[43]. In the inner ear, cadherin-23 might be involved in the formation of lateral and/or tip links that join stereocilia, as well as vezatin and harmonin. Cadherins are proteins involved in cell-cell interactions. Missense mutations in cadherin-23 gene (*CDH23*) cause nonsyndromic recessive hearing loss, while more severe mutant alleles, like nonsense or truncating mutations, are associated with Usher syndrome type 1D[44].

◆ Otoferlin (*OTOF*)

The otoferlin gene is mutated in some cases of dominant progressive hearing loss[45]. Its protein is located at the base of inner hair cells and is involved in sypnatic vesicle transport.

Extracellular matrix

The extracellular matrix is important for the integrity and proper function in many organ systems, and the inner ear is not an exception.

◆ Tectorial membrane: *TECTA*, *COL11A2*

Mutations in the gene encoding α-tectorin (*TECTA*) and collagen 11α2 (*COL11A2*) lead to ultrastructural defects of the tectorial membrane and hearing loss in humans. *TECTA* mutations are responsible for both dominant and recessive forms of hearing loss[46,47]. *COL11A2* mutations lead to late-onset autosomal dominant forms of deafness[48].

◆ Cochlin (*COCH*)

Cochlin is a major constituent of the inner-ear extracellular matrix. *COCH* mutations are implicated in an autosomal dominant form of hearing loss characterized by postlingual progressive hearing impairment, accompanied in some cases of vestibular dysfunction[49]. The presence of the mutant protein causes a loss of cells in the spiral ligament and limbus and the accumulation of acidic mucopolysaccharides in the nerve channels and supporting tissues of the Organ of Corti, perhaps leading to compression or blockage of the cochlear nerve[50].

Transcription factors

As with other biological pathways, transcription factors are essential in hearing and defects in these proteins have helped to elucidate the components of the process.

◆ *EYA* genes: *EYA1*, *EYA4*

EYA genes are critical in embryonic development. Although each *EYA* gene has a unique expression pattern, there is extensive overlap: *Eya1* and *Eya4* are both expressed in the otic vesicle and its derivatives[51,52]. In humans, *EYA1* mutations result in branchio-oto-renal syndrome[53]. In contrast, *EYA4* mutations are responsible of autosomal dominant progressive hearing loss starting between the second and fifth decade of life[52]. Thus, a role in cell survival in the mature cochlea is postulated for *EYA4*.

◆ *POU* transcription factor genes: *POU3F4*, *POU4F3*

The *POU3F4* gene is expressed in the mesenchyma of the inner and middle ear, in which it is involved in the maturation of bone[54]. Mutations in this gene are responsible of X-linked hearing loss. However, the finding of monozygotic twins with different phenotypes suggests the influence of other factors[55].

The *POU4F3* gene is expressed only in hair cells, where it is responsible for terminal differentiation and maintenance of inner hair cells[54]. Mutations in *POU4F3* gene result in dominant progressive hearing loss[56]. Further studies of this gene and related counterparts may result in valuable insight into the molecular process of hair cell degeneration as loss of hair cells is thought to be a fundamental cause of progressive age-related hearing loss (presbycusis).

In summary, investigations in hereditary deafness have revealed many lessons in genetics, foremost among them a sensory system with profound genotypic and phenotypic heterogeneity. Each discovery gets closer to a better understanding of the hearing process, which will lead to increased availability of diagnostic and presymptomatic testing options, early intervention and disease-based treatments.

2. Mitochondria and mitochondrial disease

Diseases caused by mitochondrial dysfunction were first described by Luft and coworkers in 1962[57] and since then, have become an important area in human pathology. Striking developments have been achieved in the lasts 20 years as a result of the elucidation of the structure and function of the human mitochondrial genome and the discovery of the first pathogenic mitochondrial DNA (mtDNA) mutations in the late 1980s[58]. Today, more than 100 mtDNA point mutations and rearrangements, and more recently, nuclear gene mutations have been shown to be associated with a variety of disorders affecting the skeletal muscles, the brain, the heart, the liver and the cochlea among other organs[59]. These findings have stimulated basic research aimed at an in depth understanding on mitochondria's components and function. Advances in the knowledge of basic processes, in turn, have provided new insights into the way in which alterations in these processes result in pathological or aging-related phenotypes.

2.1 MITOCHONDRIA STRUCTURE AND FUNCTION

Mitochondria are intracellular organelles characteristic of all eukaryotic cells. They are the sole sites of oxidative phosphorylation, a process where free energy from aerobic cell respiration is converted into adenosine triphosphate (ATP), the major energy currency of the cell. In addition, mitochondria are now known to play an important role in apoptosis, the orchestrated death of cells. Mitochondria were once free-living bacteria which were integrated in larger cells 2-3 billion years ago, giving rise to all complex eukaryotic cells[60]. These organelles are unusual in having their

own DNA, although many of the genes that once resided in the mitochondria have, over evolutionary time, decamped to the cell's nucleus.

2.1.2 Structure of mitochondria

Mitochondria are cylindrical structures of 0.5-1 μm diameter, but their size and shape vary between tissues, as do their number per cell. The number of mitochondria per cell ranges from none (erythrocytes) to thousands (striated muscle cells), depending on the amount of substrates and oxygen utilization that a particular cell requires. The basic structure is essentially the same in all mitochondria, with four compartments: (i) the outer membrane, (ii) the inner membrane, (iii) the intermembrane space, and (iv) the matrix (Figure 2.1).

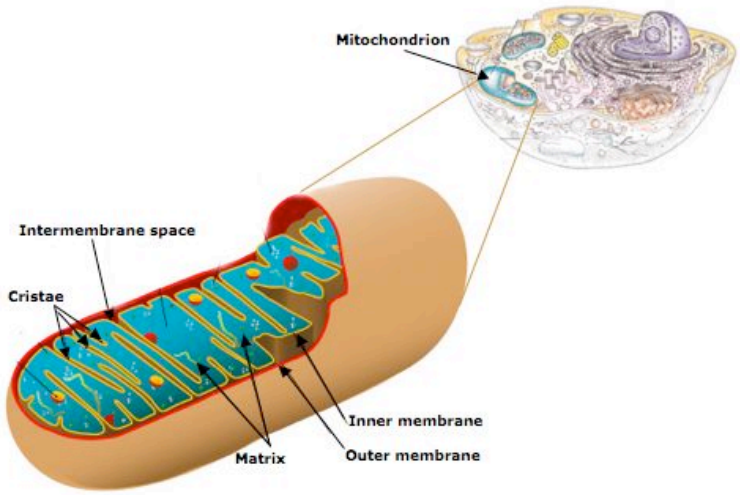

Figure 2.1. Structure of a mitochondrion.

The **outer membrane** presumably arose from the cell membrane of the ancient endosymbiont host. The lipid composition of the outer membrane is indeed similar to that of the plasma membrane. The outer membrane is relatively permeable to metabolites as well as to small molecules (< 10,000 Da), in part due to the presence of porins[61].

The **inner membrane** is a lipidic bilayer deriving from the cell membrane of the engulfed aerobic bacterium. It is highly folded into *cristae* structures that maximize its surface area, and it is built up of 70% proteins. The inner membrane is by itself quite impermeable to most molecules, including small ions, which is one of the keys of mitochondria bioenergetic function. Metabolites may penetrate only by means of specific carrier molecules or transporters located in the membrane[62]. The exceptions are gas molecules such as O_2 and CO_2 and other uncharged hydrophobic substances, which penetrate due to their apolar nature[63].

The inner membrane is the site of oxidative phosphorylation. Embedded in it are the four respiratory chain complexes as well as the ATP synthase, the key enzyme of oxidative ATP synthesis. Additionally, it also contains several other membrane proteins such as metabolites and ion carriers that link the metabolism of the cytoplasm of the cell with that of the inner mitochondrial space.

Between both membranes, there is a narrow **intermembrane space**, where other specific enzymes are located, such as cytochrome-c, which initiates apoptosis if released into the cytosol[62].

The **matrix,** inside the inner membrane, may be described as a gel due to the very high concentration of proteins and other macromolecules. Within the matrix, there are enzymes such as those of the Krebs' cycle and fatty acids oxidation, DNA-polymerases, chaperones and mRNAs, tRNAs as well as the mtDNA.

2.1.2 Function of mitochondria

Mitochondria are the site of hundreds of chemical reactions critical for the survival of all multicellular life[64]. Although the most important function of mitochondria is energy production, mitochondria play also many tissue-specific roles in cell metabolism, doing much more than making ATP[65]. The fundamental biological roles of mitochondria are: (i) ATP production by oxidative phosphorylation, (ii) mediation of cell death by apoptosis, (iii) heat production and (iv) regulation of calcium homeostasis.

ATP production

The principle function of the mitochondrion is to produce energy in the form of ATP. Oxidative phosphorylation is the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i) driven by the free energy from oxidation of foodstuffs by molecular oxygen.

Primary processing of foodstuffs encompasses a large number of enzymatic reactions of intermediary metabolism before the main oxidative reactions can begin. All these enzymatic reactions occur within the matrix space inside the mitochondrion[63]. Fatty acids, pyruvate and amino acids are transferred from the cytosol into the mitochondrion where they are metabolized to acetyl coenzyme A (AcCoA). AcCoA is further metabolized through the citrate cycle, causing the reduction of oxidized nicotinamide adenine dinucleotide (NAD^+) and flavine adenine dinucleotide (FAD). The reduced forms of these cofactors, NADH and $FADH_2$ may be directly oxidized by the respiratory chain, but the high energy electrons of the acetyl group of AcCoA are first transformed into NADH and $FADH_2$ by the reactions of Krebs' cycle, with production of CO_2 (Figure 2.2)[63].

The respiratory chain implies the oxidation of NADH by O_2. It takes place in a chain-like, sequential manner catalysed by three protein complexes bound to the inner mitochondrial membrane: Complex I, Complex III (or cytochrome bc_1 complex) and Complex IV (or cytochrome *c* oxidase). In addition, ubiquinone and cytochrome *c* participate as redox carriers that functionally connect complexes I and III, and III and IV, respectively. Reducing equivalents (electrons or hydrogen atoms) are transferred sequentially, until they reach O_2 at the active site of Complex IV, where O_2 is reduced to H_2O[63]. Apart from the oxidoreductase function, another important feature of respiratory chain enzymes is the ability to conserve free energy of the redox reactions for ATP synthesis, by pumping protons across the inner membrane from the matrix into the intermembrane space[61]. The established electrochemical proton gradient drives the ATP generation via the H^+-ATP synthase, also called Complex V.

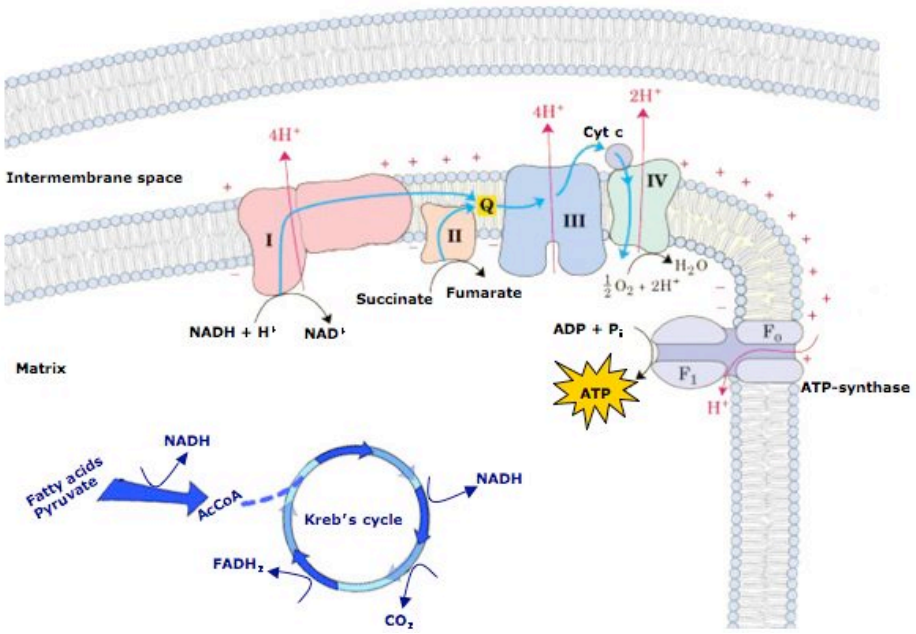

Figure 2.2. The mitochondrial respiratory chain and intermediary metabolism.

The H^+-ATP synthase allows protons to flow back into the matrix, using the released energy to synthesize ATP[66]. This enzyme consists of two parts: a trimeric orange-like structure (F_1) containing the catalytic sites for ATP synthesis; and a membrane unit (F_0) which catalyses proton translocation. The F_1F_0 complex functions as a rotatory machine[63].

Apoptosis

In the lasts years, mitochondria have been shown to play a central role in activating apoptotic cell death in response to cellular dysfunction. A variety of key events in apoptosis take place in mitochondria. At least three general mechanisms are known

by which mitochondria are capable of control cell death, and their effects may be interrelated: (i) disruption of electron transport, oxidative phosphorylation and ATP production; (ii) release of proteins that trigger activation of caspase family proteases; and (iii) alteration of cellular redox potential[67]. The different signals that converge on mitochondria to trigger or inhibit these events and their downstream effects delineate several major pathways in physiological cell death.

Heat production

Under certain conditions, protons can re-enter the mitochondrial matrix without contributing to ATP synthesis. This process is known as proton leak or mitochondrial uncoupling and is due to the facilitated diffusion of protons into the matrix, mediated by a proton channel called thermogenin. This results in the potential energy of the proton electrochemical gradient being released as heat. Thermogenin is found in brown adipose tissue (brown in color due to high levels of mitochondria) where it is used to generate heat by non-shivering thermogenesis. Non-shivering thermogenesis is the primary means of heat generation in newborn or hibernating mammals[63].

Calcium homeostasis

Mitochondria affect cytoplasmic calcium (Ca^{2+}) metabolism in two ways: indirectly via mitochondrially produced ATP that is used by Ca^{2+}-dependent ATPases to pump Ca^{2+} out of the cell or into intracellular stores; and directly through the mitochondrial membrane potential which drives the uptake of Ca^{2+} into mitochondria by a Ca^{2+}–uniporter[68].

The combined action of mitochondrial Ca^{2+} uptake and extrusion leads to the Ca^{2+}-free mitochondrial matrix responding to changes in the cytoplasmatic Ca^{2+} concentration and thus, altering the activity of matrix Ca^{2+}–dependent enzymes and of mitochondrial ATP synthesis. In addition, the uptake of Ca^{2+} by mitochondria, both buffers and modulates intracellular calcium signals, and may also act as a sink for excess cytoplasmatic Ca^{2+}, protecting the cell from calcium overload[68]. Persistently elevated cytoplasmatic Ca^{2+} not only disrupts Ca^{2+} signalling pathways, but also greatly increases the probability of cell death[68].

2.2 MITOCHONDRIAL GENETICS

Mitochondria have their own functional genome, independent of the nuclear one. Mitochondrial DNA (mtDNA) was discovered in 1963[69] and, a few years later, the

complete sequences of mouse[70] and human mtDNA[71] were published, pioneering subsequent achievements in nuclear genome sequencing.

The mitochondrial genome is a small double-stranded circle of DNA. In average, nucleated animal cells contain between 500-2000 mitochondria and each mitochondrion contains 2-10 mitochondrial chromosomes in its matrix. Therefore, a typical differentiated cell contains at least between 1000-4000 copies of mtDNA for every diploid copy of the genome in the nucleus[72].

2.2.1 The structure of mitochondrial DNA

The human mtDNA consists of 16,569 basepairs (bp) arranged in a ring of double stranded DNA. The two strands of mtDNA are designated heavy (H) and light (L) on the basis of different G+T base composition, which results in different buoyant densities in denaturing cesium chloride gradients[73]. Numbering of mtDNA nucleotides proceeds from 1 to 16,569 in a counterclockwise direction and referring to nucleotide positions on the light strand.

MtDNA replicates and is transcribed in ways reminiscent of its bacterial origin. Replication occurs simultaneously via two sites: O_H in the heavy-strand and O_L in the light strand. Transcription produces polycistronic transcripts corresponding to both mtDNA strands, which have to be posttranscriptionally processed[74].

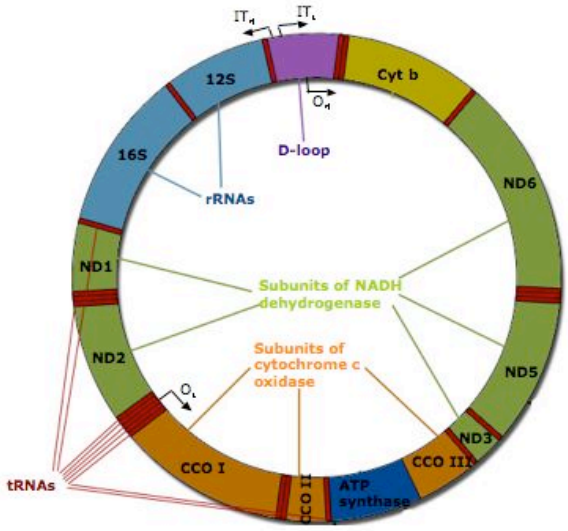

Figure 2.3. Map of the human mitochondrial DNA.

MtDNA encodes 13 polypeptides, 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA). All 13 proteins encoded by mtDNA are key components of the respiratory chain. The only long non-coding region is the D-loop or control region, which expands 1,1 kb, between nucleotides 16,024 and 576. It contains the promoters for transcription of both mtDNA strands and the initiation sites for

mtDNA replication[75]. The D-loop is the most highly polymorphic of all segments of mtDNA (Figure 2.3).

Mammalian mtDNA shows exceptional economy of organization: the genes are closely packed and lack introns; except for the D-loop region, intergenic sequences are absent or limited to a few bases; and some genes overlap. However, mitochondria are not self-supporting entities in the cell and the vast majority of mitochondrial proteins are nuclear-encoded, synthesized in the cytosol and subsequently imported into the organelle.

Somatic mutations in mtDNA are common because mitochondria have a low activity DNA repair system and are continuously exposed to oxygen radicals, leaked from the mitochondrial electron-transfer chain[76]. Unrelated individuals may differ at up to about 60 positions, making mtDNA useful for purposes of forensic identification and evolutionary biology[72].

MtDNA has often been described as "naked", lacking any equivalent of nuclear histones. However, now it is known that mtDNA is packaged into protein-DNA complexes called mitochondrial nucleoids (mt-nucleoids)[77]. Each nucleoid contains several molecules of the circular mtDNA together with proteins. Some of the proteins that are known to form part of the mt-nucleoids are bifunctional; apart from packaging and protecting mtDNA, they also function in mitochondrial metabolism[77]. The better-characterized mt-nucleoid protein in mammals is TFAM (transcription factor A, mitochondrial). TFAM is involved in packaging the mitochondrial genome but also facilitates the assembly and promoter recognition of the mitochondrial transcription machinery[78].

Although little is known regarding mt-nucleoids components, it is clear that this organization has important implications in replication, transcription and inheritance of mtDNA. Mitochondria might have evolved unique strategies for the organization and inheritance of their genomes by using proteins that also function in mithocondrial metabolism, potentially coupling these important processes[77].

2.2.2 Mitochondrial DNA expression

As mentioned above, the mitochondrial genome encodes 13 mRNA genes, as well as two rRNAs and 22 tRNAs, which are required for assembling a functional protein-synthesizing system. The 13 mRNAs are translated into proteins on mitochondrion-specific ribosomes, using a mitochondrion-specific genetic code. A modified tRNA wobble base interaction with mRNA codons allows mitochondria to translate all codons with only the 22 tRNAs coded in mtDNA genome[79].

Mitochondrial transcription and translation processes are similar to those described for *E. coli*. Although mammalian mtDNA contains the genes for a full set

of tRNAs and the two rRNAs involved in mRNA translation, all proteins which drive mitochondrial transcription and translation are nuclear-encoded.

Transcription. Transcription of human mtDNA is accomplished by a single type of nuclear-encoded RNA polymerase. There are two major initiation transcription sites (IT_{H1} and IT_L) located 150 bp of one another in the D-loop region. A promoter element encompasses the transcription initiation sites, which are located within the promoter. Moreover, enhancer elements located just upstream of the promoter regions are required for optimal transcription. Transcription initiation depends on the binding of the mitochondrial transcription factor TFAM to the enhancer regions. RNA polymerase, then, can bind to the promoters, but at least two other transcription factors (TFB1M and TFB2M) are needed for transcription to begin[79]. TFB1M transcription factor has also been involved in modulating mitochondrial translation by its rRNA methyltransferase activity[80].

Both mtDNA strands are transcribed as single polycistronic transcripts. The two rRNAs and most of the mRNA coding sequences are immediately contiguous to tRNA sequences, suggesting that the secondary structure of tRNA sequences function as punctuation marks in the reading of the mtDNA information[81]. Precise endoribonucleolytic excision of the tRNAs from the transcript yields to correctly processed rRNAs and mRNAs[81].

Mitochondrial ribosomes reside in the matrix of the organelle. In mammals, about half of the ribosomes are associated with the inner mitochondrial membrane[82]. Mitochondrial ribosomes are considered to be prokaryotic in nature for several reasons: (i) the spectrum of antibiotics inhibiting mitochondrial protein synthesis resembles that of bacteria; (ii) they use *N*-formylmethionyl-tRNA for polypeptide chain iniciation and (iii) mitochondrial translation initiation and elongation factors are also functional on bacterial ribosomes *in vitro*[79]. Nevertheless, the physiochemical properties of mitochondrial ribosomes differ considerably from both their cytosolic and their bacterial counterparts.

Mitochondrial ribosomes have a remarkable low RNA content, which is compensated by high protein content. The large (39S) and small (28S) subunits of mitochondrial ribosome contain respectively the 16S and the 12S rRNA species, encoded by the mtDNA. The majority, if not all, human mitochondrial proteins (MRP) have been identified and mapped. There are 29 distinct proteins in the small ribosomal subunit and the large subunit contains 48 different proteins, all of them nuclear-encoded[83,84]. Surprisingly, three sequence variants have been found for one of the proteins of the small mitochondrial ribosome subunit, MRPS18. In analogy to bacterial ribosomes, it is likely that each mitochondrial ribosome contains a single copy of MRPS18. Therefore, the presence of three different isoforms suggests that

there is a heterogeneous population of mitochondrial ribosomes, which may have different decoding properties[83]. Two other MRPs of the small subunit, MRPS29 and MRPS30, were earlier identified as pro-apoptotic proteins, suggesting that mitochondrial ribosomes play an important role in cellular apoptotic signalling pathways[85,86].

Translation. Mammalian mitochondrial mRNAs have no leader sequences to facilitate ribosome binding, and a cap recognition mechanism for directing the ribosome to the initiation codon is also missing. The low mitochondrial translation efficiency may, in fact, be the result of the absence of a 5'-end ribosome recognition site and therefore, abundant mRNAs are required to ensure a sufficient level of translation. The small mitochondrial ribosomal subunit has the ability to bind mRNA tightly in a sequence independent fashion. Once the small ribosomal subunit is bound to template mRNA, the subunit moves towards the initiation codon and protein synthesis begins following the classical model described in *E.coli*: the initiation tRNA binds to the peptidyl (P) site on the ribosome, while the two other sites for tRNA molecules, the aminoacyl (A) and the exit (E) sites, remain empty. Then, the large ribosomal subunit associates and the 55S initiation complex is formed. Several translation initiation factors may be involved in the initiation of translation, but most of them are still unknown[79]. The main features of elongation of translation are similar to those of *E.coli*: an aminoacid is added at a time to the growing polypeptide according to the sequence of codons found in the mRNA. First, the codon is exposed in the A-site where the codon-anticodon interaction with the corresponding aa-tRNA takes place, and upon cognate recognition, a peptide bond formation is catalysed between the incoming aminoacid and the growing peptide found in the P-site. Then, the peptidyl-tRNA in the A-site is physically translocated to the P-site, the mRNA precisely moves three nucleotides and the deacylated-tRNA is shifted to the E-site before its release, leaving the ribosome ready for the next round of elongation. Termination of protein synthesis requires the action of several release factors (MTRF; mitochondrial release factor), which recognize the stop codons resulting in the hydrolysis of the bond between the polypeptide chain and the tRNA at the P site. The detached polypeptide leaves the ribosome, followed by the tRNA and mRNA, and subsequently, the two ribosomal subunits disassemble and the small ribosomal subunit is set to start a new round of protein synthesis[79].

2.2.3 Inheritance and segregation of mitochondrial DNA

The transmission of mtDNA is a complex phenomenon that is far from understood. Mitochondrial DNA's high copy number, its compartmentalization in organelles, and

the fact that mtDNA is organized in nucleoids inside mitochondria, contribute to complexity.

In mammalian cells, the sequence of all or almost all, copies of mtDNA is identical at any given nucleotide position, a condition known as **homoplasmy**. Occasionally, sequence variations lead to a dual population of mtDNA, situation referred to as **heteroplasmy**. Heteroplasmy is a rare condition in normal individuals, but it is often associated with mtDNA disease (mixed population of wild-type and mutant mtDNA). Heteroplasmy occurs both within and between cells, and also between organs[72]. The heteroplasmy rate is the product of the apparently random segregation of wild type to mutant DNA during early embryogenesis[62].

Inheritance of mtDNA is exclusively maternal. Usually, paternally derived mtDNA is labelled with an ubiquitine tag, which invokes rapid proteolysis when it enters the oocyte[87]. This leads to the expectation that a mtDNA mutation can lead to disease equally in both sexes, but can only be transmitted through the maternal line[88]. There is however, a unique case report of a patient with mitochondrial disease in which the causative mutation arose on paternal mtDNA. It comprised 90% of mtDNA in skeletal muscle but all other tissues contained maternally derived mtDNA[89].

Very little is known about the mechanisms of mtDNA transmission in humans. It has been shown that heteroplasmic female mice transmit neutral heteroplasmic mtDNA polymorphisms to their offspring, and the variation among the offspring is largely determined by random genetic drift[90]. It is thought that a restriction in the number of mtDNA molecules in early oogenesis is behind this process, what is known as the **mitochondrial genetic bottleneck**[91]. The bottleneck theory was proposed by Ashley et al[92] who found in Holstein cows that the proportion of heteroplasmy in a mtDNA site could change in a single generation and could revert to homoplasmy in two to three generations. According to this theory, mtDNA from a few mitochondria would be selectively amplified during oogenesis and thus, a genotype can become predominant and fixed in future generations[93]. As a result, a female harbouring a pathogenic mtDNA defect might transmit low levels of mutant mtDNA to some offspring and high levels to others. Some of the offspring may be severely affected whilst some may remain asymptomatic throughout their life[94].

Both mitochondrial division and mtDNA replication are unrelated to the cell cycle or to the timing of nuclear DNA replication. Thus, a dividing cell has the potential to donate a variable number of organelles and genomes to its daughter cells, a phenomenon termed **mitotic segregation**. Mitochondrial segregation was originally believed to be a random process, but recent findings have shown that

segregation occurs not only in a tissue-specific manner but is also under nuclear genetic control[95].

The simplistic view of mtDNA inheritance – where mitochondria are distinct particles passively awaiting segregation by the cytokinesis machinery – ignores the potential role of mitochondrial dynamics, a prominent feature of these organelles[96]. Recent data suggest that mitochondria are capable of fusion and fission, and that they are organized in a dynamic architecture with transitions between a network-like structure and a more disperse one[97]. Although these issues await clarification, the fact that these organelles are dynamic and able to interchange material is likely to have important consequences for the interaction, and thereby segregation and inheritance, of mtDNA molecules.

2.3 MITOCHONDRIAL DISEASE

Mitochondria are vital components of all nucleated cells. Thus, genes affecting the mitochondrion play a central role in human health and disease. Most mtDNA genes have been linked to human diseases. However, mitochondrial disease can also arise from nuclear gene mutations, as most human mitochondrial proteins are coded in the nucleus and then imported into the organelle. These findings corroborate again the importance of fully functional mitochondria to human health.

Mitochondrial diseases can either be due to sporadic or spontaneous gene mutations in mtDNA or nuclear DNA, or alternatively to exogenous factors such as drugs, toxins and infections[62]. In children, approximately one-third of the inherited metabolic disorders are attributable to mitochondrial defects[62]. Estimates from the British population indicate that 1 in 3,500 people either suffer from some mtDNA disease or are at risk of developing it[76]. These estimates do not include the recent association of mtDNA mutations and common clinical features such as hypertension, suggesting that the incidence could be still higher[98]. In addition, there is increasing evidence that acquired mtDNA mutations and mitochondrial dysfunction are involved in aging and age-related diseases[76].

Inherited disorders of mitochondrial function produce childhood and adult diseases with notoriously diverse clinical presentations. Mitochondrial diseases affecting a single organ are rare and tend to present as multisystem disorders affecting predominantly tissues with high oxygen and energy demands like muscle, brain, heart, liver, retina, ear and guts[62]. Mitochondrial dysfunction has been linked to disorders as diverse as infertility, cancer, migraine, diabetes, heart disease,

blindness, deafness, hair and skin disorders, kidney disease, liver disease, stroke, Parkinson disease, Alzheimer and the aging process itself[72].

A striking feature of mtDNA diseases is their clinical heterogeneity. This may be in part explained by the presence of heteroplasmy (coexistence of mutant and wild type DNA within cells and tissues). The fraction of mutant mtDNA may vary from less than 1% to more than 95% in affected tissues of patients with mitochondrial disease. In addition, the amount of heteroplasmy varies from tissue to tissue and even between cells within a tissue[72], and, in some cases, heteroplasmy can change also with time[74]. The most functionally drastic mutations are always found in heteroplasmic state, since homoplasmy entails lethality. On the contrary, at modest levels of heteroplasmy even drastic mutations can have a subtle phenotypic effect. Conversely, functionally mild mutations that can segregate to homoplasmy in the germ line without compromising early development might have a profound effect in some specific tissues[99]. Nevertheless, for some mitochondrial diseases the phenotype is independent of mutant mtDNA abundance, suggesting the involvement of other factors. The threshold effect (percentage of mutation needed to manifest clinically, dependent on each particular tissue), the age and the environment can also influence the pathogenesis of mitochondrial disorders. In addition, the modulating effect of other mitochondrial and/or nuclear genes could also contribute to the diversity of clinical phenotypes[100].

Despite the clinical importance of mitochondrial diseases and the fact that the sequence, the genes and the presumed function of mitochondrial chromosome have been completely described for decades, the molecular mechanisms leading from genotype to clinical phenotype remain unsolved. The pathophysiology of mitochondrial diseases is also not well known. While disruption of oxidative phosphorylation is central to mitochondrial diseases, many other factors such as calcium dyshomeostasis, increased oxidative stress, and defective turnover of mitochondrial proteins may also contribute.

This lack of knowledge regarding genotype-phenotype correlations and the pathophysiology of mitochondrial disease has prevented useful counselling of patients and the search for better therapeutic interventions.

2.3.1 Pathological mutations of mitochondrial DNA

Pathological mtDNA mutations have been known for over a decade, yet their mechanistic is not well understood. To date, over 100 point mutations and 200 deletions and rearrangements in mtDNA have been associated with disease[59]. Unfortunately, the high degree of polymorphism in mtDNA complicates the

interpretation of "mutations" found in patients. In general, to consider a mtDNA sequence change as a mutation, three out of the following four criteria must be met: (1) it is not found in controls, (2) it is seen in unrelated pedigrees with similar disease presentations, (3) the nature and location of the mutation suggests a logical mechanism of disease and (4) it is heteroplasmic[72].

mtDNA mutations in humans can be classified into three broad categories: (i) point mutations affecting protein-coding genes (oxidative phosphorylation); (ii) point mutations affecting the protein synthetic apparatus; and (iii) large deletions[99].

Point mutations affecting respiratory chain and oxidative phosphorylation

Human diseases caused by impaired respiratory chain function and oxidative phosphorylation have been increasingly recognized. Although neurological diseases are the most usual consequence of such dysfunctions, it is now apparent that virtually any tissue in the body can be affected[101].

The most common missense mutations occur in families with Leber's hereditary optic neuropathy (LHON). Several mtDNA mutations have been described in association with LHON, but three of them, G11778A, G3460A and T14484C, are present in around 90% of the families. These mutations occur in the *ND4*, *ND1* and *ND6* genes, respectively, being all of them art of the NADH ubiquinone-oxidoreductase of complex I[102]. The incomplete penetrance of the disease and the predominance of males affected suggest that other factors (other mtDNA or nuclear mutations) play a role in the clinical manifestation of the disease[62].

Missense mutations in the ATPase genes can cause Leigh syndrome, an encephalopathy with ataxia and brain stem signs in children. The same mutations can also present later in childhood or adult life with a milder phenotype (NARP; neurogenic weakness with ataxia and retinitis pigmentosa)[94].

Mutations in cytochrome *b* gene (Cyt *b*), a subunit of complex III, have been linked to several diseases, such as LHON, mitochondrial myopathy, mitochondrial encephalopathy, exercise intolerance and myoglobinuria. Different mutations have been identified, scattered throughout the protein, but all of them characterized by impaired complex III activity, with a strikingly similar phenotype[103,104].

Point mutations affecting protein synthesis

In addition to the 13 protein coding genes, human mtDNA contains 24 genes encoding essential RNA components of the translation apparatus: 2 rRNAs and 22 tRNAs. At the present, mutations in 19 of the 22 tRNA genes and in one of the two rRNA genes have been reported in association with disease, although not every one

has been validated by mechanistic studies, or by large-scale epidemiological analysis[59].

The most common point mutation in a tRNA gene is A3243G in the $tRNA^{Leu(UUR)}$. This mutation was first described in a patient with mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), but it can also cause diabetes mellitus and deafness, chronic progressive external ophtalmoplegia (CPEO) and cardiomyopathy[94]. Numerous other point mutations involving tRNA genes have been described in association with a range of different clinical phenotypes, including MERRF syndrome (myoclonic epilepsy with ragged-red fibres). A mutation in $tRNA^{Lys}$, A8344G, is often associated with MERRF syndrome, although other mutations in the same gene have been found in other phenotypically indistinguishable cases of the disease[99].

Mutations in rRNA genes cause mainly non-syndromic sensorineural deafness. These mutations have been the focus of the work presented in this doctoral thesis, and therefore, will be discussed in more detail later on.

Large deletions and mitochondrial depletion

Small amounts of mtDNA with large-scale rearrangements can be found in normal individuals, generated by non-specific disease processes such as degeneration during aging or degenerative diseases. However, since they account for a small proportion of mtDNA present in tissues, it remains to be determined whether or not they have a pathological effect[105].

The disorders associated with mtDNA deletions have multiple clinical outcomes. Large deletions (from a few hundred bp to >10 kb) generally cause sporadic disorders such as chronic progressive external ophtalmoplegia (CPEO) and the Kearns-Sayre syndrome[94]. Duplication of mtDNA is also found in patients with mtDNA disease. Although duplications are not primarily pathogenic, they can lead to the formation of deletions that can cause disease.

Depletion of mtDNA is observed in children presenting in the first months of life with hypotonia, renal dysfunction, hepatic failure and lactic acidosis. This can lead to death within the first year of life. In less severe cases, individuals survive into late childhood[94].

2.3.2 Nuclear genes and mitochondrial diseases

Nuclear gene products account for the vast majority of mitochondrial proteins, being, therefore, candidate genes for mitochondrial disorders. However, currently the number of defined mitochondrial disorders caused by defects of nuclear genes is still small.

Nuclear gene mutations associated with mitochondrial disorders can be classified into three groups based on the function of the mutated protein in the mitochondrion: (i) mutations leading to loss or instability of mtDNA; (ii) mutations affecting mitochondrial functions, mainly oxidative phosphorylation; an (iii) mutations affecting mtDNA expression.

Nuclear gene mutations leading to loss or instability of mtDNA

Practically any defective protein involved in mtDNA replication, maintenance, segregation or mtDNA organization within mitochondria, could precipitate loss or instability of mtDNA, although many such defects would be lethal at an early developmental stage.

The most significant advances of nuclear-mitochondrial disorders involving mtDNA instability came from the study of families with autosomal recessive mitochondrial neurogastrointestinal encephamolyopathy (MNGIE) and autosomal dominant chronic progressive ophtalmoplegia (CPEO). Both disorders are associated with the formation of multiple mtDNA deletions in postmitotic tissues, which cause the clinical phenotype[106].

MNGIE is caused by mutations in nuclear-encoded thymidine phosphorylase (*TP*) gene, which is likely to have an important role in nucleoside metabolism by regulating the availability of thymidine for DNA synthesis[62,106]. In the case of CPEO, most patients have mutations in one of three nuclear genes: *POLG1*, encoding the catalytic subunit of the mtDNA-specific polymerase-γ; *SLC25A4* encoding ANT1, the muscle-heart specific mitochondrial adenine nucleotide translocator; or *C10orf2* encoding Twinkle, a mtDNA helicase[106,107].

Multiple mtDNA deletions arise either from mutations in *POLG* or indirectly due to disturbed deoxi-nucleotide (dNTP) pools, as they also reduce the fidelity of the polymerase. Alternatively, a dNTP imbalance may affect other mitochondrial processing enzymes, such as Twinkle, involved in mtDNA replication and repair. When generated, the partially deleted mtDNA molecules replicate faster than larger mtDNA molecules and the mutant mtDNA may thus increase in proportion over time. When a critical, possibly tissue-specific threshold in the level of mutant mtDNA is exceeded, clinical phenotype develops[105].

Nuclear gene mutations affecting oxidative phosphorylation

Deficiences in oxidative phosphorylation are associated with a diverse array of multisystem disorders that are often referred to as mitochondrial encephalomyopathies, because of the prominent involvement of the nervous systems and striated muscle[108].

The clinical presentation of defects of the respiratory chain is heterogeneous, with onset ranging from neonatal to adult life[109]. In children, the most common clinical presentation is that of Leigh syndrome, a fatal progressive neurodegenerative disease[109]. All defects described in Leigh syndrome patients affect oxidative metabolism, likely impairing energy production. Thus, the typical neuropathological findings are the expression of the damage produced by faulty oxidative metabolism on the developing brain[109]. Leigh syndrome is genetically heterogeneous; it is caused by mutations in genes encoding respiratory chain subunits but also in mtDNA[62].

Deficiencies in Complex I are the most common respiratory chain defects, and about half of the patients present with Leigh syndrome. Less common presentations of Complex I deficiency are hepatophaty, cardiomyopathy, cataracts and lactic acidosis. Several disease-associated mutations in nuclear-encoded subunits of Complex I have been discovered, but no major mutation hotspot has been identified. To date, mutations in six different structural components of Complex I have been reported: *NDUFS4*, *NDUFS7*, *NDUFS8*, *NDUFV1*, *NDUFS1* and *NDUFS2*[108].

Complex II subunits are all nuclear-encoded. Mutations of complex II are a rare cause of Leigh syndrome or late-onset neurodegenerative disease. However, several complex II mutations have been reported in association with inherited paragangliomas and non-familial phateochromocytomas[109]. Three of the genes encoding structural subunits of Complex II (*SDHB*, *SDHC* and *SDHD*) have been implicated as tumour suppressors and suggested to play a role in oxygen sensing and hypoxia response[108].

Complex IV (COX) deficiency is possibly the most frequent biochemical abnormality in mitochondrial disease. All of the nuclear-gene defects of COX are due to mutations in enzyme assembly factors. These include five genes: *SURF1*, *SCO1*, *SCO2*, *COX10* and *COX15*[109]. Mutations in *SURF1* account for the majority of COX deficiency Leigh syndrome cases. Absence of SURF1 causes the accumulation of early assembly intermediates and the drastic reduction of fully assembled COX[109]. Mutations in other COX-assembly genes are much rare. SCO1 and SCO2 are copper-binding proteins involved in the insertion of copper into the holoenzyme. Mutations in *SCO* genes usually present as an early-onset fatal cardio-encephalo-myopathy[109].

In addition to defects in respiratory chain complexes, some neurodegenerative disorders have been attributed to mutations in mitochondrial proteins indirectly related to respiratory and energy production. The clearest example is Friedreich's ataxia, which is caused by the expansion of intronic repeats

in the *frataxin* gene[108]. Although the exact function of frataxin remains controversial, it plays a role in some aspect of mitochondrial iron handling, and a deficiency in the protein leads to marked reductions in the activity of mitochondrial enzymes with Fe-S clusters[108].

Nuclear gene mutations affecting mitochondrial gene expression

Loss or mutation of nuclear genes encoding components of the mitochondrial protein-synthesis machinery can also result in mitochondrial disease, as well as its mitochondria-encoded components, tRNAs and rRNAs. Many nuclear genes can be considered as suitable candidates, including those that encode nuclear proteins of mitochondrial ribosome and those encoding mitochondrial aminoacyl-tRNA synthetases, tRNA modification enzymes and translation factors[110].

Although the genes for human mitochondrial ribosomal proteins (MRPs) have been recently identified, a homozygous nonsense mutation in *MRPS16* has been already associated with a case of fatal neonatal encephalopathy[111]. Many of the MRPs map to loci associated with various developmental and sensorineural disorders, including some deafness loci, being candidate genes for such disorders[112].

Another class of mitochondrial-protein-synthesis defects involves tRNA modification, which seems to be important for efficient and accurate decoding. One example is the human pseudouridylate synthase 1 gene (*PUS1*), in which a mutation has been identified in a patient with mitochondrial myopathy and sideroblastic anemia[110].

2.3.3 Mitochondria in neurodegenerative disorders and aging

Neurons appear particularly vulnerable to mitochondrial dysfunction, and many mitochondrial disease results in neurodegeneration. Thus, it is not unexpected that increasing evidences relate common neurodegenerative diseases and aging process with mitochondrial respiratory chain function and mtDNA mutations.

Mitochondrial respiratory chain function is impaired in many neurodegenerative diseases, but the underlying causes of this dysfunction and the role it plays in the disease's pathogenesis may be considerable variable in different diseases. Mutations in nuclear genes encoding mitochondrial respiratory chain subunits have been identified in patients with Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS)[113]. However, there is no evidence that they are important in the majority of patients with these diseases. It has been also suggested that increased excitotoxicity (Huntington's disease, ALS), altered mitochondrial biogenesis and increased oxidative stress and damage (ALS,

PD and Friedreich's ataxia), may account for the changes in mitochondria respiratory chain function found in neurodegenerative diseases[113].

In 1986, Miquel and Fleming presented the "oxygen radical-mitochondrial injury hypothesis of aging" and since then, several lines of investigation have implicated mitochondria in the naturally occurring process of aging[74]. The biochemical reactions of mitochondrial respiratory chain have inherent danger because of electron leakage leading to the production of reactive oxygen species (ROS). Because mtDNA is spatially close to the source of ROS, it is thought to be particularly vulnerable to ROS-mediated mutations. Together with impairment of respiration, mtDNA mutations can have a second, more insidious consequence: mtDNA mutations that reduce the accuracy of electron transfer increase the likelihood of ROS production and further mtDNA lesions, leading to a so-called "vicious cycle". This scenario is the basis for the hypothesis that mitochondrial dysfunction plays a critical role in the aging process. In this view, aging is caused by the ROS-accelerated accumulation of mtDNA damage, leading to a progressive decline in respiratory function over time[96].

In support of this hypothesis, many tissues from aged individuals have found to have lower respiratory function compared to those from younger individuals[114-116]. Both mtDNA point mutations and deletions are more prevalent in aged tissues and cells[117,118]. Aging has also been associated with clonal mtDNA deletions and respiratory incompetence in single neurons in the substantia nigra[119,120].

Although these studies show that aging is associated with declining respiratory function, accumulation of variable levels of mtDNA deletions and point mutations, and accumulation of oxidative damage to mtDNA, there remain substantial concerns about whether these changes are causal in the aging process[96]. First, some studies suggest that respiratory decline is due to physical inactivity rather than to chronological age[121]. Second, the functional significance of mtDNA alterations is unclear. The levels of mtDNA mutations found in most studies are too low to affect respiratory function. In cell culture and animal model systems, no defect in respiration is found until pathogenic mtDNA genomes reach very high levels[122-124]. Finally, although specific point mutations can reach high levels in aged individuals, the effect of these mutations on mitochondrial function remains to be determined[117].

3. Mitochondria and hearing loss

Although most hereditary hearing loss is due to nuclear gene defects, in recent years, it has become clear the important contribution of mitochondrial genes. Actually, the first genetic defect causing nonsyndromic hearing loss was detected in 1993 and was a mitochondrial mutation[88]. Various mutations in mtDNA causing both syndromic and nonsyndromic hearing loss, have been identified since then[76,125-127]. The relatively large number of mtDNA mutations identified in hearing loss patients, suggests an important role for mitochondria in the function of the inner ear (Table 3.1).

Several findings suggest that mitochondrial mutations are a frequent cause of hearing impairment. Causative mtDNA mutations were found in 5% of patients in both southern Italy and UK populations with postlingual nonsyndromic hearing loss[128]. Deleterious mtDNA point mutations and/or abnormal mtDNA content or multiple deletions were identified in 20 of 31 patients with mitochondrial syndromic hearing loss[129]. In addition, there is increasing evidence that acquired mtDNA mutations and mitochondrial dysfunction are involved in aging process and age-related diseases such as presbycusis[88]. Most mtDNA mutations identified to be responsible for hearing loss cases are located in tRNA and rRNA genes.

The study of mitochondrial mutations in different diseases has helped to describe and catalogue the spectrum and frequency of oxidative phosphorylation disorders, but it has not led to an understanding of the factors contributing to the two major clinical and biological issues: penetrance and tissue specificity, which may allow the establishment of genotype-phenotype correlations. MtDNA mutations linked to nonsyndromic hearing loss are generally homoplasmic and therefore,

provide a greatly simplified model to understand the pathophysiology of mitochondrial diseases in general.

Table 3.1. Mitochondrial DNA mutations associated with hearing loss.

GENE	MUTATION	PHENOTYPE
12S rRNA	A1555G	Nonsyndromic SNHL
	C1494T	Nonsyndromic SNHL
	961 mutations	Nonsyndromic SNHL
	T1095C	Nonsyndromic SNHL or with parkinsonism and neuropathy
	A827G	Nonsyndromic SNHL
tRNA$^{Ser(UCN)}$	A7445G	Nonsyndromic SNHL or with palmoplantar keratoderma
	7472insC	Nonsyndromic SNHL or with neurological dysfunction
	T7510C	Nonsyndromic SNHL
	T7511C	Nonsyndromic SNHL
	T7512C	Progressive myoclonic epilepsy, ataxia and hearing impairment
	G7444A	Nonsyndromic SNHL
tRNA$^{Leu(UUR)}$	A3243G	MELAS and MIDD
	C3256T	MERRF
tRNA^{Lys}	A8344G	MERRF
	T8356C	MERRF
	A8296G	MIDD
tRNA^{Glu}	T14709C	MIDD
Several	Large deletions	KSS
	Large deletions/duplication	MIDD
	Random	Presbyacusis

SNHL; sensorineural hearing loss, MELAS; mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes, MERRF; myoclonic epilepsy with ragged-red fibres, MIDD; maternally inherited diabetes and deafness, KSS; Kearns-Sayre syndrome

3.1 MITOCHONDRIAL DNA MUTATIONS AND SYNDROMIC HEARING LOSS

Hearing loss often occurs as one of several symptoms in syndromic diseases caused by mitochondrial defects. The most frequent forms of mitochondrial syndromic hearing loss are part of classic mitochondrial disorders, such as Kearns-Sayre syndrome, MELAS and MERRF. Excluding large rearrangements involving several genes, all mitochondrial mutations leading to syndromic hearing loss are point mutations in tRNA genes. In these cases, the heteroplasmic mtDNA mutations, either rearrangements or point mutations, can generally be found with the highest abundance in nervous tissues and muscle. Because of the higher energy requirements of these tissues, and the fact that small numbers of dysfunctional nerve and muscle cells can interrupt the function of many neighbouring normal cells, mtDNA mutations in these tissues are thought to be particularly harmful. Thus, it is not unexpected that generalized neuronal dysfunction is also expressed in the auditory system.

The first mitochondrial tRNA point mutation shown to be associated with human disease was the A8344G substitution in the gene for tRNA^{Lys} [130]. This heteroplasmic mutation was found to segregate matrilineally with MERRF

syndrome, a disease in which the degree of hearing loss is variable. Other mutations in the same $tRNA^{Lys}$, like the T8356C, have been found in cases of an essentially indistinguishable disease[131].

The second pathological tRNA mutation to be discovered, A3243G in the gene for $tRNA^{Leu(UUR)}$, has also become the most intensively studied[132]. The major reasons of interest in A3243G mutation are its high prevalence and its extremely variable clinical phenotype. The original syndromic disorder in which the mutation was found is MELAS[132], but the majority of patients with the A3243G mutation present other clinical symptoms: maternally inherited diabetes and deafness (MIDD), progressive external ophthalmoplegia (PEO) or other symptoms which include kidney disease, cardiomyopathy, neuropathy or endocrinopathies different from diabetes[99]. No fewer than 15 other mutations in $tRNA^{Leu(UUR)}$ has been linked to human disease, all of them found in heteroplasmy and deafness is present in a significant proportion of the associated syndromes (Table 3.1)[59].

Heteroplasmic point mutations T14709C in $tRNA^{Glu}$ and A8296G in $tRNA^{Lys}$ were also found to be associated with MIDD[88]. In these cases, hearing loss is sensorineural and usually develops after the onset of diabetes[88].

Finally, mutations affecting $tRNA^{Ser(UCN)}$ are prominently associated with syndromic disorders in which sensorineural deafness appears to be the predominant feature. Most of these mutations differ from those in $tRNA^{Lys}$ or $tRNA^{Leu(UUR)}$ in that clinically affected individuals are typically homoplasmic for the mutation[99]. Mutation A7445G is associated with palmoplantar keratoderma in addition to hearing loss and, the phenotype linked to mutations 7472insC and T7512C includes ataxia and myoclonus[99].

3.2 MITOCHONDRIAL DNA MUTATIONS AND NONSYNDROMIC HEARING LOSS

Hearing loss can also be the sole symptom of mitochondrial disease, suggesting that hearing is strongly dependent on mitochondrial function. Inherited deafness-associated mtDNA mutations usually occur in the genes encoding components of the protein-synthesizing apparatus: rRNAs and tRNAs[125].

Various mtDNA mutations causing progressive nonsyndromic hearing loss have been identified. Mutations in 12S rRNA gene and $tRNA^{Ser(UCN)}$ gene account for most cases of maternally inherited nonsyndromic deafness[125]. Nonsyndromic deafness-linked mutations are often homoplasmic or at high levels of heteroplasmy, indicating a high threshold for pathogeneicity[127]. Phenotypic expression of these

mtDNA mutations requires the contribution of other factors such as nuclear modifier genes, environmental factors, or mitochondrial haplotypes[125]. Hearing loss due to mtDNA mutations is usually of late childhood or early adulthood onset and progressively worsens with advancing age[126].

Recently, acquired mtDNA mutations have been proposed to be involved in the development of presbyacusis, the hearing loss that occurs with age in a significant proportion of individuals[127].

3.2.1 tRNA mutations

Mitochondrial tRNA genes are one of the hot spots for mutations of maternally inherited deafness, as a number of mutations in four tRNA genes have been identified. However, $tRNA^{Ser(UCN)}$ is the only one involved in nonsyndromic hearing loss cases.

In the mitochondrial $tRNA^{Ser(UCN)}$ gene, five nonsyndromic deafness-linked mutations have been identified: A7445G[133,134], 7472insC[128,135,136], T7510C[137,138], T7511C[139-142] and G7444A[143-145], all of them often found in homoplasmy. The primary defect in $tRNA^{Ser(UCN)}$ gene mutations appears to be a failure in tRNA metabolism, thereby leading to a decrease in the steady-state levels of affected tRNAs, which is subsequently responsible for the reduced rate of mitochondrial protein synthesis and the respiration defects[146].

The **A7445G** mutation was first described in a Scottish family[133] and confirmed and established in two unrelated pedigrees from New Zealand[147] and Japan[134]. The penetrance of this mutation in the Scottish pedigree is quite low, while in the New Zealand and Japanese pedigrees, is very high. Thus, mutation A7445G itself does not seem to be sufficient to cause hearing loss, but requires additional genetic or environmental factors, which seem to be rare in the Scottish pedigree and common in the New Zealand and Japanese pedigrees. Complete sequencing of mtDNA in the New Zealand pedigree revealed three additional sequence changes in complex I genes, two of which have also been labelled as secondary Leber's hereditary optic neuroretinopathy mutations[147]. Since these or similar changes are not present in the Scottish pedigree[133], mitochondrial haplotype appears to account for the differences in penetrance in this case.

The **7472insC** mutation was described in a Sicilian family with hearing loss, with some members presenting also a widespread neurological disease[135]. Later, it was identified in a Dutch family and several sporadic subjects only presenting progressive nonsymdromic hearing loss[136]. The mutation is heteroplasmic, although most individuals have over 90% of abnormal mitochondrial chromosomes in the

tissues examined[136]. Mutation 7472insC alone is usually sufficient to cause hearing loss, and when present at very high levels can also lead to neurological dysfunction.

The **T7510C** mutation has been identified in a British and a Spanish family with nonsyndromic hearing loss[137,138]. The mutation is predicted to disrupt base pairing in the acceptor stem of the tRNA, causing mitochondrial dysfunction.

The **T7511C** mutation is found associated with nonsyndromic hearing loss in several families from different ethnic groups, including African[139,148], French[141] and Japanese subjects[140]. This mutation often exists in homoplasmy or high level of heteroplasmy, but the degree of heteroplasmy does not correlate with the severity and age-of-onset of deafness. Despite sharing some common phenotypic features, subjects with T7511C mutation exhibit variable severity, age of onset and progression of hearing loss[139-142,148]. The high phenotypic variability and different penetrance between pedigrees suggest the existence of additional genetic or environmental factors modifying the expression of the mutation. Interestingly, two homoplasmic mutations, T3308C in *ND1* gene and T5655C in $tRNA^{Ala}$ gene, were found in all maternal members of an African American pedigree[139]. Functional studies indicated that cybrid cell lines derived from affected individuals showed a decreased mitochondrial function. This observation imply that a combination of the T7511C mutation with other mtDNA mutations can lead to significant biochemical defects in mutant cell lines and may account for the high penetrance of deafness in the African American family[149].

The **G7444A** mutation was first identified coexisting with the 12S rRNA gene A1555G mutation in two different Asian pedigrees[143]. The pathogenic role of this variant has not been established until recently, when it has been identified alone in homoplasmy and in two additional Chinese families with aminoglycoside-induced and nonsyndromic hearing loss[145]

3.2.2 rRNA mutations

Mitochondrial rRNA mutations associated with nonsyndromic hearing loss have been identified in the 12S rRNA gene linked with both, aminoglycoside-induced and nonsyndromic hearing loss. Different mutations have been identified: A1555G[150-152], C1494T[153], T1095C[154-156], A827G[157,158] and 961 mutations (961delT+C(n), T961G, T961C, 961insC)[159-161]. No deafness-associated mutations have been detected in the 16S rRNA gene.

The most common 12S rRNA mutation is A1555G, which has been identified in several families of various ethnic backgrounds. Due to the relevance for the work performed during this doctoral thesis, A1555G mutation and its related C1494T mutation will be discussed in a separate section.

The **T1095C** mutation was first identified in an Italian family with deafness, neuropathy and Parkinsonism[154]. Later on, this mutation has been found associated with nonsyndromic hearing loss in another Italian family[155], a Chinese woman with auditory neuropathy[156] and four Chinese patients with aminoglycoside-induced nonsyndromic sensorineural hearing loss[153,162]. This T-to-C transition disrupts an evolutionarily conserved base-pair at stem loop of 12S rRNA[163], resulting in impaired translation of mitochondrial protein synthesis and a significant decrease of cytochrome c oxidase activity, causing mitochondrial dysfunction[154].

Several mutations at position **961** in the 12S rRNA gene have been found in sporadic subjects and unrelated families with aminoglycoside-induced and/or nonsyndromic sensorineural hearing loss. These include 961delT+C(n) and 961insC mutations in Caucasian and Asian subjects, T961G mutation in Caucasian subjects and T961C in Chinese patients[159-161]. The region where nucleotide 961 is located is not very evolutionarily conserved and its function is not well defined. It has been postulated that alteration of the tertiary or quaternary structure of the 12S rRNA by mutations at position 961, may affect the binding of aminoglycosides and result in a mitochondrial translation defect[125].

More recently, two Chinese pedigrees affected of maternally inherited sensorineural nonsyndromic hearing loss have been identified, both carrying in homoplasmy the **A827G** mutation [157,158]. In one family, hearing loss was of congenital or early-onset[157], whereas in the other case deafness developed only after use of aminoglycosides[158]. This mutation was also previously detected in several sporadic individuals with aminoglycoside-induced and/or nonsyndromic hearing loss[162]. The A827G mutation is located in the A-site of the mitochondrial 12S rRNA gene, which is highly evolutionary conserved.

3.2.3 Presbyacusis

A condition associated with acquired heteroplasmic mtDNA mutations and hearing loss is presbyacusis. Presbyacusis is the hearing loss that occurs with age in a significant proportion of individuals. Since mtDNA mutations and the resulting loss of oxidative phosphorylation activity seem to play an important role in the aging process, it is not unlikely that acquired mtDNA mutations are, in the auditory system, a cause of presbyacusis.

In different studies, screening of cochlear tissue from temporal bones of patients with presbyacusis have identified mtDNA mutations, which could be related to age-related hearing loss. The common **4977-bp deletion** is found in a significantly higher proportion of patients with presbyacusis than in hearing controls[164]. In addition, mutations in the mitochondrially-encoded cytochrome

oxidase II gene were found to be more frequent in presbyacusis patients compared to controls[165].

However, with the existing data it is still unclear to what extent a certain mutant mtDNA load in the cochlea is translated into detectable pathology. First, it is difficult to correlate the number of mitochondrial mutations with functional deficits, particularly in the brain. Mitochondrial haplotype and nuclear factors can also influence the functional consequences of acquired mtDNA mutations. Moreover, it has not been established whether the identified mitochondrial mutations are primary causing the tissue dysfunction or whether some of the degenerative disease of older age lead to the acquisition of mtDNA mutations as a secondary event[166]. In this sense, two different models are possible. The first model assumes that genetic and/or environmental factors combine to cause acquired mtDNA mutations that lead to hearing loss, implying a primary causative role for mtDNA mutations. Alternatively, other genetic or environmental factors could cause presbyacusis directly, resulting in cell death, which will lead to increased oxygen radicals and an increased rate of mtDNA mutations. In this case, mtDNA mutations would be only a sign of presbyacusis but not a cause[88].

3.3 THE A1555G MUTATION

The first mutation shown to cause non-syndromic hearing loss in humans was the A1555G mutation in the small ribosomal RNA gene (12S rRNA). Nowadays, it has been found in hundreds of families throughout the world, making it one of the most common genetic causes of hearing loss currently identified.

Mutation A1555G was first described in a large Arab-Israeli pedigree[150] were most of the deaf family members had onset of severe to profound sensorineural hearing loss during infancy, but a minority of family members had onset during childhood or even adulthood. Mutation A1555G was subsequently found in other families, initially only in conjunction with previous exposure to aminoglycosides[150]. However, afterwards, pedigrees were described in Zaire, Spain and Italy with family members who went deaf with and without aminoglycosides[151,152,167,168]. In particular, a study by Estivill et al, is remarkable for indicating a higher than previously expected frequency of this mutation[152]. First, it describes 19 families with the A1555G mutation out of a total of 70 collected families with sensorineural hearing loss, representing an unexpectedly high frequency of familial sensorineural hearing loss due to A1555G mutation. Second, the fact that the mutation was identified on different haplotypes, a finding supported by a study from Torroni and

colleagues[169], indicates that it is likely that this mutation exists in other populations as well, and may not be rare[169,170]. In addition, and different from the Arab-Israeli pedigree, the age of onset of hearing loss in the Spanish and Italian families was rarely congenital.

Mutation A1555G resides in a highly conserved region and a functionally well-characterized domain, which is an essential part of the decoding site of the small ribosomal subunit[171,172]. The mutation has now been detected in many families of different ethnic backgrounds[141,152,162,167,168,173,174]. It can be found in 0.6-2.5% of the Caucasian clinical population with nonsyndromic hearing loss, although the frequency is particularly high in Spanish population[152]. In Asian populations, the incidence of A1555G mutation is also high: 2.9% in Chinese[162], 3% in Japanese[175] and 5.3% in Indonesia[173]. Usually, mutation A1555G occurs in homoplasmy, but in some families the heteroplasmic state is also identified[167,176]. However, there is not a clear correlation between the mutation load and the severity of hearing loss[176].

Phenotypic expression of mutation A1555G is extremely variable, including cases of normal hearing carriers[148]. Hearing loss, in affected cases, shows a varied age of onset and severity, some individuals being deaf from birth, whereas others show a slow, progressive hearing loss of adult-onset. Such variation may imply the involvement of other factors, which influence the age of onset and progression of hearing loss. Aminoglycoside antibiotics, mitochondrial haplotypes and nuclear modifying genes have been proposed as the three major modulators for the phenotypic expression of the deafness-associated 12S rRNA mutations[125].

In the absence of exposure to aminoglycosides, the A1555G mutation produces a clinical phenotype that varies considerably among family members and ranges from severe congenital deafness, to moderate progressive hearing loss of later onset, to completely normal hearing[125]. The penetrance differs between families carrying A1555G mutation. In some families, most of the individuals carrying mutation A1555G develop hearing loss, but in others, the penetrance can be extremely low[174,177]. Functional characterization in the Arab-Israeli family demonstrate that more severe biochemical defects were observed in mutant lymphoblastoid cell lines derived form symptomatic individuals that in cell lines derived from asymptomatic individuals[178]. However, under a constant nuclear background, a nearly identical degree of mitochondrial dysfunction was observe in cybrid cell lines derived from symptomatic and asymptomatic individuals from this family[179]. These genetic and biochemical data strongly point out that the A1555G mutation is a primary factor underlying the development of deafness and that nuclear modifier genes play a role in modulating the phenotypic expression of the hearing loss associated wit the A1555G mutation.

Recently, a number of families affected of nonsyndromic hearing loss carrying a homoplasmic C to T transition at position 1494 (**C1494T** mutation) in the mitochondrial 12S rRNA gene have been identified[153,180,181]. Similar to A1555G mutation, the phenotype observed in these families is characterized by a variable severity and age of onset of hearing impairment, and aminoglycosides can induce or worsen deafness in carriers of C1494T mutation. Functional studies using lymphoblastoid cell lines derived from C1494T carriers showed a significant decrease in the rate of total oxygen consumption[181]. The wild-type nucleotide at position 1555 is A, but when mutated to a G, it would pair with the C at position 1494[125] (Figure 3.1). Thus, the identification of families carrying C1494T mutation with an essentially non-distinguishable phenotype from those with A1555G mutation, gives further evidences of the important role of this specific site of the 12S rRNA gene in the onset of deafness.

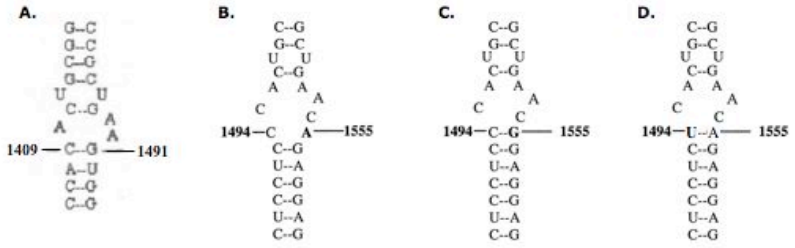

Figure 3.1. The site of the A1555G and C1494T mutations in the decoding region of mitochondrial 12S rRNA. **A.** E. coli 16S rRNA corresponding region **B.** Human wild-type 12S rRNA; **C.** A1555G mutation; **D.** C1494T mutation.

3.3.1 Aminoglycoside ototoxicity and A1555G mechanism of action

Aminoglycoside antibiotics, such as gentamicin, streptomycin, kanamycin and tobramycin, are clinically important drugs. They are mainly used in the treatment of aerobic gram-negative bacterial infections, particularly chronic infections such as tuberculosis or infections associated with cystic fibrosis[182]. These drugs are known to exert their antibacterial effects by binding to 16S ribosomal RNA in the small subunit of the bacterial ribosome, causing mistranslation or premature termination of protein synthesis[183]. Use of these drugs, even at conventional treatment doses, can frequently lead to toxicity, which involves the renal, auditory and vestibular systems, because they are concentrated in renal tubular cells and in the perilymph and endolymph of the inner ear[182,184]. The renal damage is usually reversible, but the auditory and vestibular ototoxicity frequently is not. Although all aminoglycosides can affect cochlear and vestibular functions, some (streptomycin and gentamicin) produce predominantly vestibular damage, whereas others (neomycin and kanamycin) cause mainly cochlear damage[182].

Mitochondrial ribosomes share more similarities with bacterial ribosomes than do cytosolic ribosomes[185]. Therefore, it is thought that the ototoxic site of action of aminoglycoside antibiotics is the mitochondrial ribosome. The fact that aminoglycoside hypersensitivity is often maternally transmitted suggests that mtDNA mutations are involved in aminoglycoside ototoxicity, as it has been demonstrated for different mutations in the 12S rRNA gene.

As previously mentioned, mutation A1555G is the most common cause of aminoglycoside-induced hearing loss, especially in familial cases[126,186]. This mutation accounts for 33% of a Japanese population with a history of exposure to aminoglycosides[175]. The A1555G mutation is also present in 13% of Chinese pediatric subjects with aminoglycoside ototoxicity[162]. In Caucasian populations, 17% and 17.7% of cases in U.S. and Spanish cohorts with aminoglycoside ototoxicity carry the A1555G mutation respectively[152,187]. These indicate that the mitochondrial genome, and especially the 12S rRNA gene, is a hot spot for aminoglycoside-induced hearing loss.

It is known that nucleotide 1555 is located in a highly conserved region of the 12S rRNA, which is an essential part of the decoding site of small ribosomal subunit and is important for the action of aminoglycosides. In particular, the new nucleotide pair formed in the presence of A1555G or C1494T mutations (1555G-1494C and 1494U-1555A, respectively) makes the secondary structure of the RNA resemble more closely the corresponding region of *E. coli* 16S rRNA and consequently leads to defects in mitochondrial translation[188]. The new nucleotide pair in the 12S rRNA is also expected to create a binding site for aminoglycosides, which facilitates interaction with these drugs[189]. Thus, exposure to aminoglycosides causes hearing loss in individuals carrying A1555G or C1494T mutations (Figure 3.1).

Different aspects of mitochondrial oxidative phosphorylation are important in the pathogenesis of aminoglycoside ototoxicity: (i) mitochondria generate cellular energy in the form of ATP by the process of oxidative phosphorylation, which is essential for cellular function; (ii) oxidative phosphorylation is the major endogenous source of reactive oxygen species, which are toxic by-products of respiration; and (iii) mitochondria provide a major switch for the initiation of apoptosis. Based on this, the following mechanism of action has been proposed for aminoglycoside ototoxicity[125]. Aminoglycosides accumulate in cochlear mitochondria, where they inhibit mitochondrial protein synthesis by interacting with the 12S rRNA, especially when it carries A1555G or C1494T mutations. These mitochondrial translational defects result in a decline in ATP production in the cochlear cells. At the same time, these defects in oxidative phosphorylation lead to

increased generation of reactive oxygen species, thereby damaging mitochondrial and cellular proteins, lipids and nucleic acids. Consequently, mitochondrial permeability transition pore opens and activates apoptosis. This causes a loss of cochlear cell function or cell death and gives rise to hearing impairment.

A similar mechanism would take place in absence of exposure to aminoglycosides. In this case, other additional factors would lead to a decreased mitochondrial function and initiate the chain of events which compromise cochlear cell survival.

3.3.2 Nuclear modifying factors

Genetic and biochemical data have strongly pointed out that the A1555G is a primary factor underlying the development of deafness and that other environmental and/or genetic factors play a role in modulating the phenotypic expression of the hearing loss associated with the A1555G mutation[125].

The Arab-Israeli and some of the Spanish and Italian pedigrees are good examples of the role of nuclear modifying genes. The entire Arab-Israeli family lives in the similar environmental surroundings of a small Arab village in Israel, and all maternal relatives share the same mitochondrial haplotype. Biochemical differences between lymphoblastoid cell lines of hearing and deaf family members with identical mitochondrial chromosomes provide direct support for the role of nuclear factors[178,188]. An extensive genome wide search has led to the conclusion that this nuclear effect is unlikely to be due to the effect of a single nuclear locus, but involves a number of modifier genes[190,191] (Table 3.2).

Table 3.2. Results of multipoint sib-pair analysis in Spanish/Italian set of families and the Arab-Israeli kindred[191]. A LODscore of 3.1 was obtained for marker D8S277, combining all the data.

SPANISH/ITALIAN CANDIDATE LOCI IN ARAB-ISRAELI KINDRED			ARAB-ISRAELI CANDIDATE LOCI IN SPANISH/ITALIAN FAMILIES		
	Multipoint nonparametric LOD score			Multipoint nonparametric LOD score	
Marker	Spanish/Italian	Arab-Israeli	Marker	Arab-Israeli	Spanish/Italian
D17S949	1.7	0.4	D14S280	2.1	0.4
D8S277	**1.4**	**1.6**	**D8S277**	**1.6**	**1.4**
D1S312	1.3	0.0	D15S107	0.9	0.0
D9S175	1.3	0.0	D18S462	0.7	0.2
D6S305	1.1	0.1	D1S234	0.7	0.3
D3S1300	0.9	0.0	D4S1627	0.6	0.0
D7S550	0.9	0.6	D12S269	0.6	0.0
D2S347	0.7	0.3	D7S507	0.5	0.0
D10S597	0.6	0.0	D20S470	0.5	0.0
D18S64	0.5	0.2	D5S471	0.5	0.1

The shared locus is underlined

The chromosomal location of one of these modifier genes has been identified in chromosome 8p23.1, and linkage disequilibrium has been obtained in families from varied ethnic backgrounds[191,192] (Figure 3.2). Although the modifying locus

was mapped to a relatively small chromosomal region, no obvious candidate genes are found in the region and, despite intensive study of the region, the identification of the putative modifier nuclear gene remains elusive. In addition, not all A1555G families tested were linked to 8p23.1 locus, giving further evidences of the involvement of multiple nuclear modifying genes[192,193]. Thus, the modifier gene on chromosome 8 is not a disease deterministic gene, but a disease susceptibility gene, meaning that individuals with susceptibility alleles in this gene will have increase risk for hearing loss, but this may not be sufficient to cause hearing loss.

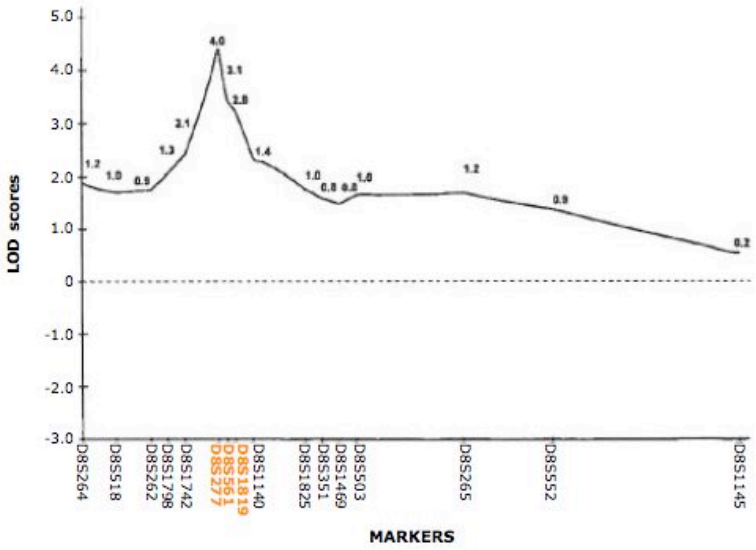

Figure 3.2. Multipoint output for markers in 8p23.1 region around marker D8S277[192]. The distance between the linked markers (shown in orange) was 300-kb, a sharp linkage peak rarely identified for a complex genetic disease.

Additional nuclear-encoded putative modifier genes have been identified using a candidate gene approach. Since all the mitochondrial mutations associated with nonsyndromic hearing loss involve rRNAs or tRNAs, genes encoding proteins involved in mitochondrial RNA processing and translation constitute excellent candidates for being nuclear modifiers. Following this approach different genes have been identified as modifiers of the deafness phenotype linked to mtDNA mutations: *MTO1*[194,195], *GTPBP3*[194,196], TFB1M[194,196,197], and *TRMU*[198,199].

MTO1 and ***GTPBP3*** are evolutionary conserved proteins that play a role in the mitochondrial tRNA modification. In yeast, null mutations in *MTO1* or *GTPBP3* genes express a respiratory-deficient phenotype only when their mtDNA carry the P^{R}_{454} mutation, corresponding to the deafness-associated A1555G mutation[200,201]. Studies of genetic linkage and linkage disequilibrium analysis performed in 214 samples from Spanish, Italian and Arab-Israeli families resulted in highly suggestive linkage for *MTO1* and *GTPBP3* genes[194]. The exact nature and contribution of these genes as nuclear modifier of the deafness phenotype is not clear. *MTO1* gene would

be prominently involved in the phenotype, strongly affecting families of all ethnic backgrounds, while the effect of the *GTPBP3* gene would be less significant[194]. The precise mutation(s) and/or variant(s) in *MTO1* and *GTPBP3* genes responsible for the modifier effect remain to be identified.

Human mitochondrial transcription factor B1 (***TFB1M***) has been proposed as a candidate for being a modifier of the deafness phenotype, since it methylates adenine residues in the adjacent loop of the A1555G mutation in the *12S rRNA* gene[197]. Similar to *MTO1* and *GTPBP3*, linkage and linkage disequilibrium studies strongly suggested *TFB1M* as a modifier gene for maternally transmitted deafness[194]. Absence of the disease-associated mutation in the coding region and location of linkage disequilibrium at a marker 146-kb 3' to the gene, suggested a possible regulatory mutation affecting gene expression[194].

TRMU encodes a highly conserved mitochondrial protein related to tRNA modification[198]. Genotyping analysis of *TRMU* in subjects carrying the A1555G or the C1494T mutation revealed a missense mutation (G28T) altering an invariant amino acid residue (A10S) in the evolutionarily conserved N-terminal region of TRMU protein. All matrilineal relatives carrying both the *TRMU* A10S and 12S rRNA A1555G mutations exhibited prelingual profound deafness. In addition, functional analysis showed that the homozygous A10S mutation leads to a marked failure in mitochondrial tRNA metabolism, specifically reducing the steady-state levels of mitochondrial tRNA suggesting that these defects contribute to the impairment of mitochondrial protein synthesis[199].

In summary, the nuclear background may determine the expression of the disease, but genetic susceptibility may be heterogeneous and have a complex model of inheritance. Different findings indicate that mitochondrial rRNA and tRNA modification are likely to play an important role in determining the phenotype linked to A1555G mutation. Thus, the model that emerges for explaining penetrance of A1555G mutation is a threshold model, where a combination of environmental, mitochondrial and nuclear factors can push a cell over a threshold, with dramatic clinical differences on either side of the threshold. In this model, a number of nuclear variants push the oxidative phosphorylation capability of the cell below a critical threshold in the deaf individuals, while remaining above in the hearing ones. This situation would be similar to the additive effects of secondary mitochondrial mutations in LHON and in families with the A7445G nonsyndromic hearing loss mutation[147].

3.4 PATHOPHYSIOLOGY OF HEARING LOSS DUE TO MITOCHONDRIAL DNA MUTATIONS

Audiological evaluations of individuals with mitochondrial disease suggest cochlear damage due to a loss of outer hair cell function[202]. A number of patients have been successfully fitted with cochlear implants indicating the presence of intact acoustic nerve[203]. Histological studies of the inner ear from mitochondrial deafness patients and experimental animals showed advanced cochlear degeneration including organ of Corti, stria vascularis and supporting cells[126,204]. No apparent pathological changes were observed in the vestibule, semicircular canal, or among the vestibular nerve fibres[204].

The cells most likely involved by a mitochondrial defect are the hair cells and those of the stria vascularis in the cochlea. However, the mechanisms by which mtDNA mutations induce sensorineural hearing loss are unclear. Deficiencies in mitochondrial oxidative phosphorylation appear to be the main pathogenic factor, although the reactive oxygen species generation and altered apoptotic signalling may also play a role[95]. One possibility could be the large dependence of the energy metabolism of the organ of Corti and stria vascularis on mitochondrial oxidative phosphorylation. The progressive accumulation of the mutant mtDNA with age leads to a decline in the oxidative phosphorylation capacity. Energy-dependent ATPase and the release of neurotransmitters in the cochlea would be suppressed by a lowering of the ATP production. Another possibility is a disturbance in ion transport, leading to a reduction in the efficiency of acoustic transduction[126]. The stria vascularis is the most metabolically active site in the cochlea, being its primary function to maintain the ionic environment of hair cells[205]. This requires the secretion of ions, particularly K^+, into the endolymph, often against the ionic gradient, in a process requiring ATP-dependent pumps. Thus, the most likely effect of a decline in ATP production due to mitochondrial dysfunction is a slowing down of these pumps, disturbing ionic balance in the inner ear, and reducing the capacity of the inner ear to detect and transmit sound waves entering the ear[126].

Although the energetic hypothesis is a possible explanation of the pathophysiological pathways leading from the mitochondrial mutation to hearing loss, two major biological questions need to be answered: why does the same mutation cause severe hearing loss in some family members but not in others, and why is the ear the only organ affected taking into account that the cochlea is not the most energy-dependent organ in the body? Study of the mitochondrial mutations leading to hearing loss has led to three possible precipitating factors

modulating phenotypic expression: environmental agents, such as aminoglycosides, mitochondrial haplotype and nuclear modifier genes.

The second major biological question relates to tissue specificity as it is unclear how a homoplasmic mutation affecting oxidative phosphorylation leads to a clinical defect exclusively confined in the cochlea, rather than affecting every tissue. Cochlea-specific isoforms or splice-variants involved in mitochondrial RNA processing or translation could interact abnormally with the mutated rRNA, tRNA or polycistronic mRNA and lead to qualitative or quantitative changes in the protein products. Different processing of mitochondrial RNA and protein, leading to tissue specific defects of functions have been described. In this sense, several examples of tissue specificity in oxidative phosphorylation and of tissue specific secondary functions of mitochondrial RNAs exist. In addition, tissue-specific subunits for oxidative phosphorylation have been described.

In summary, how mitochondrial dysfunction contributes to the pathogenesis of hearing loss is still unclear. The essential role of mitochondrial oxidative phosphorylation in cellular energy production, the generation of oxygen reactive species, and the initiation of apoptosis may suggest a number of novel mechanisms for mitochondrial deafness. In addition, the identification of nuclear modifier genes through genetic positional cloning of candidate gene testing will shed light on the pathophysiological pathways leading from the mitochondrial mutation to hearing impairment, and provide targets for prevention and therapy.

4. CHROMOSOME 8p23.1

Chromosome 8p23.1 has been proposed by genome-wide linkage analysis as a putative localization for a modifier locus of the phenotype associated to A1555G mutation[179,188,190-192]. However, the identification of the modifier genetic factor has been greatly complicated due to the genomic features of the region, involving polymorphic duplications and inversions.

4.1 STRUCTURAL VARIATION IN THE HUMAN GENOME

Genomic variability can be present in many forms, including single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs), presence/absence of transposable elements (e. g. *Alu* elements) and structural alterations (e. g. deletions, duplications and inversions). Until recently, SNPs were thought to be the predominant form of genomic variation and to account for much of the normal phenotypic variation[206]. However, the availability of human genome sequence for genome-wide analysis has revealed a type of DNA variation involving small DNA segments, ranging from ~1 kb to 3 Mb in size, called submicroscopic structural variants[207]. This submicroscopic structural variation can include copy number variations (CNVs), inversions, deletions and other complex rearrangements, most of which are not detected by standard cytogenetics or DNA sequencing[207].

Now, it is known that submicroscopic structural genomic variants are at least as important as SNPs, in their contribution to genome variation[207-209]. Moreover, these type of variants can encompass millions of bases of DNA containing entire genes and their regulatory regions. Although structural variants in some genomic regions have no obvious phenotypic consequence, others influence gene dosage,

which might cause genetic disease, either alone or in combination with other genetic or environmental factors[207].

4.1.1 Segmental duplications and genomic rearrangements

Genomic rearrangements include duplications, deletions and inversions, as well as translocations, marker chromosomes, isochromosomes and other complex rearrangements, both microscopically visible or not. These rearrangements are not random events but instead reflect the involvement of higher-order architectural features of the human genome[210].

The architectural features that appear to render genomic regions susceptible to rearrange are region-specific repeated sequences, known as segmental duplications (SD)[211]. SDs can be defined as repetitive regions in the genome, ranging from 1-kb to 400-kb in size and sharing a sequence identity greater than 90%. SDs can contain one or multiple genes, pseudogenes, gene fragments, retroviral sequences, regulatory regions or other paralogous segments. SDs are often found in pericentromeric and subtelomeric regions of human chromosomes, but they may be found in interstitial regions as well. The size, relative orientation, distance between copies, and shared percent identities of the SDs are factors that make the genomic region susceptible to rearrange and may influence the type of rearrangement which occurs[211].

Rearrangements are classified as either recurrent or nonrecurrent, depending on whether the same rearrangement can be identified in unrelated individuals. In general, recurrent rearrangements, or those of common size and having clustered breakpoints, frequently result from a mechanism of nonallelic homologous recombination (NAHR) between region-specific SDs. NAHR is a process whereby SDs on the same chromosome can facilitate copy number changes of segmental duplicated regions along with intervening sequences[208].

4.1.2 Submicroscopic structural variation

In the past few years, several studies have identified a previously uncharacterized prevalence of submicroscopic structural variants, mainly copy number variations and inversions[209].

Deletions, insertions, duplications and complex multi-site variants, collectively termed **copy number variations (CNVs)**, are found in all humans[207]. A CNV is defined as a DNA segment of 1-kb or larger present at variable copy number in comparison with a reference genome[207]. A recent study of CNVs in the HapMap samples has defined over 1400 CNV regions[209]. On average, each individual varies at over 100 CNVs, representing about 20 Mb of genomic DNA

difference[209]. Thus, CNVs account for a significant proportion of human normal phenotypic variation, as well as they may also have an important role in the pathological variation in the human population[208,212].

Higher-order genomic features have been suggested to function mediating normal variation in the human genome. CNVs often occur in regions reported to contain, or be flanked by SDs, which may be susceptible to structural chromosomal rearrangements via NAHR. Thus, SDs may play an important role both in normal and pathological structural variation due to CNVs[208]. CNVs are generally located in gene-rich regions and may include genes for which dosage is critical. Analyses of the functional attributes of currently known CNVs reveal a remarkable enrichment for genes that are relevant to molecular-environmental interactions and genes that influence response to specific environmental stimuli, such as genes involved in immune response and inflammation[208].

Inversions represent another class of structural variation, but knowledge on their prevalence in general population is more limited, as until recently there has not been a robust method for detecting balanced, submicroscopic variants of this type[207,212]. In most of known inversion polymorphisms, the inversion has no detectable effect in parents, but increases the risk of a disease-associated CNV in the offspring. Moreover, in a high proportion of the identified inversions, their breakpoints are associated with SDs, highlighting again the propensity of these regions to mediate structural changes[207].

4.1.3 Implications for phenotype and disease

Structural variants can lead to phenotypic variation or disease in several ways. They can affect gene dosage directly (in the case of CNVs), or can indirectly alter gene expression through position effects. As well as directly causing disease, structural variants might function as susceptibility alleles in complex genetic diseases. Although some large variants might seem to be benign and are prevalent in certain populations, in combination with other genetic or environmental factors, they might contribute to a disease phenotype[207]. The presence of a structural variant might also predispose to further, potentially harmful structural changes[207].

CNVs that do not directly result in early onset, highly penetrant genomic disorders may play a role in later onset genomic disorders or common diseases. For example, inter-individual and inter-populational differences in CNVs of the gene encoding *CCL3L1*, a HIV-1 suppressive chemokine and ligand for the HIV coreceptor CCR5, were recently reported[213]. Carrying a *CCL3L1* copy number that is lower than the population average is associated with markedly increased HIV susceptibility, highlighting how a CNV can function as a susceptibility allele that is involved in a

complex phenotype[213]. The high prevalence of CNVs in normal individuals together with the abundance of functional sequences in CNV regions indicates that they may account for a significant portion of normal phenotypic variation[208,209]. Furthermore, CNVs are not confined to a single population, indicating that either these variants were present early in human history or that they are highly recurrent events[209].

In the case of polymorphic inversions, a reduced recombination frequency, resulting from the different orientation of chromosomal segments, might increase the chance of misalingment between non-allelic SDs. Therefore, carriers of an inversion might be at higher risk of a *de novo* deletion or other chromosomal rearrangement during meiosis[214].

Finally, there is emerging evidence that structural variants might contribute to the phenotypic variation that has a role in determining fitness, with potential evolutionary implications. There is a particular enrichment of genes that are involved in general defense response, including responses to bacterial and external biotic stimuli, xenobiotic metabolism and regulation of cell organization and biogenesis. These observed enrichments indicate that genes involved in structural variation might have roles in the adaptability and fitness of an organism in response to external pressures. In general, these genes are thought to be more plastic, having a greater potential to evolve quickly, implying that structural variation might be important for the dynamics of genes and organisms evolution[207].

4.2 GENES AND GENOMIC STRUCTURE OF CHROMOSOME 8p23.1

Complexity and variability are essential genomic features of human chromosome 8p23.1. Chromosome band 8p23.1 is known to be a frequent site of chromosomal rearrangements mediated by segmental duplications (SDs). The presence of SDs difficult sequence assembly and encompass the existence of two gaps in 8p23.1 at 7.5 Mb and 12 Mb of the March 2006 human reference sequence (Figure 4.1).

Homologous recombination between SDs in the region is responsible for four chromosome rearrangements: a deletion, two inverted-duplications and a submicroscopic inversion[215]. Multiple patients with rearrangements of the short arm of 8p23.1 have been reported[216]. It has also been described that as many as one in four individuals from the general population carry a 4.7 Mb inversion of the region[215,217,218]. The presence of the inversion polymorphism in the parent transmitting the disease-related chromosome, mediates some recurrent 8p rearrangements[215].

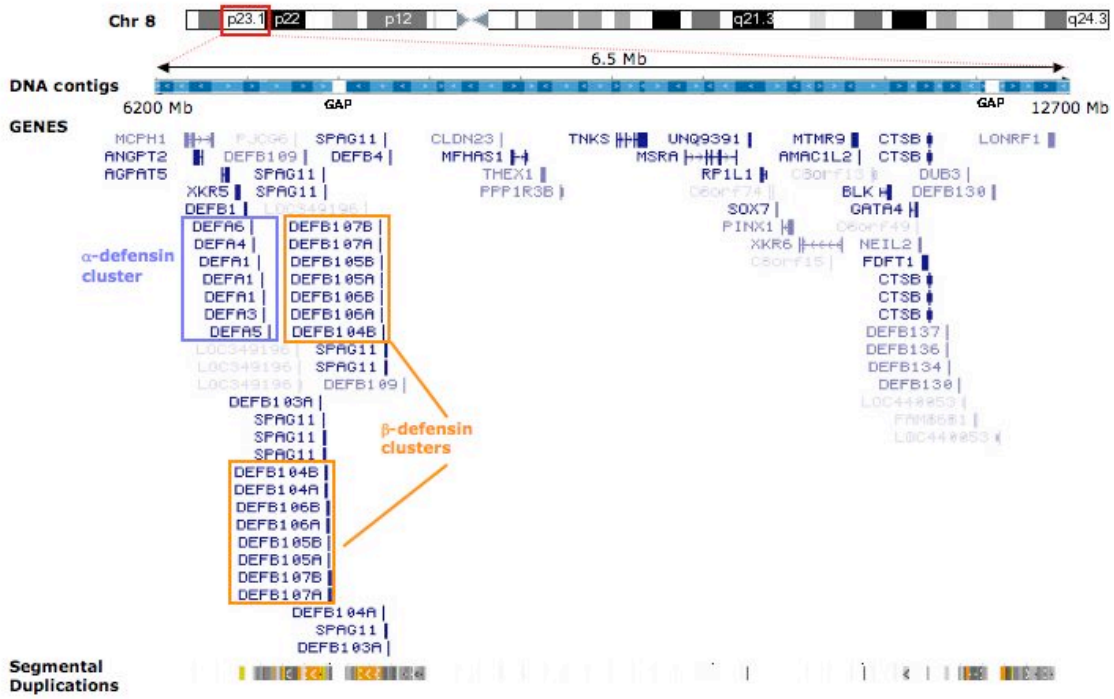

Figure 4.1. Chromosome band 8p23.1. The sequence gaps and segmental duplications are depicted as well as the genes annotated in the region (based on human march 2006 assembly).

Chromosome band 8p23.1 is a gene rich region, where most of human defensin genes are located. Defensin genes encode a family of small cationic peptides that act as antimicrobial mediators of the innate immune system[219]. The two main defensin subfamilies, α- and β-defensins, differ in the length of the peptide segments between cysteine residues and in the arrangement of disulphide bonds that link them. β-defensins have been found in most vertebrate species, whereas α-defensins are specific to mammals[220]. Defensin genes within 8p23.1 region are organized in different clusters: a telomeric cluster mostly containing α-defensin genes (*DEFB1*, *DEFA6*, *DEFA4, DEFA1, DEFT1, DEFA3* and *DEFA5*) and at least two centromeric clusters of β-defensin genes (*DEFB109p*, *DEFB108*, *DEFB4*, *DEFB103*, *DEFB104*, *DEFB106*, *DEFB105* and *DEFB107*)[221].

Copy number variability involving both α-defensin (*DEFA1* and *DEFA3)* and β-defensin (*DEFB4*, *DEFB103* and *DEFB104)* genes in chromosome 8p23.1 has been well detected and characterized[222-225]. The number of *DEFA1* and *DEFA3* gene copies has been reported to range from 4 to 11 in a sample of 111 subjects, the *DEFA3* allele being completely absent in 10% of them[223]. In another study, Linzmeier and colleagues determined copy numbers of the *DEFA1* and *DEFA3* alleles in 27 subjects and found between 5 and 14 copies per diploid genome, with *DEFA3* being absent in 26% of them[225].

The repeated unit of the β-defensin CNV expands 240-kb[224]. Carriers of an apparent chromosomal duplication or euchromatic variant visible cytogenetically which has no clinical phenotypic effect have 9 to 12 copies of the β-defensin CNV, whereas most of other normal people have 2 to 7 copies[224,226,227].

The exceptional genomic complexity and heterogeneity of human 8p23.1 locus must be appreciated when interpreting SNP or linkage data from this region. The combination of genomic structural variations and paralog differences and polymorphisms can produce extremely diverse repertoire of genetic and genomic variants. Careful characterization of the region is essential for performing clinical association studies.

Objectives

Objectives

The main objective of this doctoral thesis has been the understanding of the molecular basis of deafness due to mitochondrial DNA mutations. The work has been focused on the study of factors influencing the deafness phenotype associated to 12S rRNA gene A1555G mutation, the most prevalent mtDNA mutation leading to hearing loss.

Different aspects of mitochondrial and nuclear genetics have been studied as possible contributors to the phenotypic variation linked to A1555G mutation. Later on, the study of one of such putative nuclear modifying locus derived to a population-based approach to study the region's variability. This has supposed a step towards a non-disease oriented and more global vision of common human genetic variation.

The specific objectives faced can be summarized in:

1. Identification of deafness-related mutations in the mitochondrial 12S rRNA gene in a cohort of Spanish hearing impaired patients.
2. Clinical characterization of deaf and hearing subjects carrying mutation A1555G.
3. Identification and characterization of mitochondrial and nuclear genetic factors responsible of the phenotypic heterogeneity associated to mtDNA mutations:

    3.1 Study and quantification of mtDNA heteroplasmy.

    3.2 Characterization of chromosome 8p23.1 as a modifier locus for the deafness phenotype linked to mutation A1555G.

4. Study of the population variability of the alpha-defensin locus on chromosome 8p23.1.

Results

Results

This section summarizes the scientific results obtained during the present doctoral thesis. Most of the results either have been already published or are submitted for publication in international peer reviewed journals. This section is organized in different chapters, each of them corresponding to a research article, conceived to answer one of the objectives which guided this doctoral thesis. A brief introduction is given to each of the papers, in order to highlight the aim of the work, how it has been developed and its conclusions.

The different chapters are:

1. Mitochondrial 12S rRNA gene mutations affect RNA secondary structure and lead to variable penetrance in hearing impairment
2. Cochlear alterations in deaf and unaffected subjects carrying the deafness-associated A1555G mutation in the mitochondrial 12S rRNA gene
3. Low-level mtDNA heteroplasmy in a deafness pedigree co-segregating 1555A>G 12S rRNA and 15287T>C cytochrome *b* mutations give new insights into mtDNA transmission
4. Detailed analysis of 8p23.1 putative modifying genetic factors for the deafness-linked A1555G mutation
5. Inter-population variability of *DEFA3* gene absence: correlation with haplotype structure and population variability

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

Ester Ballana, Estela Morales, Raquel Rabionet, Bàrbara Montserrat, Marina Ventayol, Olga Bravo, Paolo Gasparini and Xavier Estivill

In this study, a mutational screening of mtDNA 12S rRNA gene has been performed in a cohort of 443 families with hearing impairment. As a result, 69 unrelated cases carrying mutation A1555G and two novel nucleotide changes (T1291C and T1243C) have been identified. This work confirmed the high prevalence of mutation A1555G in deafness cases and the major role of the 12S rRNA gene in hearing. Only 63% of subjects with mutations A1555G developed hearing impairment and treatment with aminoglycosides was reported for 22% of the 183 A1555G deaf subjects, indicating that A1555G is not a fully penetrant change.

From the two novel changes reported only mutation T1291C fulfilled the criteria of a disease-causing change, based on segregation of the variant with deafness, abnormal prediction of 12S rRNA secondary structure, and absence of the mutation in a cohort of 443 hearing loss cases and 95 Spanish controls. From the data obtained, the role of variant T1243C could not be clearly stated.

In a commentary on our report, Abreu-Silva et al. proposed T1291C to be an African non-pathogenic polymorphism as they found the substitution in 5/203 deaf Brazilian subjects (2 familial, 3 isolated deafness cases) and 2/300 unrelated hearing controls[228]. Six T1291C carriers belong to the macrohaplogroup L1/L2 that indicates an African origin of their mtDNA[228]. Therefore, the mitochondrial haplogroup of the family we described carrying T1291C mutation was analysed, showing that it also belong to L1 haplogroup and indicating an African origin of the mtDNA. This would support the idea that T1291C mutation is an African rare polymorphism.

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

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Running title: 12S rRNA alterations and deafness

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ABSTRACT

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

KEYWORDS: nonsyndromic hearing loss, sensorineural hearing impairment, mitochondrial DNA**,** 12S rRNA gene, RNA secondary structure.

INTRODUCTION

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

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

Código de campo cambiado

Con formato: Francés (Francia)

Con formato: Francés (Francia)

Código de campo cambiado

In contrast with other deafness-associated mtDNA mutations, reported only in a few number of families, the A1555G mutation in the small ribosomal RNA gene (12S rRNA) has been associated with aminoglycoside-induced and adult onset nonsyndromic deafness in many families of different ethnic origins [20-24], with a prevalence of 0.5-2.4% in European sensorineural deafness patients and 3% in Japanese patients [11, 25, 26]. The resulting phenotype varies considerably among matrilineal relatives within families or among different families carrying the A1555G mutation, ranging from

severe deafness, to moderate progressive hearing loss or even completely normal hearing. Incomplete penetrance and variable expressivity of hearing loss associated with mutation A1555G are thought to be due to the interaction between genetic factors, such as nuclear modifier genes or mitochondrial haplotype, and environmental factors, such as aminoglycoside antibiotics [27-31]. Although aminoglycosides are known to trigger deafness onset, their role in the development of hearing loss in subjects carrying the A1555G mutation has been estimated to be as low as 20% when a large number of patients and families have been studied [20-24]. On the other hand, nucleotide 1555 maps to a phylogenetically conserved and a functionally well-characterized domain of the small subunit rRNA, in the decoding site of the ribosome. The mutation is predicted to alter the secondary structure of the 12S rRNA molecule, in a way that it resembles more closely its bacterial counterpart, being the deafness-associated phenotype the consequence of this structural change [20-24].

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

MATERIALS AND METHODS

Patients and families

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

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

Mutational analysis of DFNB1 locus and 12S rRNA gene

After obtaining written informed consent, total DNA was extracted from peripheral blood using standard procedures. The samples were tested for the presence of mutations in the coding region of *GJB2*, the two deletions affecting *GJB6* and the A1555G mutation in the 12S rRNA gene, prior to the analysis of the mtDNA 12S rRNA.

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

The analysis of the 12S rRNA gene was performed by direct sequencing. Once the new variants were identified, the genotyping of other deaf patients and control samples was performed using different methods. The detection of the A1555G and T1291C mutations were performed by PCR amplification of a 340-bp fragment (Forward 5'-GCTCAGCCTATATACCGCCATCTTCAGCAA-3' and Reverse 5'-TTTCCAGTACACTTACCATGTTACGACTTG-3'), followed by the digestion with restriction endonuclease *Hae*III, as both changes introduce a cleavage site for this enzyme. Screening of the T1243C variant was performed using the Pyrosequencing™ technology (PSQ96MA) (Biotage AB, Sweden). Specific SNP assays were designed by Pyrosequencing (Forward 5'-TAAACCCCGATCAACCTCAC-3', Reverse 5'-TCCACCTTCGACCCTTAAGTT-3' and Sequencing 5'-GATCAACCTCACCACC-3'). Sequence identification was performed automatically by the SQA software.

Secondary structure prediction

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

RESULTS

A1555G mutation is a common cause of deafness in Spanish patients

Among the 313 families from our cohort, 215 (69%) of the pedigrees showed an autosomal recessive segregation pattern, 97 (31%) were considered autosomal dominant and one (0.3%) was classified as X-linked. Among them, 107 pedigrees (34%) showed a segregation pattern likely to correspond to maternal transmission. These pedigrees had information for at least three generations and the inheritance pattern was characterized by the presence of affected maternal relatives and the lack of father to son transmission.

The affected subjects of the 107 families compatible with maternally inherited deafness showed bilateral and sensorineural hearing loss as the sole clinical symptom. There was a wide variability in the age at onset of deafness within and between families, although most of the patients presented late-onset/progressive deafness.

All the samples from our cohort, independently of their inheritance pattern, were analysed for mutations in the *DFNB1* locus and for the A1555G mutation in the 12S rRNA gene. The A1555G mutation was found in 65 families (61%) from the 107 deafness pedigrees compatible with maternal transmission and in 4 of the sporadic cases, resulting in a total of 69 unrelated samples with the mtDNA A1555G mutation, indicating that 15% of our cohort of Spanish deaf patients is a carrier of the A1555G mutation. The 69 families or sporadic cases positive for the A1555G mutation included a total of 290 individuals, of which 183 were deaf A1555G carriers (63%) and 107 were asymptomatic carriers of the mutation (37%).

Twenty-four out of the 69 families or sporadic cases positive for the A1555G mutation reported previous exposure to aminoglycoside antibiotics, either for the index case or for some of the family members. This represented only 35% of the families. All the patients who received aminoglycosides (n=40) became deaf, but these patients

represent only 22% of the total of deaf carriers of the A1555G mutation. The role of aminoglycosides in the group of pedigrees here studied is limited, indicating that other environmental or nuclear factors would determine the onset and severity of hearing loss.

Two novel nucleotide changes in the mtDNA 12S rRNA gene

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

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

The T1291C variant was identified in a three-generation family of Cuban origin, with members affected of sensorineural hearing impairment. DNA was obtained from six individuals, four affected and two non-affected (Figure 2). Clinical characterization

included family history of hearing loss, which was consistent with maternal inheritance, use of aminoglycosides, age of onset and pure tone audiometry. All affected individuals exhibited bilateral, sensorineural progressive hearing impairment as the sole clinical symptom. None of them reported a previous history of aminoglycoside exposure. Audiometric studies showed a more severe loss of hearing at high frequencies and a wide range in the age at onset of hearing impairment, varying from 7 years (III-4 and IV-1) to 40 years (II-1 and II-3). The offspring of the deaf father II-1 does not report any hearing problems. Although mutation T1291C was present in homoplasmy in all affected individuals, they differ in the severity and the age of onset, suggesting the involvement of environmental and/or genetic factors in the phenotype. The T1291C has been tested in the rest of our cohort of deaf patients and 100 control samples with negative results in all cases, suggesting that it may be a deafness-related variant.

Conservation and secondary structure of 12S rRNA nucleotide variants

It has previously been reported that nucleotide 1555 is a phylogenetically conserved position located in a domain which plays a key role in both transcriptional fidelity and interaction of the ribosome with aminoglycoside antibiotics. The A1555G mutation is predicted to alter the secondary structure of the 12S rRNA molecule [20-24]. Under this assumption, we analyzed the newly identified sequence changes T1243C and T1291C, as well as the known pathogenic mutation A1555G, for their possible effects on secondary structure of the rRNA.

The comparison between human mitochondrial 12S rRNA and *E. coli* 16S rRNA showed that the T at position 1243 and the A at position 1555 are highly conserved nucleotides. In the case of T at 1291, it is localized in an helix not present in the *E. coli* 16S rRNA (Figure 3a) [34]. The sequence concordance for T1243 and A1555, and the

discordance for T1291 between the human mitochondrial 12S rRNA and *E. coli* 16S rRNA was also confirmed by comparison in different species (Figure 3b).

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

DISCUSSION

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

Since mutations in mtDNA account for a high number of familial and sporadic sensorineural hearing loss cases [5], we considered the possibility that other mutations in the 12S rRNA gene could also be causing deafness. The search for additional mutations in the 12S rRNA gene, lead to the identification of two changes with possibly different roles as deafness-related variants.

T1243C was present in homoplasmy in five cases and one control, thus likely representing a polymorphism. However, a wide variety of symptoms have been reported in deaf patients with mitochondrial mutations, including cases of normal hearing [20-24]. Moreover, the A1555G mutation is thought to be a predisposing mutation, which

needs the combined action of environmental factors and/or nuclear modifying genes to cause hearing impairment [27-31]. Taking this into account, we could not exclude a similar role for the T1243C variant, which in conjunction with other factors may lead to hearing loss.

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

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

variant the more severe for the T1291C. This suggests a more important functional effect of mutation T1291C, which would eventually lead to a more severe phenotype.

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

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

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Figure Legends

Figure 1. mtDNA variant T1243C in the 12S rRNA in available nuclear pedigrees and its audiometric alterations. **(a)** Pedigrees of families with the T1243C variant and sequence chromatograms from an affected individual and a control, showing the T to C nucleotide change. **(b)** Available audiometries of affected patients with the T1243C variant.

Figure 2. mtDNA variant T1291C in the 12S rRNA in a Cuban genealogy with hearing impairment and its audiometric alterations. **(a)** Pedigree of the Cuban family and ethidium bromide-stained gel showing the PCR-RFLP analysis used for the detection of the T1291C variant. This change specifically creates a novel site for the restriction enzyme *Hae*III, resulting in three fragments. **(b)** Audiometric evaluation of a mildly affected (III-4) and a severely affected (II-3) family members.

Figure 3. Conservation analysis of the identified 12S rRNA changes. **(a)** Comparison of the secondary structure of human mtDNA 12S rRNA and the corresponding region of *E. coli* 16S rRNA. Note that T at position 1243 and A at 1555 are conserved nucleotides. On the other hand, the different structure of the helix where the T1291C is localized made impossible the comparison. **(b)** Sequence alignment of the 12S rRNA gene in different species. The T at 1243 and the A at 1555 position are highly conserved throughout evolution, but not the T at position 1291.

Figure 4. Secondary structures predicted by the RNAfold software. The three variants resulted in a change of the folding compared to the wild-type prediction, however the most severe change is the one caused by the T1291C variant.

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

Patient Num.	Age	Sex	Hearing loss	Age of onset	T1243C	Others
E19	10	F	+	congenital	+	No pedigree information
S25.1	7	M	+	congenital	+	No response in ABR
S25.2	37	M	-	-	-	
S25.3	32	F	-	-	+	
S41.1	22	M	+	19 years	+	Tinnitus
S41.2	55	F	-	-	+	
S100.1	11	M	+	2 years	+	
S100.2	40	M	-	-	-	
S100.3	39	F	+	unknown	+	
S303	1	F	+	congenital	+	No affected relatives

Figure 1

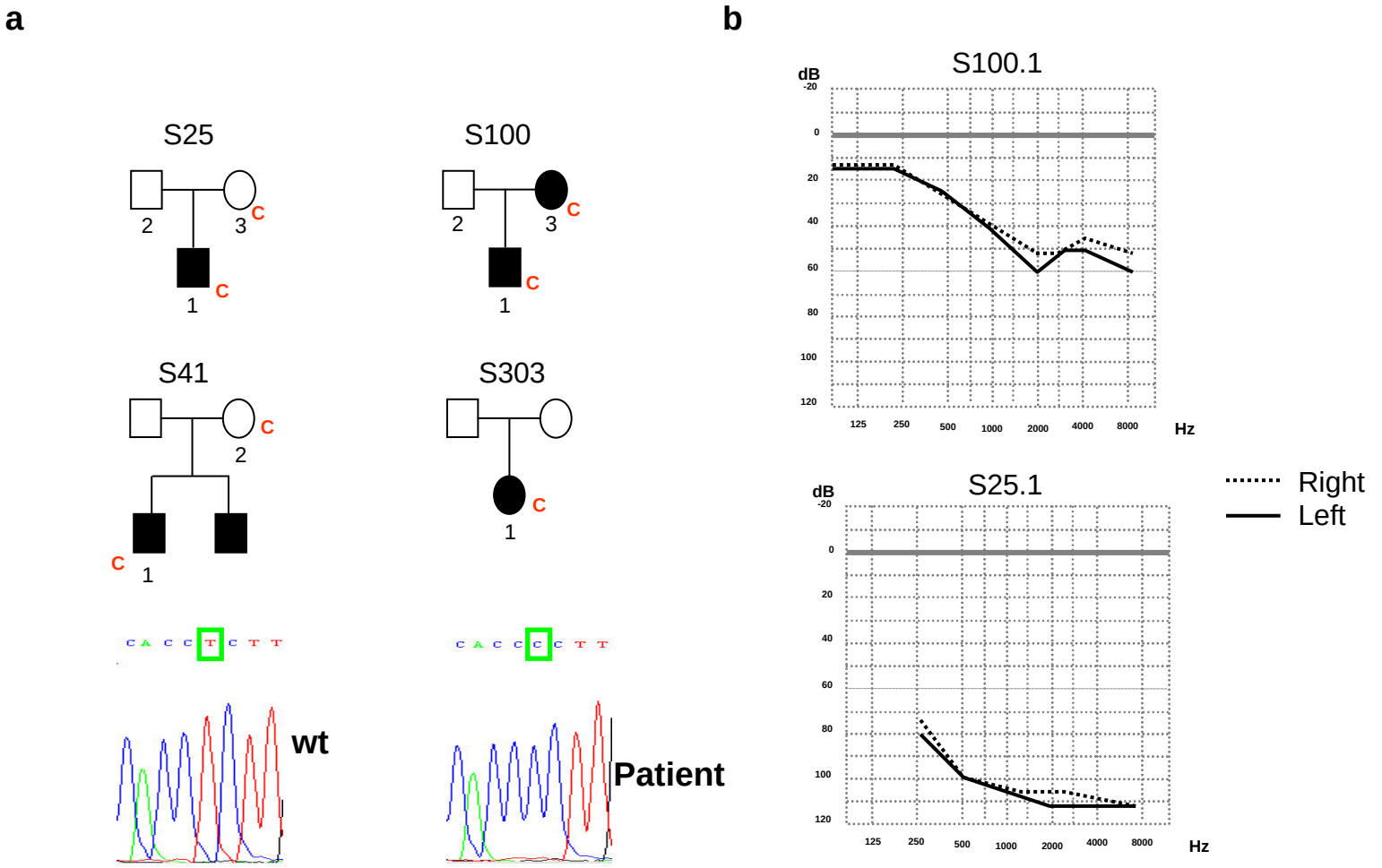
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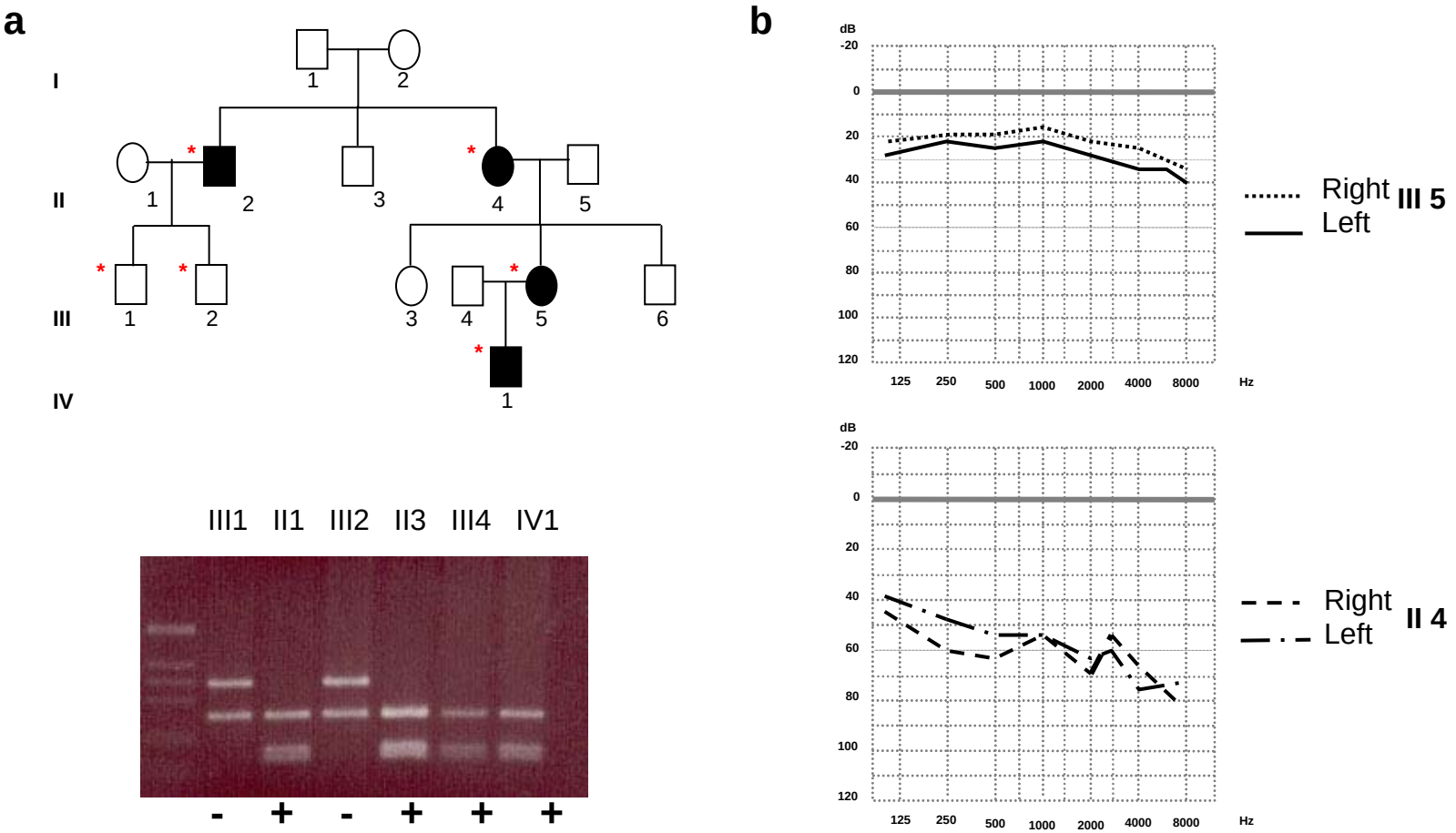
Figure 2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III1 II1 III2 II3 III4 IV1
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Figure 3

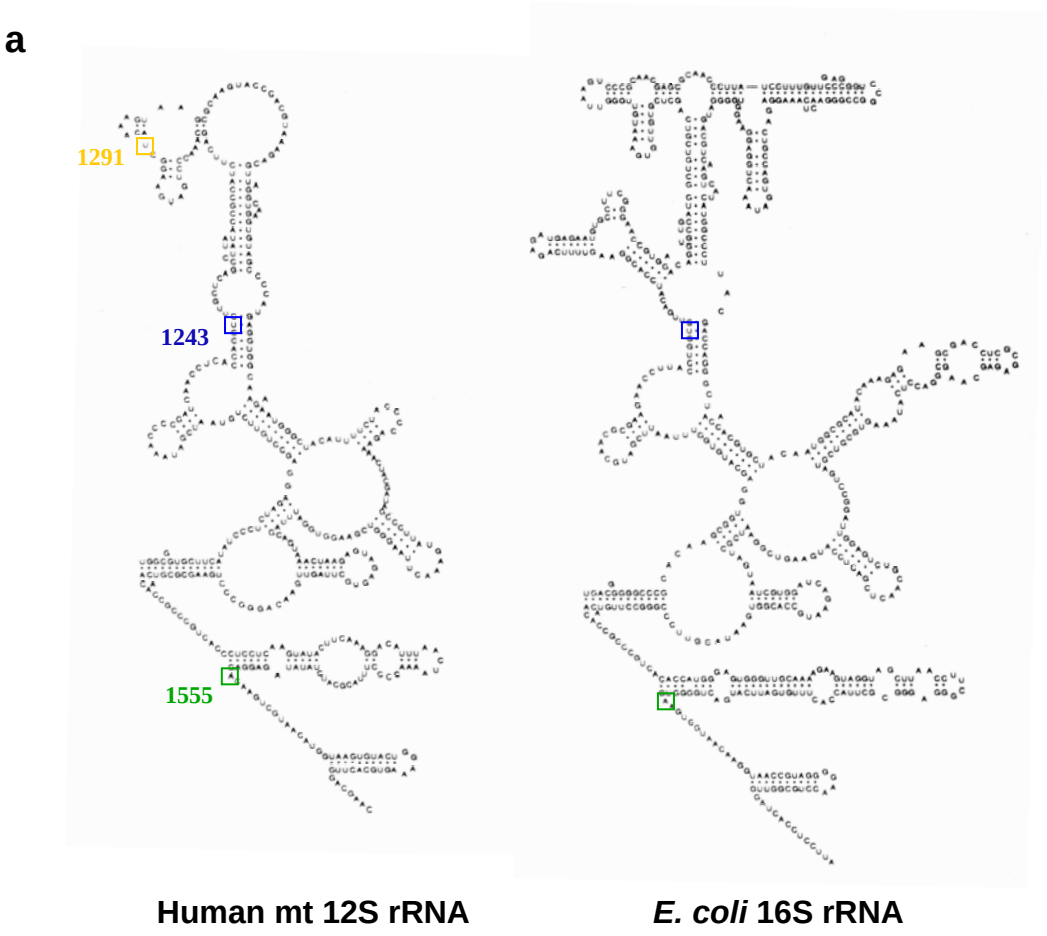

Figure 4

Original article

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Reply to correspondence by Abreu-Silva et al. regarding Ballana et al.: Mutation T1291C in the mitochondrial 12S rRNA gene involved in deafness in a Cuban family belongs to the macrohaplogroup L1 of African origin.

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Reply to correspondence by Abreu-Silva et al., regarding Ballana et al.,

Ballana E, Morales E, Estivill X.

Mutation T1291C in the mitochondrial 12S rRNA gene involved in deafness in a Cuban family, belongs to the macrohaplogroup L1 of African origin

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Running title: Mutation T1291C is of African origin

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We described mitochondrial DNA (mtDNA) T1291C mutation in a Cuban family affected of nonsyndromic sensorineural hearing impairment based on segregation of the variant with deafness, abnormal prediction of 12S rRNA secondary structure and absence of the mutation in a cohort of 443 hearing loss cases and 95 Spanish controls [1]. Abreu-Silva et al proposed T1291C to be an African non-pathogenic polymorphism as they found the substitution in 5/203 deaf Brazilian subjects (2 familial, 3 isolated deafness cases) and 2/300 unrelated hearing controls. Six T1291C carriers belong to the macrohaplogroup L1/L2 that indicates an African origin of their mtDNA [2]. We have analyzed the mitochondrial haplogroup of our Cuban family carrying T1291C mutation showing that they also belong to L1 haplogroup, thus indicating an African origin of their mtDNA. This would support the idea that T1291C mutation is an African rare polymorphism. However, taking into account our data [1,3] and previous reports regarding phenotypic variability associated to deafness-causing mtDNA mutations [4-6], the role of T1291C mutation as a genetic susceptibility factor to develop hearing impairment could not be totally excluded, at least in the Cuban family we reported [1].

It is well known that phenotypic variability is a hallmark of mitochondrial disorders in general [7]. Mutation T1291C is located in the mitochondrial 12S rRNA gene, where several variants have been identified associated with aminoglycoside-induced and non-syndromic hearing loss, suggesting that the mitochondrial 12S rRNA gene is a hot-spot for deafness associated mutations [4]. Mutation A1555G, the most common mtDNA variant associated to hearing loss, has been linked with different clinical phenotypes, ranging from severe deafness to moderate progressive hearing loss or even completely normal hearing [3,8-11]. Thus, pathogenicity of mutation T1291C cannot be excluded only based on the phenotypic variability found in T1291C carriers.

Incomplete penetrance and variable expressivity of hearing loss associated with mutation A1555G seem to require the contribution of additional genetic factors, such as nuclear modifier genes or mitochondrial haplotype, and/or environmental factors, such as aminoglycoside antibiotics [12-16]. A similar scenario could explain the phenotypic variability observed regarding the T1291C substitution. Moreover, it would be important to clinically describe the Brazilian cohort described by Abreu-Silva et al., especially regarding exposure to environmental factors that may modulate the phenotype observed. A detailed clinical examination could shed light on the variability observed in the T1291C associated phenotypes.

In conclusion, further mutational data of mtDNA from different populations would help to determine the exact contribution of the T1291C in the onset of hearing impairment and possibly of other mtDNA variants. From our experience, we would like to encourage the reporting of new identified variants and when available, the origin of the corresponding samples. Such information is extremely important to determine the nature of genetic variants possibly related to disease: mutation or polymorphism.

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Cochlear alterations in deaf and unaffected subjects carrying the deafness-associated A1555G mutation in the mitochondrial 12S rRNA gene

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* These two authors contributed equally to this work

In the present study, an audiological evaluation of a group of deaf patients and hearing carriers of mutation A1555G was performed. The aim of the work was to assess the prevalence of A1555G mutation and determine the associated cochlear alterations. Fifty-four patients affected of nonsyndromic hearing loss were screened for the presence of the A1555G mitochondrial mutation. Nine of the familial cases (21%) carried the A1555G mutation, whereas the mutation was not found in any of the sporadic cases. The positive cases and some of their family members underwent a clinical study consisting in a clinical evaluation and audiological testing. Audiologic evaluation included otoscopic examination, pure-tone audiometry (PTA), acoustic reflexes, tympanometry, evaluation of auditory brainstem responses (ABRs), and measurement of distortion products of otoacoustic emissions (DPOAE).

The phenotype of A1555G patients varied in age of onset and severity of hearing loss, ranging from profound deafness to completely normal hearing. The present work demonstrated that A1555G mitochondrial mutation causes a cochlear form of deafness, characterized by a more severe loss of hearing at high frequencies. Although the expression of the mutation is variable, cochlear alterations are present in all carriers of mutation A1555G.

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Cochlear alterations in deaf and unaffected subjects carrying the deafness-associated A1555G mutation in the mitochondrial 12S rRNA

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Running title: Audiological findings in A1555G patients

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ABSTRACT

The A1555G mutation in the mitochondrial small ribosomal RNA gene (12S rRNA) has been associated with aminoglycoside-induced, nonsyndromic hearing loss. However, the clinical phenotype of A1555G carriers is extremely variable. In the present study, we have performed an audiological evaluation of a group of deaf patients and hearing carriers of mutation A1555G with the aim to assess the prevalence of the mutation and determine the associated cochlear alterations. Fifty-four patients affected of nonsyndromic hearing loss were screened for the presence of the A1555G mitochondrial mutation. Nine of the familial cases (21%) carried the A1555G mutation, whereas the mutation was not found in any of the sporadic cases. The positive cases and some of their family members underwent a clinical study consisting in a clinical evaluation and audiological testing. The phenotype of A1555G patients varied in age of onset and severity of hearing loss, ranging from profound deafness to completely normal hearing. The audiometric alterations showed bilateral hearing loss, being more severe at high frequencies. Otoacoustic emissions were absent in deaf A1555G carriers, and auditory brainstem response indicated a prolonged Wave I, suggesting a cochlear dysfunction without any effect of the auditory nerve. Moreover, all hearing carriers of A1555G also presented alterations in cochlear physiology. In conclusion, the A1555G mitochondrial mutation causes a cochlear form of deafness, characterized by a more severe loss of hearing at high frequencies. Although the expression of the mutation is variable, cochlear alterations are present in all carriers of mutation A1555G.

KEYWORDS: nonsyndromic hearing loss, pure tone audiometry, auditory brainstem response, otoacustic emissions, 12S rRNA mutation.

INTRODUCTION

Hearing loss is a common sensory disorder affecting one in 1000 newborns. It is estimated that about 50% of cases have a genetic origin or predisposition basis [1]. Genetically determined hearing impairment can follow an autosomal dominant, autosomal recessive, X-linked or mitochondrial pattern of transmission [2]. Deafness can also be caused by environmental factors, including perinatal infection, acoustic or cerebral trauma affecting the cochlea, or ototoxic drugs, such as aminoglycoside antibiotics, or by the interaction between genetic and environmental factors [3, 4].

Mutations in the mitochondrial DNA (mtDNA) have been associated with both syndromic and nonsyndromic deafness [5, 6]. In particular, the A1555G mutation in the mitochondrial small ribosomal RNA gene (12S rRNA) has been associated with aminoglycoside-induced, nonsyndromic hearing loss in various families of different ethnic backgrounds [7-12]. Aminoglycoside antibiotics enhance the susceptibility for the mitochondrial ribosome carrying the A1555G mutation to develop hearing loss [13, 14]. However, in the absence of aminoglycoside exposure, the mutation leads to a phenotype that ranges from severe deafness, to moderate hearing loss or even to apparently normal hearing [7, 10]. Thus, expression of the deafness phenotype in carriers of the A1555G mutation appears to require the contribution of additional genetic and/or environmental factors. The factors that modulate the expressivity of this mutation in hearing impairment have not yet been resolved.

Prevalence, onset and severity of the A1555G mutation associated hearing loss have been characterized in the Spanish population [10]. However, there is no information about the cochlear function in hearing carriers of the mutation, which could exhibit a subclinical phenotype. In the present study, a group of deaf patients attended in the same otolaryngology department, were studied for the presence of A1555G

mutation with the aim to assess the prevalence of this mutation and to evaluate the degree of hearing impairment in unaffected carriers. The study has allowed definition of the spectrum of cochlear alterations associated with the A1555G mutation.

MATERIALS AND METHODS

Subjects

A total of 54 adult unrelated patients affected by postlingual nonsyndromic deafness have been selected from the Otolaryngology Department of the "Ciutat Sanitària i Universitària de Bellvitge" and Hospital "Universitario Sagrat Cor". Informed consent was obtained from all participants prior to their participation in the study in accordance with the Institute Review Board and Ethics Committee. Forty-two patients had a familial history of hearing impairment, while 12 were sporadic cases. Clinical information such as severity and age of onset of hearing impairment, exposure to some kind of ototoxic substances, especially aminoglycosides, and any other medical diagnoses were evaluated.

Mutation detection

DNA of patients and relatives who agreed to participate in the study was extracted from peripheral blood using standard procedures. A DNA fragment of 339 bp containing part of the 12S rRNA gene was amplified by PCR (Forward primer 5'-GCTCAGCCTATATACCGCCATCTTCAGCAA-3' and Reverse primer 5'-TTTCCAGTACACTTACCATGTTACGACTTG-3') and analyzed by direct sequencing to detect the A1555G mutation in the index case of each family. Screening for the A1555G mutation in all family members was performed by polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) using endonuclease HaeIII, as described elsewhere [10].

Audiologic examination

Audiologic evaluation included otoscopic examination, pure-tone audiometry (PTA), acoustic reflexes, tympanometry, evaluation of auditory brainstem responses (ABRs), and measurement of distortion-products of otoacoustic emissions (DPOAE).

Pure tone audiograms (PTA) were performed using Amplaid 460 (Amplifon, Milan, IT). Pure tone hearing thresholds were determined for 500, 1000, 2000, 4000 and 8000 Hz, measured in dB nHL (based on normal hearing subjects). The PTA was calculated from the sum of audiometric thresholds at 500, 1000, 2000, 4000 and 8000 Hz. The degree of hearing loss was defined according to the mean hearing loss as follows: normal ≤20 dB (6 subjects); mild = 20-40 dB (1 subject); moderate = 41-70 dB (6 subjects); severe = 71-95 dB (5 subjects); and profound >95 dB (6 subjects).

The DPOAE were recorded and analysed using an otodynamic analyser ILO292 (Otodynamics Ltd, Hatfield, Herts, UK). Following the sealing of the acoustic probe into the ear canal, two primary tones with a frequency ratio (f1/f2) kept at 1.22 were presented at 70 dB SPL [15]. The DPOAE audiogram was built registering the distortion products in half octave steps. Control values were obtained using the same protocol from normally hearing individuals randomly selected of the same range of ages.

ABR recordings were performed using a Medelec Synergy instrument (Oxford Instruments plc, Oxon, UK). Alternating polarity clicks were given to the patients with a monoaural stimulus at intensities of 95 dB, being the contralateral ear masked with a stimulus of –20 dB. Sweeps of 2000 stimulus per second with frequency amplitude of 2000-4000 Hz were performed. The electrical activities were recorded as the potential difference between the electrodes placed Cz on the forehead and A1, A2 electrodes placed on the mastoids, according to international System of electrodes 10-20. Two different measures were performed for stimulation. Evaluation of results was done according to the absolute latency of the waves and the interpeak latencies.

Statistical analysis

Predicted mean effects of covariates analysed (wave, group and ear) were estimated using linear mixed-effect models. These models deal with the correlation within patient, which is produced due to paired design. All analyses were performed with S-PLUS functions using the *nlme* library [16].

RESULTS

12S rRNA A1555G Mutation

The A1555G mutation was detected in homoplasmy in nine of the 54 patients screened (17%). All nine positive patients for the A1555G mutation showed a familial history of hearing loss, resulting in a prevalence of 21% among familial cases. A1555G was not found among the sporadic cases. Determination of the A1555G mutation was also performed in the family members who agreed to participate in the study. A clear maternal inheritance pattern of A1555G was observed in all the pedigrees analysed (males and females were equally affected, the trait was always transmitted by females and the affected males did not transmit the disorder).

Twenty-four A1555G carriers (9 index cases and 15 relatives) underwent the clinical study. Of these 24 individuals, 6 presented normal hearing and 18 were hearing impaired with different degrees of hearing loss (1 mild, 6 moderate, 5 severe and 6 profound hearing loss). Four subjects developed hearing impairment after aminoglycoside exposure. These subjects had moderate to profound hearing loss with an age at onset correlating with the period of drug administration (Table 1).

The age of onset varied from one year to 20 years, with an average of 9 years. However, the majority of the unaffected subjects were older than the average age of onset observed in the affected group of patients, thus suggesting the presence of other factors that may influence the development of hearing loss.

Medical records of A1555G carriers showed no other clinical abnormalities such as diabetes, muscular disease, visual problems or neurological disorders.

Audiologic evaluation

All 24 subjects underwent clinical examination consisting in otoscopy and timpanometry, which were normal in all cases. Tinnitus was noted in 11/24 subjects (9

deaf and two hearing individuals). Dizziness was present in two of the hearing impaired subjects, accompanied in one case by an altered electronystagmography, indicating a vestibular dysfunction (data not shown).

Pure-tone audiometries (PTA) indicated variable degrees of hearing loss between individuals, characterized in all cases by a sensorineural, bilateral hearing loss more severe at high frequencies.

Only one of the 18 affected subjects tested displayed acoustic reflexes at high-frequencies, whereas when low frequencies were tested, only the patients affected of mild or moderate hearing loss presented acoustic reflexes. In addition, some of the normal hearing carriers tested did not display acoustic reflexes at 4.0 kHz, indicating a subclinical alteration (Table 2).

Otoacoustic emissions were absent in all affected patients and present in the non-affected ones. Distortion products of otoacoustic emissions (DPOAE) were also measured. The mean amplitude of DPOAE in both deaf and non-affected groups was calculated and compared with general population values. Most of the affected subjects displayed non-recordable DPOAEs across all frequencies (Fig 1). In the case of hearing A1555G carriers DPOAE measures were within normal values, with the exception of the highest frequencies tested where decreased amplitude was observed (Fig 1). Since DPOAE are an objective measure of cochlear function, these results indicate a cochlear dysfunction.

Auditory brainstem responses (ABR) were evaluated in 8 subjects affected by different degrees of hearing loss. All 8 subjects had symmetric ABR recordings with prolonged latency of Wave I and preservation of interpeak latencies. These results imply a cochlear dysfunction with no affectation of the auditory nerve (Table 3).

DISCUSSION

The A1555G mutation in the mitochondrial 12S rRNA gene is one of the most common causes of sensorineural hearing loss and aminoglycoside-induced deafness. This mutation was first discovered in a large Arab-Israeli family [17] and subsequently found in various ethnic groups from Europe, Asia and Africa, with a variable prevalence [10-12, 18-21]. In the absence of aminoglycosides exposure, the phenotype observed is extremely variable by means of the severity of hearing loss and age of onset. Moreover, a significant portion of individuals has normal hearing for their entire life. This confirms the fact that the A1555G mutation alone is not sufficient to produce the clinical phenotype. Furthermore, we have observed that the mean age of normal hearing A1555G carriers is much higher than the mean age at onset of deaf patients, suggesting the implication of other factors that determine the development of hearing loss.

Different hypotheses have been proposed to explain the role of the A1555G mutation as a deafness predisposing mutation. Mitochondrial haplotypes may explain some of the differences between families and ethnic groups [22, 23], but different A1555G penetrance could be the result of a combined action of other susceptibility genes and environmental factors. Biochemical and genetic data suggest that the nuclear background may modulate the phenotypic expression of the mutation [24, 25]. Finally, extensive genome-wide search 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 [26-28].

The present study has focused on a detailed evaluation of the auditory function of hearing loss associated to the A1555G mutation, taking especially into account the characterization of normal hearing carriers of the mutation. General physical examination of all patients disclosed normal general health and no other pathologies.

Tinnitus was present as an additional symptom in half of the hearing impaired patients (9/18) but also in two of the normal hearing individuals. It has previously been reported that approximately 50% of patients who initially experienced tinnitus alone developed hearing loss at a later time [29], indicating that tinnitus is a clinically significant warning sign for future hearing loss. Vestibular abnormalities were only detected in one case.

Pure-tone audiometry and acoustic reflexes showed a bilateral sensorineural hearing loss more severe at high frequencies in all affected patients, being consistent with previously reported audiometric features [30-32]. Absence of acoustic reflexes at 4.0 kHz was noted in two hearing carriers, suggesting a subclinical alteration. The more severe phenotype observed at high frequencies indicates a topographic effect in the cochlea, being the basal turn the most damaged one.

Otoacoustic emissions are generated by the outer hair cells of the cochlea and are well known as an objective measure of cochlear function [33, 34]. Distortion products of otoacoustic emissions (DPOAE) were absent at all frequencies in the vast majority of hearing impaired individuals, which is consistent with the idea that hearing loss due to the A1555G mutation is a cochlear form of deafness [31, 35]. In the non hearing-impaired group, recording of DPOAE showed lower amplitude of response at high frequencies as compared to normal reference values. These findings indicate a deficit in cochlear physiology of all A1555G carriers, not severe enough to lead to hearing impairment in normal hearing individuals with this mutation. Auditory brainstem evoked potentials (ABR) showed a delay in wave I with preservation of interpeak latencies, indicating that hearing loss associated to the A1555G mutation is due to a cochlear defect without retrocochlear dysfunction. Thus, the subclinical alterations support the recommendation of preventive measures against environmental

hearing loss factors (i.e., avoiding loud noises and ototoxic drugs and undergoing periodic hearing tests) for A1555G hearing carriers. Moreover, the observation that there are cochlear alterations in asymptomatic subjects is consistent with data that there was severe mitochondrial dysfunction in cells derived from asymptomatic subjects in the arab-israeli pedigree carrying the A1555G mutation [36].

In the present study, the A1555G mutation was found in 9 out of 54 adult patients (17%) affected of nonsyndromic hearing loss. When only familial cases were considered, the prevalence was 21%, thus confirming the A1555G mutation as a principal factor leading to familial nonsyndromic hearing loss of postlingual onset in Spanish patients [7-11]. Aminoglycoside exposure was reported in four affected individuals, giving further evidences of the strong relationship between the A1555G mutation and an increased susceptibility to aminoglycoside antibiotics. Genetic screening for mutation A1555G among deaf patients with a familial history of hearing loss should be considered. The early detection of the mutation would enable prevention of the onset of hearing loss (such as avoiding exposure to aminoglycosides) and/or mitigation of the progression of hearing impairment.

In summary, our findings indicate that hearing loss associated with the A1555G mutation is a cochlear form of deafness resulting from hair cell dysfunction. These results agree with the proposed mechanism of action for the A1555G mutation in the cochlea, where hair cells are damaged as a consequence of a mitochondrial translational defect of ribosomes with the A1555G mutation. This defect results in a decline in ATP production and an increase in the generation of reactive oxygen species, which leads to hair cell apoptosis [37]. It is not clear why the A1555G mutation commonly causes hearing loss without additional abnormalities, such as vestibular dysfunction, even though the mutation is present in all tissues. The identification of subclinical alterations

in normally hearing A1555G carriers is a step further in understanding the clinical consequences of this relatively common mutation responsible for hearing impairment.

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FIGURE LEGENDS

Figure 1. Distortion-products of otoacoustic emissions (DPOAE) measurements in subjects that carry the A1555G 12S rRNA mutation in the mitochondrial genome. Mean and confidence interval 95% of the response in milliseconds (ms) for the different frequencies. A comparison of the means for the general population, deaf A1555G carriers and non-affected A1555G carriers is presented. Note that differences between non-affected A1555G carriers and general population values were statistically significant only in the highest frequencies tested.

Table I. Clinical features of the 24 patients carrying the A1555G mutation in the 12S rRNA gene.

	Patient No.	Sex	Age	Age of onset	Aminoglycoside administration	PTA (dB)		Tinnitus	Dizziness	Degree of hearing loss
						Right ear	Left ear			
DEAF	**1**	F	21	8	none	25	25	-	-	mild
	2	F	46	8	streptomycin	110	110	-	-	profound
	3	M	46	16	none	75	75	+	-	severe
	4	M	40	9	none	110	116	+	+	profound
	5	M	37	2	none	50	61	-	-	moderate
	6	M	25	10	streptomycin	44	40	+	-	moderate
	7	F	36	3	none	85	70	-	-	severe
	8	M	31	15	none	60	60	+	-	moderate
	9	F	39	10	none	100	100	+	-	profound
	10	F	35	13	none	50	55	-	-	moderate
	11	F	37	1	none	80	90	-	-	severe
	12	M	32	1	none	55	60	-	-	moderate
	13	F	25	7	none	90	90	-	-	severe
	14	M	27	14	none	100	100	+	-	profound
	15	F	42	10	streptomycin	85	80	+	+	severe
	16	F	46	8	none	95	95	+	-	profound
	17	F	27	4	streptomycin	nr	nr	-	-	profound
	18	F	57	20	none	45	40	+	-	moderate
HEARING	**19**	F	9	-	none	5	5	-	-	normal
	20	F	53	-	none	15	15	-	-	normal
	21	M	12	-	none	10	10	-	-	normal
	22	F	28	-	none	15	15	+	-	normal
	23	F	30	-	none	10	10	+	-	normal
	24	F	30	-	none	10	10	-	-	normal

PTA. Pure Tone Audiometry; dB. decibels; nr. non recordable.

Table II. Acoustic reflexes and otoacoustic emissions (OAE) in subjects carrying the 12S rRNA A1555G mutation

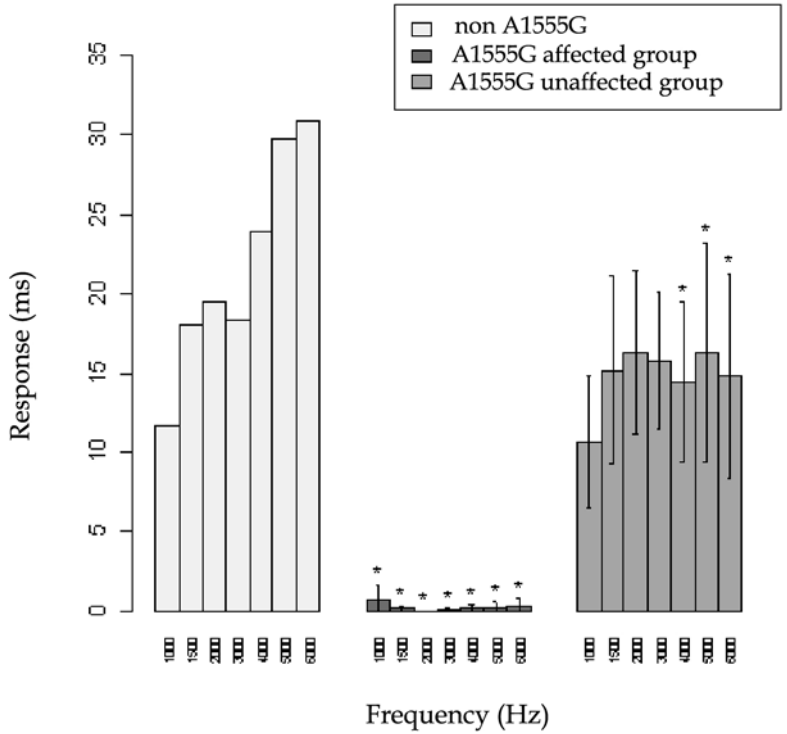
	Acoustic reflexes (kHz)						OAE	
	0.5		1.0		4.0			
	R	L	R	L	R	L	R	L
Symptomatic (n=18)	11/18	14/18	3/18	4/18	1/18	0/18	0/18	0/18
Non symptomatyc (n=6)	6/6	6/6	6/6	6/6	4/6	4/6	6/6	6/6

R. right ear, L. Left ear

Table III. Auditory Brainstem Response recordings for eight symptomatic carriers of the 12S rRNA A1555G mutation

Patient No.	Wave I		Wave III		Wave V		Interval I-III		Interval III-V		Interval I-V	
	R	L	R	L	R	L	R	L	R	L	R	L
3	1920	2120	4480	4490	6460	6620	2560	2820	1980	1680	4540	4500
5	1960	1800	3860	3700	5620	5300	1900	1900	1760	1600	3660	3500
6	1660	1520	3960	3740	6380	6160	2480	2440	2640	2200	4800	4880
7	1620	1540	3720	3560	6420	6420	2100	2020	2860	2700	4720	4640
8	2160	2060	4120	4480	6180	6220	1960	2420	2100	1700	4060	4120
9	2260	1900	4860	3620	7340	5630	2600	1720	2480	2000	5080	3720
13	2160	1960	4660	4220	7340	6460	2500	2460	2680	2240	5180	4500
15	2060	1900	2740	2920	6840	7080	2180	2700	2600	2480	4780	5180
Mean	2024.2	1800.8	4057.3	3834.0	6516.0	6292.7	2285	2310	2387,5	2075	4602.5	4380
p value*	0.0002	0.0326	0.0012	0.0965	<0.0001	<0.0001	ns	ns	ns	ns	ns	ns

Values are in milliseconds (ms); ns, non significant; R, right, L, left: *p value corresponding to comparison of means to reference values $\bar{x}_{\text{Wave I}} = 1500$, $\bar{x}_{\text{Wave III}} = 3600$, $\bar{x}_{\text{Wave V}} = 5500$.


non A1555G
A1555G affected group
A1555G unaffected group
Response (ms)
0
5
10
15
20
25
30
35
1000
1500
2000
3000
4000
5000
6000
Frequency (Hz)

Low-level mtDNA heteroplasmy in a deafness pedigree co-segregating 1555A>G 12S rRNA and 15287T>C cytochrome *b* mutations give new insights into mtDNA transmission

Ester Ballana, Nancy Govea, Rafael de Cid, Cecilia Garcia, Carles Arribas, Jordi Rosell and Xavier Estivill

In this work, two assays for reliably and accurately detecting low-level mtDNA heteroplasmy were developed: DHPLC and Pyrosequencing. The developed assays were used to screen a group of deaf samples of unknown aetiology for the presence of heteroplasmic mtDNA variants.

Two heteroplasmic 1555A>G samples, previously considered as homoplasmic, and a three-generation heteroplasmic family were identified. The three-generation family segregates two distinct mtDNA mutations in heteroplasmy: the deafness-related 1555A>G mutation and 15287T>C, a novel variant in mitochondrial cytochrome *b* gene. The mutation load for both mutations was assessed in two different tissues: blood and saliva, allowing the comparison between them. Both heteroplasmic mtDNA point mutations are transmitted through generations in a random manner, showing differences in mutation load between siblings within the family, in accordance with the bottleneck theory of mtDNA inheritance. The analysis of mtDNA inheritance in this pedigree showed rapid shifts in mtDNA haplotypes, suggesting a small developmental bottleneck, which was calculated to be between 2-46 segregating units.

To gain insight into the role of the novel described 15287T>C variant a group of 181 control samples and 190 index deaf cases were tested, finding the variant in one control and two deaf samples. Thus, the role of 15287T>C variant is unclear although a specific role in the inner ear when found together with other 12S rRNA mutations could not be discarded, as suggested by the fact that 15287T>C in deaf cases has been found associated with 1555A>G or 1243T>C 12S rRNA variants.

Low-level mtDNA heteroplasmy in a deafness pedigree co-segregating 1555A>G 12S rRNA and 15287T>C cytochrome *b* mutations provide new insights into mtDNA transmission

Ester Ballana[1], Nancy Govea[2], Rafael de Cid[3], Cecilia Garcia[3], Carles Arribas[3], Jordi Rosell[2] and Xavier Estivill[1,3,4]

ABSTRACT

Mitochondrial DNA (mtDNA) mutations are an important cause of human disease, which in most cases present a high phenotypic heterogeneity. Heteroplasmy may underlie some of the phenotypic variability observed in mitochondrial disorders. In the present report, we have developed two assays, using DHPLC and Pyrosequencing, 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 1555A>G mutation and a new variant (15287T>C) in the cytochrome *b* gene. Both heteroplasmic mtDNA mutations are transmitted through generations in a random manner, thus showing differences in mutation load between siblings within the family, in accordance with the bottleneck assumption of mtDNA inheritance. The observed rapid shifts in mitochondrial haplotypes in this family suggested also a small developmental bottleneck, which was calculated to be between 2-46 segregating units. The developed assays were used to screen a group of deaf samples of unknown aetiology for the presence of heteroplasmy for both mtDNA variants. Two additional heteroplasmic 1555A>G samples, previously considered as homoplasmic, and two deaf samples carrying 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.

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 phosphorilation, 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).

Mitochondrial DNA (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 and Alzheimer's and Parkinsons' 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 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.

Very little is known about the mechanisms of transmission of heteroplasmic mtDNA mutations. Heteroplasmic female mice transmit neutral heteroplasmic mtDNA polymorphisms to their offspring, and the variation among the offspring is largely determined by random genetic drift (Jenuth et al., 1996). It is thought that a restriction in the number of mtDNA molecules in early oogenesis is behind this process, which is known as the mitochondrial genetic bottleneck (Poulton et al., 1998). This restriction and amplification of mtDNA leads to different levels of mutant mtDNA in the female's offspring. As a result, a female harbouring a pathogenic mtDNA defect might transmit low levels of mutant mtDNA to some offspring and high levels to others. Some of the offspring may be severely affected whilst others may remain asymptomatic throughout their life (Chinnery and Turnbull, 2000).

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 new assays for the detection and quantification of mtDNA mutations, based on denaturing high performance liquid chromatography (DHPLC) and Pyrosequencing technologies. By using these approaches we have identified a three-generation heteroplasmic family harbouring two mtDNA variants, the deafness-related 1555A>G mtDNA mutation and the novel 15287T>C variant in the mitochondrial *cytochrome b (MTCYB)* 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 non-syndromic hearing loss and control samples.

MATERIALS AND METHODS

Patients and families

Familial cases of sensorineural hearing loss have been collected from different Spanish clinical centres with the aim to study the molecular basis of hearing loss. The Spanish control samples were unrelated blood donor, all of Caucasian origin. Informed consent was obtained from all participants prior to their participation in the study, in accordance with the Institutional Review Board and Ethic 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, 2000, 4000 and 8000 Hz, measured in 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 two ml of saliva, obtained using the Oragene DNA self-collection kit (Genotek®).

mtDNA sequencing

In order to confirm 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). The sequences obtained were compared with the revised Cambridge reference sequence (Genbank accession number ACJ01415).

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.

Detection and quantification of mtDNA mutations

PCR-RFLP analysis. Detection of 1555A>G mutation was first performed by PCR amplifying a 340 bp DNA fragment containing the mutation site (forward primer 5'-GCTCAGCCTATATACCGCCATCTTCAGCAA-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 (denaturing high-performance liquid chromatography) was performed using the 3500-HT WAVE nucleic acid fragment analysis system (Transgenomic). For 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 95ºC and then gradually reannealed by decreasing sample temperature from 95ºC to 24ºC at a rate of -0.04ºC 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 which allows the separation of nucleic acids in a size and conformation-dependent manner. The column is kept in the L-7300+ oven 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/min). 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 milivolts and analyzed using de D-7000 program (Transgenomic) and the Transgenomic Navigator Software (Version 1.6.1).

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 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 mutation load for 1555A>G and 15287T<C carriers specific SNP assays using Pyrosequencing were developed (for 1555A>G assay forward 5'-CGACATTTAACTAAAACCCCTACGC-3', reverse 5'-GTTGGGTGCTTTGTGTTAAGCT-3' and sequencing 5'-CACTTACCATGTTACGACT-3' primers) (for 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, and percentage of mutation load was determined using the quantification function of the software.

Threshold detection of 1555A>G and 15287T>C mutation

DNA from a wild-type individual and a carrier of each mutation were amplified using primers 5'-TGCTCGCCAGAACACTACGA-3' and 5'-TGGACAACCAGCTATCACCA-3' for 1555A>G mutation, and 5'-ACATCGGCATTATCCTCCTG-3' and 5'-AGTAAGCCGAGGGCGTCT-3' for 15287T>C. The resulting PCR-amplified fragments were cloned into pGEM-T vector (Promega®) 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%-100%. Each sample was analysed 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 1555A>G

A three-generation family with various members affected of sensorineural hearing loss was ascertained through the Genetic Service of Hospital Son Dureta (Figure 1a). Thirteen subjects (7 deaf and 6 hearing) were analysed 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 out of 7) exhibited bilateral, sensorineural hearing impairment with clinical manifestations varying from mild to total deafness (Figure 2). In one case (individual 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.

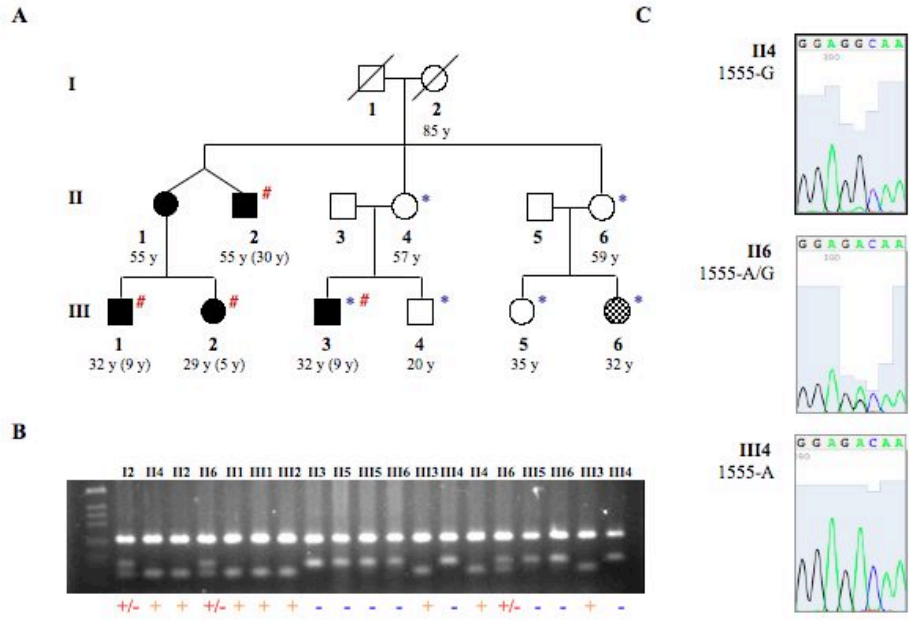


Figure 1. Identification of mtDNA mutation A1555G in a family with hearing impairment. **A/** Pedigree segregating mtDNA mutations in heteroplasmy. Solid symbols indicate clinically deaf individuals, checkered symbol unilateral affected individual and open symbols unaffected individuals. Age in years and age of onset (in brackets) is shown below of subject symbols. # denote individuals who had a history of exposure to aminoglycosides. *Indicate subjects from which two independent blood samples and saliva were obtained. **B/** PCR-RFLP analysis of A1555G mutation. In wild type allele, digestion results in 2 fragments; whereas the mutant DNA shows a different restriction pattern, resulting in 3 fragments. **C/** Sequence of a mutant (sample II4), a heteroplasmic (sample II6) and a wild-type subject (sample III4).

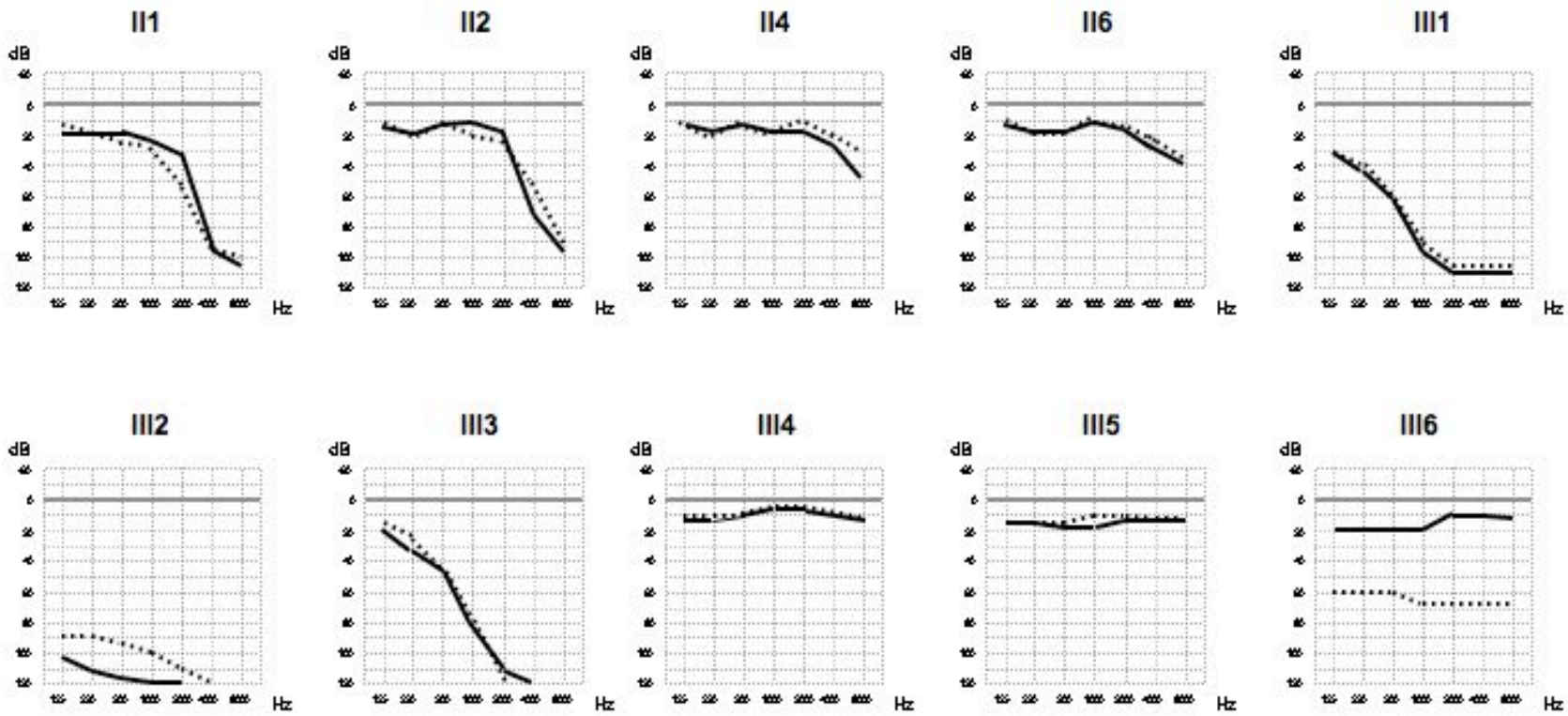


Figure 2. Pure tone audiometries of the three-generation pedigree subjects segregating mtDNA mutations in heteroplasmy. Hearing level (in dB) is plotted versus sound frequency (in Hz). Pure tone hearing thresholds were determined for 250, 500, 1000, 2000, 4000 and 8000 Hz, measured in dB nHL (based on normal hearing subjects). The PTA was calculated from the sum of audiometric thresholds at 500, 1000, 2000, 4000 and 8000 Hz. Solid lines, right ear; dashed lines, left ear.

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; del Castillo, et al., 2002), with negative results (data not shown). The 1555A>G mutation in the 12S rRNA gene was also tested by PCR amplification of a 340-bp fragment followed by the digestion with restriction endonuclease *Hae*III. Eight out of the eleven maternally-related family members were positive for mutation 1555A>G, with two of them being heteroplasmic. Surprisingly, in three of the third-generation maternally related subjects as well as in the two non-related fathers, digestion with *Hae*III failed to identify the mutation 1555A>G (Figure 1b), which could neither be identified by direct sequencing (Figure 1c). Remarkably, the two sons of mother II4 which was detected as a homoplasmic carrier of mutation 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.

In order 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, where 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, 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 leukaemia (zur Stadt et al., 2001), among other disorders. DHPLC has also been used for mutation scanning of the whole human mitochondrial genome of 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 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 were subjected to DHPLC analysis as well as the patients' samples. By analysing 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% (Figure 3a).

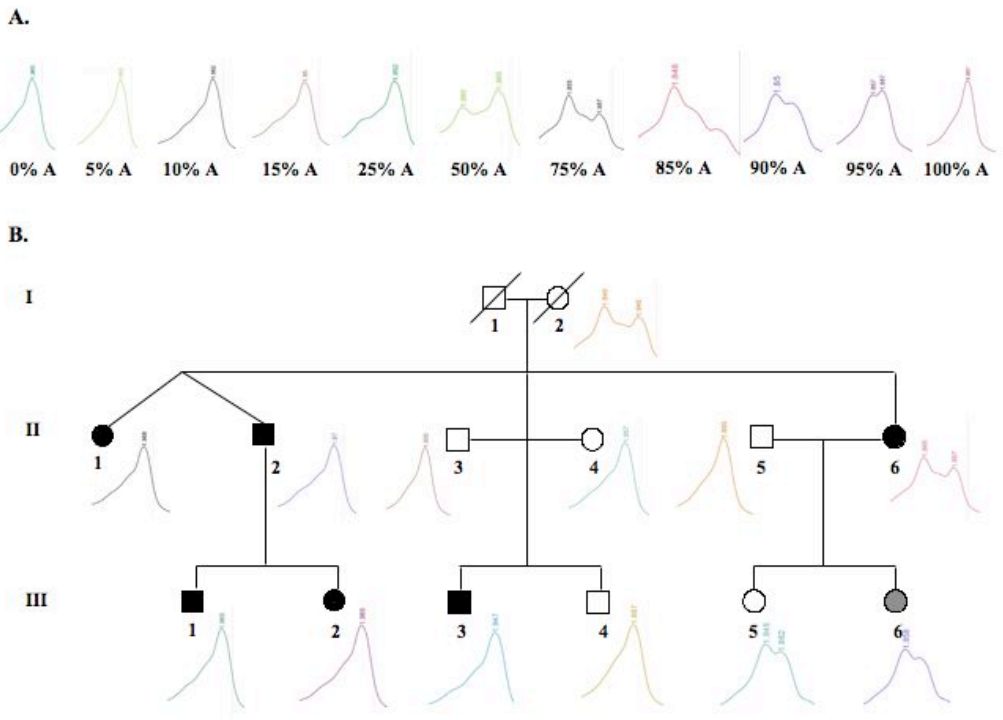


Figure 3. DHPLC profiles for mutation 1555A>G. **A/** DHPLC patterns of standard samples with known mutation load. **B/** DHPLC patterns of the three-generation pedigree segregating 1555A>G mutation in heteroplasmy.

When the family samples were analysed, clear heteroduplex patterns were observed for individuals I2, II6, III5 and III6. This confirms the presence of mutation 1555A>G in heteroplasmy in two cases (I2 and II6) and allows the identification of two additional heteroplasmic samples (III5 and III6) (Figure 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

harbour low-level heteroplasmic mtDNA, especially in the case of II1, II2, II4 and III3 (Figure 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, being then suitable for mutation identification through DNA sequencing as fragment collection enriched them with the low-level mtDNA genome (Supplementary figure 1). Using this approach, the presence of heteroplasmy, even at very low-levels, was confirmed in all maternally related subjects, except for subject 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 (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 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 figure 2). A level of 5% heteroplasmy was reliably detected (standard deviation 1.7). Quantification of three replicate experiments showed a linear regression with a regression coefficient of 0.991x, an intercept of 0-465 and a 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 wt DNA ranging from 8% to almost 100%, except for subject 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, in order 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 subject II4. However, this is a common situation in carriers of mutation 1555A>G. Deaf family subjects had less than 20% wild-type mtDNA, with

the exception of subject II6. Thus, no clear genotype-phenotype correlation could be drawn from these data.

Table 1. Results of heteroplasmic mtDNA mutations quantification in a three-generation pedigree with hearing impairment using Pyrosequencing specific assays. Values are the mean of two independent assays with three replicate measurements each.

Sample	1555A>G (%A)		15287T>C (%T)	
	Blood	Saliva	Blood	Saliva
I2	76.9	-	65.8	-
II1	10.1	-	4.7	-
II2	18.9	-	12.9	-
II3	98.7	-	99.7	-
II4	9.8	10.9	9.3	9.8
II5	98.7	-	98.7	-
II6	66.7	75.3	63.2	74.3
III1	10.6	-	7.3	-
III2	8.9	-	4.7	-
III3	3.9	4.3	4.7	4.7
III4	98.6	97.7	97.4	96.9
III5	94.9	89.4	94.1	93.8
III6	93.9	88.2	92.2	87.1

mtDNA heteroplasmy quantification in other tissues and samples

In order 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 lineal 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 1555A>G mutation in 190 deaf samples of unknown cause, with negative results. Sixty-nine additional pedigrees known to carry the 1555A>G mutation (Ballana et al., 2006) were also analysed, with the aim to detect 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 1555A>G mutation is inherited with 15287T>C in *cytochrome b*

Hearing loss associated with mutation 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; Bykhovskaya et al., 1998; Bykhovskaya et al., 2001; Guan et al., 2000; Guan et al., 2001).

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

Table 2. mtDNA sequence variants and amino acid changes identified in the two brothers III3 and III4 compared to the human mtDNA consensus Cambridge sequence.

GENE	SAMPLE	
	III3	**III4**
MT-RNR1	750A>G	750A>G
	1438A>G	1438A>G
	1555A>G	
MT-ND1	3915G>A (Gly203Gly)	3915G>A (Gly203Gly)
MT-TI	4314T>C	4314T>C
MT-ND2	4727A>G (Met86Met)	4727A>G (Met86Met)
	4769A>G (Met100Met)	4769A>G (Met100Met)
MT-ATP6	8860A>G (Thr112Ala)	8860A>G (Thr112Ala)
MT-CO3	9380G>A (Trp58Trp)	9380G>A (Trp58Trp)
MT-ND4	11253T>C (Ile165Thr)	11253T>C (Ile165Thr)
MT-ND6	14356C>T (Val106Val)	14356C>T (Val106Val)
MT-CYB	15287T>C (Phe181Leu)	
	15326A>G (Thr194Ala)	15326A>G (Thr194Ala)
MT-DLOOP	239T>C	239T>C
	263A>G	263A>G
	302ins(CC/CCC)	302ins(CC/CCC)
	310insC	310insC
	16362T>C	16362T>C
	16482A>G	16482A>G

The only sequence differences between them are mutations 1555A>G and 15287T>C; Cambridge sequence (Genbank accession number ACJ01

All the family members were analysed for the presence of the 15287T>C variant by direct sequencing and pyrosequencing. The pyrosequencing assay was

used to quantify the 15287T>C mtDNA variant with the same strategy as for mutation 1555A>G. Variant 15287T>C was identified in heteroplasmy in all family members that also carried the 1555A>G mutation, but not in the non-carriers. The specificity, sensitivity and accuracy of the 15287T>C assay were also assessed pyrosequencing known mutation load mixtures standards (Supplementary figure 2). 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 1555A>G assay, sensitivity and accuracy for mutation load quantification are still very high.

The segregation pattern and quantification results obtained for variant 15287T>C are very similar to that observed for mutation 1555A>G (Table 1). These results indicate that both changes may have arisen in the same maternal ancestor and were then co-segregated. The analysis of 181 Spanish unrelated control samples resulted in the identification of one sample carrying 15287T>C in homoplasmy. This sample did not carry the 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 aetiology, were analysed for the presence of variant 15287T>C, identifying two additional nuclear families in homoplasmy (Supplementary figure 3). One of them was previously reported to carry the 1243T>C variant in 12S rRNA gene, which also has an unknown role in the pathogenesis of deafness (Ballana, et al., 2006).

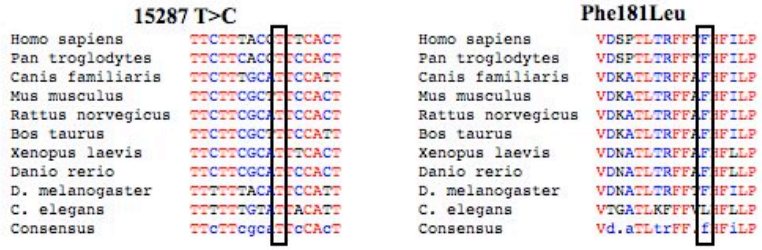


Figure 4. Conservation of variant 15287T>C across species at the DNA and protein levels. At both levels, T at position 15287 and Phe at position 181 are conserved throughout evolution; with the exception of C. elegans in which Phe is replaced by Leu.

Random genetic drift drives heteroplasmic mtDNA transmission through generations

To gain insight into the mechanisms that drive transmission and segregation of mtDNA heteroplasmic mutations in humans, a detailed analysis of mtDNA inheritance in this heteroplasmic pedigree was performed. The pedigree is characterized by a co-segregation in heteroplasmy of the 1555A>G and 15287T>C variants. Children of heteroplasmic mother I2 either have high rates of mutant mtDNA at both sites (subjects II1, II2 and II4) or, alternatively, the heteroplasmy

levels for both substitutions are maintained (subject II6). However, both substitutions tend to segregate to homoplasmy by the third generation, where three of the subjects are almost homoplasmic for the wild type allele at both sites (III4, III5 and III6) and three for the mutant allele (III1, III2 and III3). Such rapid and variable shifts in heteroplasmic lineages can be explained because of the mtDNA genetic bottleneck during oogenesis (Jenuth et al., 1996). The fact that half of the third-generation descendants are wild type and half mutant may indicate that selective pressures do not influence segregation of the mutations. Thus, the transmission pattern in this pedigree suggests that random genetic drift in the female germline would drive the rapid segregation of heteroplasmic mtDNA.

The segregation of mtDNA in cells and organisms has been modelled using the population-genetic-bottleneck model developed by Wright (Birky et al., 1983; Howell et al., 1992; Jenuth et al., 1996; Poulton et al., 1998). This model assumes that a population of infinite size is instantaneously restricted to a minimum bottleneck size (N) for a number of generations (g) before the population instantaneously expands back to an infinite size. By measuring the change in variance of mtDNA genotype frequencies between generations, one can estimate the effective number of mtDNA molecules that are sampled at each generation from a large pool, assuming that segregation occurs by genetic drift. We calculated the effective number of segregating units (N) for mtDNA using such a model which related the variance (V_n) in the *n*th generation to the initial frequency of one of the mtDNA genotypes (p_0) taking into account the number of cell divisions in the female germline (g) as follows:

$$V_n = p_0(1- p_0)[1-(1-1/N)^{gn}]$$

In humans an estimated six million primary oocytes are produced which would require ~24 mitotic divisions from a single founder cell (Jenuth et al., 1996). An alternative approach is to assume that a single sampling event occurs once during development (Bendall et al., 1996), which greatly simplifies the calculation as g = 1. We have applied both models to the pedigree described, considering the initial allele frequency, p_0, the mutation load observed in woman I2 (p_0 = 0.78) and V_n, the variance in mutation load between first generation siblings (V_n = 0.07). When equation (1) with g = 24 is used, the effective number of segregating units (N) for mtDNA calculated is 46; when the single-sampling approach is used, the estimated number of segregating units is 2. These figures are smaller than other reported values for human pedigrees (Howell et al., 1992; Jenuth et al., 1996; Poulton et al., 1998) but similar to that reported for 8993T>G mutation (Blok et al., 1997). This could be explained based on the different nature and functional consequences of particular mtDNA mutations.

DISCUSSION

Mutations in mtDNA are recognised 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 harbouring mtDNA mutations (Chinnery and Turnbull, 2000). Human mtDNA has also become a useful tool in anthropological and evolutionary research, as well as in forensic studies, mainly because of the presence of multiple polymorphisms that can be used to distinguish non-maternally-related individuals. However, an important handicap for such studies is the presence of heteroplasmy, which may lead to false negative or ambiguous results (Sekiguchi et al., 2003). In the present study we have used two technologies for the reliable measurement of heteroplasmy in different tissues: DHPLC and pyrosequencing. The performance of both approaches was examined with two different mtDNA variants: the deafness-associated 1555A>G mutation and a novel variant in the *MTCYB* gene, 15287T>C, both identified in a three-generation heteroplasmic pedigree affected of nonsyndromic hearing loss.

The 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, 1555A>G is usually homoplasmic. Heteroplasmy for mutation 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 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 1555A>G heteroplasmic subjects (Sekiguchi et al., 2003). On the other hand, it has been reported that mtDNA mutations can segregate and accumulate in certain tissues, being the level of heteroplasmy frequently lower in blood leukocytes compared to affected tissues (Meierhofer et al., 2005). We obtained mtDNA from saliva, which is a non-invasive technique that has demonstrated a good performance in the assays and allowing 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.

Similarly to our results, only in a few of the previously described 1555A>G heteroplasmic cases 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 1555A>G mutation seems to be small in most of the cases. Other genetic factors may determine the differences in the phenotypic expression of mutation 1555A>G. The search of additional mtDNA factors that could influence the phenotype associated with 1555A>G mutation in this three-generation heteroplasmic pedigree resulted in the identification of a novel mtDNA variant in the mitochondria-encoded *MTCYB* gene, 15287T>C, co-segregating with the 1555A>G mutation. The most likely explanation for this co-segregation is that the two sequence changes occurred simultaneously, or nearly so, within the same mtDNA molecule in a recent maternal ancestor and were then co-segregated in the matrilineal progeny.

Although position 15287 is conserved both, at DNA and protein levels, the pathogenic role of variant 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 15287T>C carriers; none of them presenting evidence of respiratory complex III deficiency. Thus, 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 15287T>C in deaf cases has been found associated with 1555A>G or 1243T>C. Further studies are needed to determine the role of this 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, the variable proportions of heteroplasmy observed in the descendants of a single heteroplasmic woman are in accordance with the bottleneck theory of mtDNA inheritance. The bottleneck theory was proposed by Ashley et al (1989) who found in Holstein cows that the proportion of heteroplasmy in a mtDNA site could change in a single generation and could revert to homoplasmy in two to

three generations. According to this theory, mtDNA from a few mitochondria would be selectively amplified during oogenesis and thus, a genotype can become predominant and fixed in future generations (Chinnery et al., 2000). In humans, studies of heteroplasmic mtDNA segregation have accumulated considerable data to support the existence of a developmental bottleneck, although its size is disputed and may vary among different human mtDNA lineages (Bendall et al., 1996; Bendall et al., 1997; Blok et al., 1997; Howell et al., 1992; Howell et al., 1996; Parsons et al., 1997). In the present study, third generation individuals are effectively fixed either for wild type or mutant mtDNA, which is compatible with the effects of random genetic drift operating through a small developmental bottleneck, containing 2-46 segregating units.

Similar reports of rapid shifts in mitochondrial haplotypes within a single generation suggested also a small bottleneck, whereas other estimations of bottleneck size concluded that it might not always be small (Bendall et al., 1996; Bendall et al., 1997; Blok et al., 1997). The discrepancies regarding mtDNA transmission and bottleneck size could be partially explained by the nature and functional consequences of the particular mutation studied. 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; Howell et al., 1996; Parsons et al., 1997). Mutation 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 small bottleneck size calculated for this pedigree, in comparison with other mutations showing more severe functional effects (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 the first step for a better management of mtDNA diseases. Issues of sensitivity, specificity and labour 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. Moreover, the calculation of the bottleneck size along with the knowledge of the functional consequences of mtDNA mutations can be used to better predict the risk of transmitting heteroplasmic mutations and developing the disease. The identification and study of other pedigrees harbouring heteroplasmic mtDNA mutations would help to solve the reported discrepancies regarding transmission and bottleneck size of heteroplasmic mtDNA, which together with the phenotypic heterogeneity typically found in mitochondrial diseases, emphasize the difficulty of genetic counselling.

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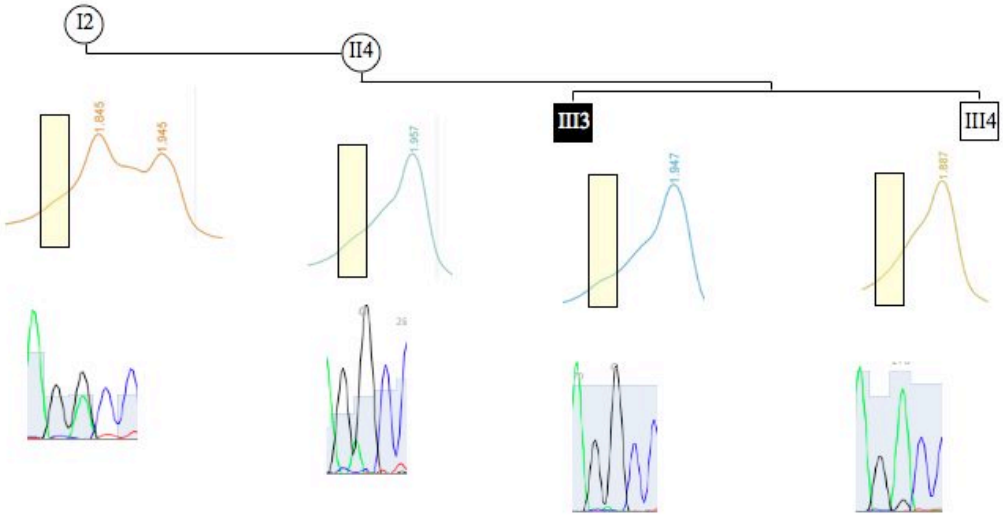
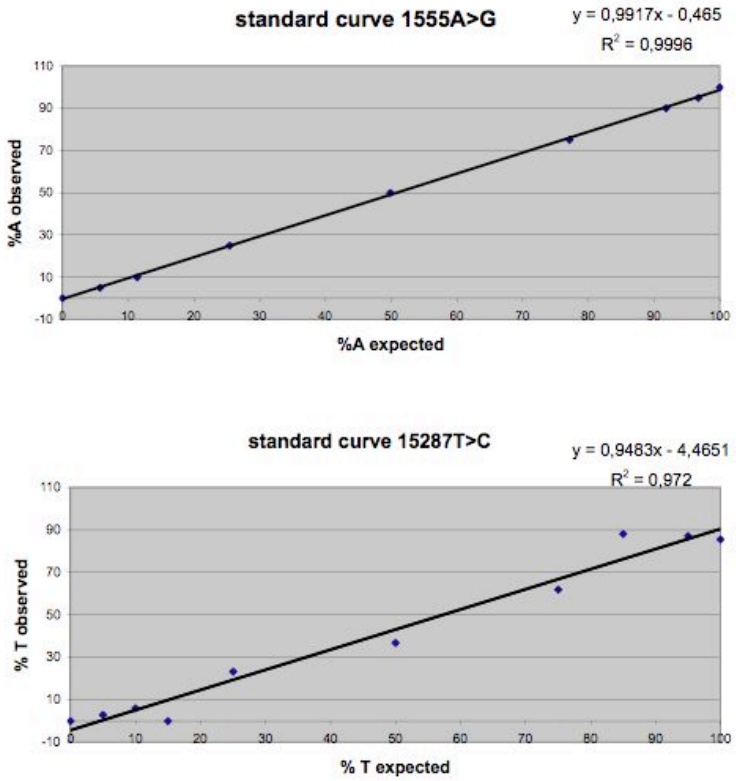
SUPPLEMENTARY INFORMATION

Supplementary figure 1



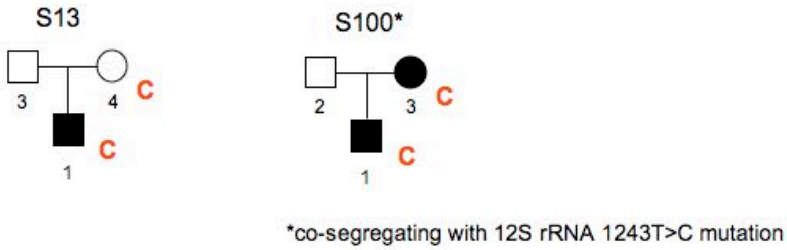
DNA fractions collected with DHPLC Fragment Collector (Transgenomic). The different fractions collected, which correspond to heteroduplex peaks, and their corresponding sequences are depicted.

Supplementary figure 2



Pyrosequencing standard curves for mutations 1555A>G and 15287T>C.

Supplementary figure 3.



Pedigrees of the two additional families affected of nonsyndromic hearing loss carrying mutation 15287T>C. Family S100 was previously reported to carry the 1243T>C variant in the 12S rRNA gene (Ballana, et al., 2006).

Detailed analysis of 8p23.1 putative modifying genetic factors for the deafness-linked A1555G mutation

Ester Ballana, Josep Maria Mercader and Xavier Estivill

Chromosome 8p23.1 was identified as the location of a major modifying locus for A1555G mutation. Despite the efforts of different research groups to identify the gene responsible of A1555G phenotypic variability, none of them has been successful. In this work, different 8p23.1 genomic features, which might be involved in the pathogenesis of A1555G mutation, have been characterized in detail. This includes genomic structural variants such as CNVs, and mutational screening of genes and pseudogenes that could play a role in cochlear function.

Chromosome 8p23.1 is a frequent site of chromosomal rearrangements. A common polymorphic 4.7-Mb inversion mediated by flanking pairs of segmental duplications has been reported in the region. The interpretation of linkage studies of human disease needs to take into account this structural genomic variation. Using family based association tests we have analysed the contribution of the *DEFA1A3* CNV, *CLDN23* gene and *MRPS18CP2* pseudogene in the distinct phenotypes associated to A1555G mutation. Although in none of the cases a clear phenotype-modifying effect has been detected, weak associations between *DEFA3* gene absence and a SNP in *MRPS18CP2* have been found. These associations may have two different interpretations: (i) it gives further evidences in favour of being chromosome 8p23.1 a modifying locus for A1555G phenotype, or (ii) both *DEFA3* gene absence and/or *MRPS18CP2* could be directly or indirectly influencing the deafness onset. Further studies in additional families are needed, but in any case, this is the first time that genomic structural variation is considered in relation with A1555G mutation and represents a promising field to follow up.

Detailed analysis of 8p23.1 putative modifying genetic factors for the deafness-linked A1555G mutation

Ester Ballana[1], Josep Maria Mercader[1] and Xavier Estivill[1,2,3,§]

ABSTRACT

Mitochondrial DNA (mtDNA) mutations at least account for 5% of cases of postlingual, nonsyndromic hearing impairment. Between them, mutation A1555G is frequently found associated with aminoglycoside-induced and/or nonsyndromic hearing loss in families presenting with extremely variable clinical phenotypes. Biochemical and genetic data have suggested that nuclear background is the main factor involved in modulating the phenotypic expression of mutation A1555G. Although a major nuclear modifying locus was located on chromosome 8p23.1 and regardless intensive screening of the region, the gene involved has not been identified. With the aim to gain insights into the factors that determine the phenotypic expression of A1555G mutation, we have analysed in detail different genetic and genomic elements on 8p23.1 region (*DEFA3* gene absence, *CLDN23* gene and *MRPS18CP2* pseudogene) in a group of 213 A1555G carriers. Family based association studies identified a weak positive association for a polymorphism on *MRPS18CP2* pseudogene and an overrepresentation of *DEFA3* gene absence in the deaf group of A1555G carriers. Although any of the factors analysed seem to have a major contribution in the phenotype, our findings provide further evidences of the involvement of 8p23.1 region as a modifying locus for A1555G 12S rRNA gene mutation.

INTRODUCTION

Mitochondrial DNA (mtDNA) mutations are an important cause of human disease and have been associated with many clinical abnormalities, including various forms of syndromic and nonsyndromic hearing loss [1]. It has been reported that at least 5% of cases of postlingual, nonsyndromic hearing impairment are attributable to known mtDNA mutations, representing one of the most frequent causes of hearing impairment [2]. The most commonly reported nonsyndromic deafness-causing mtDNA mutations are a C insertion or deletion at position 961 [3-5], C1494T [6, 7] and A1555G [8-12] in the 12S rRNA gene, and mutations A7445G [13-15], 7472insC [16, 17], T7510C [18] and T7511C [4, 19, 20] in the $tRNA^{Ser(UCN)}$ gene.

In particular, the A1555G mutation has been associated with aminoglycoside-induced and/or nonsyndromic hearing loss in various families of different ethnic backgrounds [8-12]. Remarkably, in Spain it accounts for about 15% of all familial and sporadic cases of hearing loss, irrespective of their mode of inheritance and age of onset [21]. The phenotype associated to A1555G mutation varies considerably among matrilineal relatives, ranging from severe deafness, to moderate progressive hearing loss or even completely normal hearing. Biochemical and genetic data suggest that nuclear background may be the main factor involved in modulating the phenotypic expression of the mutation [22-24]. Extensive genome wide search revealed that nuclear modifying factors are likely to be numerous, but a region in chromosome 8p23.1 has been proposed as a putative localization for a modifier locus [22, 23, 25-27]. However, the gene involved has not been identified yet.

Chromosome band 8p23.1 is known to be a frequent site of chromosomal rearrangements mediated by low copy repeats (LCRs) or segmental duplications (SDs). It has been described that as many as one in four individuals from the general population carry a 4.7 Megabase (Mb) inversion of the region [28-30]. A high density of genes are present in the region, and copy number variability involving both α-defensin (*DEFA1* and *DEFA3*) and β-defensin (*DEFB4*, *DEFB103* and *DEFB104*) genes has been well detected and characterized [31-34].

The objective of the present work was to analyse in detail the contribution of different 8p23.1 genetic elements to the phenotypic variability observed in deaf patients with mitochondrial 12S rRNA A1555G mutation. The analysis has focused in three different genomic features: *DEFA3* gene absence, claudin23 (*CLDN23*) mutational analysis and the putative function of a ribosomal mitochondrial protein pseudogene (*MRPS18CP2*).

MATERIALS AND METHODS

Patients and samples

Familial cases of sensorineural hearing loss have been collected from different Spanish clinical centres with the aim to study the molecular basis of hearing loss associated to mtDNA A1555G mutation. The analysis was performed on 213 patients, from 55 pedigrees with A1555G mutation and 336 Spanish controls. The Spanish control samples were unrelated blood donor controls, all of Caucasian origin. Informed consent was obtained from all participants prior to their participation in the study, in accordance with the Institutional Review Board and Ethic Committee.

Clinical information such as the severity and age of onset of hearing impairment, the exposure to some kind of ototoxic substances, specifically aminoglycosides, and any other medical diagnoses were evaluated from at least one member of each pedigree.

Detection of A1555G mutation

The detection of the A1555G mutations was either performed by PCR amplification of a 340-bp fragment (Forward 5'-GCTCAGCCTATATACCGCCATCTTCAGCAA-3' and Reverse 5'-TTTCCAGTACACTTACCATGTTACGACTTG-3'), followed by the digestion with restriction endonuclease *Hae*III, or alternatively using Pyrosequencing™ technology (PSQ96MA) (Biotage AB, Sweden). A specific SNP assay was designed for Pyrosequencing (Forward 5'-CGACATTTAACTAAAACCCCTACGC-3', Reverse 5'-GTTGGGTGCTTTGTGTTAAGCT-3' and Sequencing 5'-CACTTACCATGTTACGACT-3' primers) and sequence identification was performed automatically by the SQA software.

DEFA3 determination

A PCR amplification assay followed by restriction enzyme digestion (PCR-RFLP) has been used to discriminate *DEFA1* and *DEFA3* gene alleles differing by a single nucleotide. A fragment of 304 bp around C3400A PSV was PCR amplified with fluorescently labelled primers (Forward 5'-TGAGAGCAAAGGAGAATGAG-3', Reverse 5'-GCAGAATGCCCAGAGTCTTC-3') and digested with *Hae*III enzyme. About 2 μl of digestion product was added to 10 μl HiDi formamide containing ROX500 marker (Applied Biosystems) and run on an ABI 3100 capillary system (Applied Biosystems). Peaks were analysed using Genemapper software (Applied Biosystems).

Mutational screening

The genetic screening of *CLDN23* gene and *MRPS18CP2* pseudogene was performed by direct sequencing. The entire coding sequence of *CLDN23* gene was PCR-amplified in two different fragments of 483 bp (Forward 5'-CCAGGAGGGAACTAGCCTAA-3' and Reverse 5'-AGCGAGGTGACCATGAGTG-3') and 679 bp (Forward 5'-GACGAGCCCAACTTCGTG-3' and Reverse 5'-AGGCAGATTTCCATCCACAC-3'). The *MRPS18CP2* pseudogene was PCR amplified in a single fragment spanning 543 bp (Forward 5'-CTCTGTTTACAGAAGACCTGG-3', Reverse 5'-TTTTAATCTAAAATCCATGTAGCAAA-3'). The resulting PCR products were sequenced using an ABI PRISM® 3730*xl* DNA Analyzer and ABI PRISM® BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems).

Analysis of *MRPS18CP2* expression

Analysis of *MRPS18CP2* expression was assessed by RT-PCR. We used total RNA isolated from lymphoblastoid cell lines of general population subjects as well as total adult RNA from ovary, liver, spleen, lung, placenta, kidney, thymus, heart, skeletal muscle, testes, colon (Stratagene) and brain (Ambion). We employed 1µg of total RNA for reverse transcription using SuperScript First Strand Synthesis System (Invitrogen). Reverse transcribed RNA was then PCR amplified using specific primers for *MRPS18CP2* (Forward 5'-TGTTACAACCTTTAGGGTCCTTG-3', Reverse 5'-AGAGGTTGTTCACAATATAAAC-3').

Statistical analysis

To compare the proportion of *DEFA3* absence in the different groups, between groups chi-square test was performed. Family based association tests were performed using FBAT package [35]. FBAT decomposes large pedigrees into individual nuclear families, which are treated as independent in most of the calculations. The analysis was performed with 111 nuclear families, which belong to 33 large pedigrees, from which we have detailed phenotypic information and were suitable for being analysed with FBAT package. The phenotype of subjects with a reported aminoglycoside exposure was considered unknown. Bonferroni correction was used to account for multiple testing, correcting for the number of tests performed by the FBAT software.

RESULTS

A α-defensin cluster is located in the positive linkage region on chromosome 8p23.1

Bykhovskaya and colleagues identified chromosome 8p23.1 as a major modifying locus for hearing loss phenotype associated to A1555G mutation [26, 27]. Neither a gene nor a genetic factor has been found to be involved in the phenotype associated with A1555G mutation, regardless of intensive screening of the region.

Genomic organization of chromosome 8p23.1 is characterized by the existence of blocks of segmental duplications flanking the region, which are known to mediate a 4.7 Mb inversion [28]. The microsatellite markers with highest lodscores in the linkage analysis are located telomerically with respect to the inverted region and within a cluster of α-defensin genes (Figure 1).

The α-defensin cluster consists of five α-defensin genes (*DEFA6*, *DEFA4, DEFA1*, *DEFA3* and *DEFA5*), five α-defensin pseudogenes (*DEFA8P*, *DEFA9P*, *DEFA10P*, *DEFA11P* and *DEFA7P*) and one θ-defensin pseudogene (*DEFT1P*) [36]. Three copies of a 19-kb repeat unit or copy number variant (CNV) were identified within the α-defensin cluster, which correspond to the *DEFA1A3* CNV (based on May 2004 genome assembly). Each of the 19-kb repeats contained a copy of the *DEFA1* or *DEFA3* genes, but *DEFA3* gene is known to be completely absent in a significant proportion of the population [32, 34]. The description of these genomic features is of high relevance for the search of genetic modifying factors for A1555G mutation. Both, the presence of the polymorphic inversion and the CNVs involving the α-defensin gene cluster could influence the phenotypic manifestation of deafness linked to A1555G mutation.

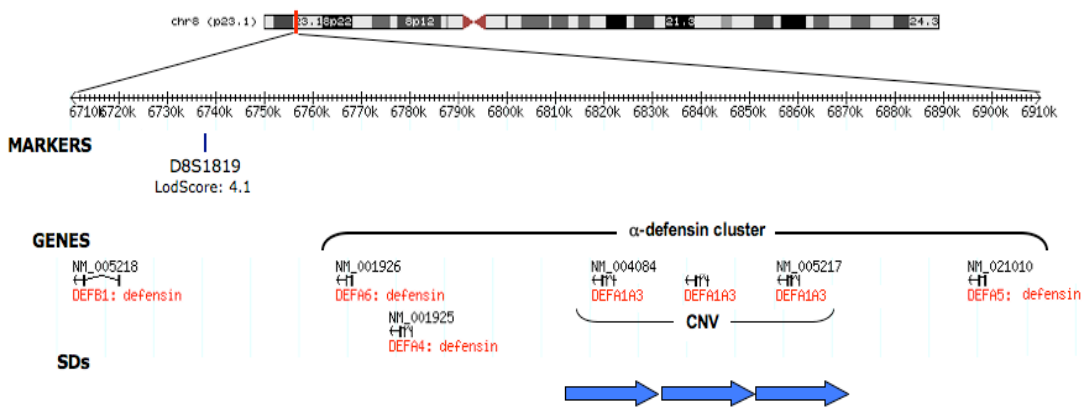


Figure 1. Schematic representation of the α-defensin cluster on chromosome 8p23.1. The marker with higher lodscore in the linkage analysis is localized as well as all the genes in the region and the segmental duplications (positions are based on hg17, May 2004 genome assembly).

With the aim to investigate the role of *DEFA3* absence in the phenotypic manifestation of A1555G mutation, we analysed the absence of *DEFA3* gene in a group of 55 hearing impaired families or sporadic subjects with A1555G mutation (213 subjects; 135 deaf and 78 hearing) and 336 unrelated blood donor controls, all of Caucasian origin. Twenty-one of the families analysed were previously included in the whole-genome linkage analysis performed by Bykhovskaya and colleagues [26, 27]. In this study, the families with non-parametric lodscore (GeneHunter) above 0.8 were considered linked to chromosome 8p23.1, and below 0 unlinked. Using these criteria, seven of the families tested (55 subjects; 31 deaf and 24 hearing) were considered linked to 8p23.1 and 14 (48 subjects; 30 deaf and 18 hearing) considered unlinked.

Table 1. *DEFA3* gene absence in A1555G and control subjects.

SAMPLES	Phenotype	*DEFA3*	NO *DEFA3*	p-value*
A1555G carriers (n=213)	Deaf (n=135)	115 (85%)	20 (15%)	0,678
	Hearing (n=78)	69 (88%)	9 (12%)	
A1555G carriers (n=213)	Deaf & Hearing	184 (86%)	29 (14%)	0,283
A1555G index cases (n=55)	Deaf	45 (82%)	10 (18%)	0,697
A1555G linked samples (n=55)	Deaf & Hearing	52 (95%)	3 (5%)	0,171
Controls (n=336)		294 (87,5%)	42 (12,5%)	

* Between groups chi-square p-value resulting from the comparison of deaf vs hearing carriers or carriers vs. control population subjects.

The frequency of individuals lacking *DEFA3* in a control population was determined. A group of 336 subjects were tested for the absence of *DEFA3*, and found 42 individuals in whom *DEFA3* gene was absent (12.5%). No differences were found in the rate of *DEFA3* absence between deaf and hearing subjects in any of the situations considered: whole set of families, index cases versus control population individuals or subjects from families linked to 8p23.1 region versus controls (Table 1). The data were also analysed using a family based association test [35] under a recessive mode of inheritance, as *DEFA3* complete absence is the only situation which could be unambiguously determined with our assay. In this case, an over-representation of *DEFA3* absence was found in the affected group ($Z = 2.36$; $p = 0.018$) (Table 2). No distinction between linked and unlinked families was possible in this case, because of lack of statistical power to perform the calculations, as FBAT is based in the analysis of large sample groups.

Table 2. Family based association study of *DEFA3* gene absence in A1555G families.

Marker	Genotype	Freq	Fam#	S	E(S)	Var(S)	Z	P
DEFA3	present	0.689	9	8.00	6.83	2.69	0.71	0.477
	absent	0.311	9	11.00	6.67	3.36	2.36	0.018**

[#] Number of informative families; S, observed transmission of genotype to affected offspring; E(S), expected transmission under Mendelian inheritance; Var(S), variance; P, two-tailed P value; **Significant P value after Bonferroni correction ($P < 0.025$).

***CLDN23* gene is not involved in the phenotypic manifestation of A1555G mutation.**

Claudins are a multigene family consisting of more than 20 members. They function as cell adhesion molecules working at tight junctions. An important function in the inner ear has been postulated for several claudin genes [37, 38]. Taking into account the function of other claudin family members and the fact that *CLDN23* gene is located nearby (1,8 Mb) the defined linkage region in chromosome 8p23.1, it was selected for mutational screening as a modifier candidate gene for A1555G deafness phenotype.

Sequencing of *CLDN23* gene coding sequence and flanking regions in A1555G pedigrees, resulted in the identification of eight sequence variants or polymorphisms, five of them already reported in public databases (Figure 2A). Three of the changes resulted in an aminoacid change, but none of them was identified in homozygosis, neither the variants were found to segregate with the phenotype in the pedigrees where they were identified. In addition, a deletion of 12 bp in the 5' untranslated region of the gene was identified in heterozygosis in one deaf sample. However, the pedigree was not informative enough to state whether it has a role in the deafness phenotype.

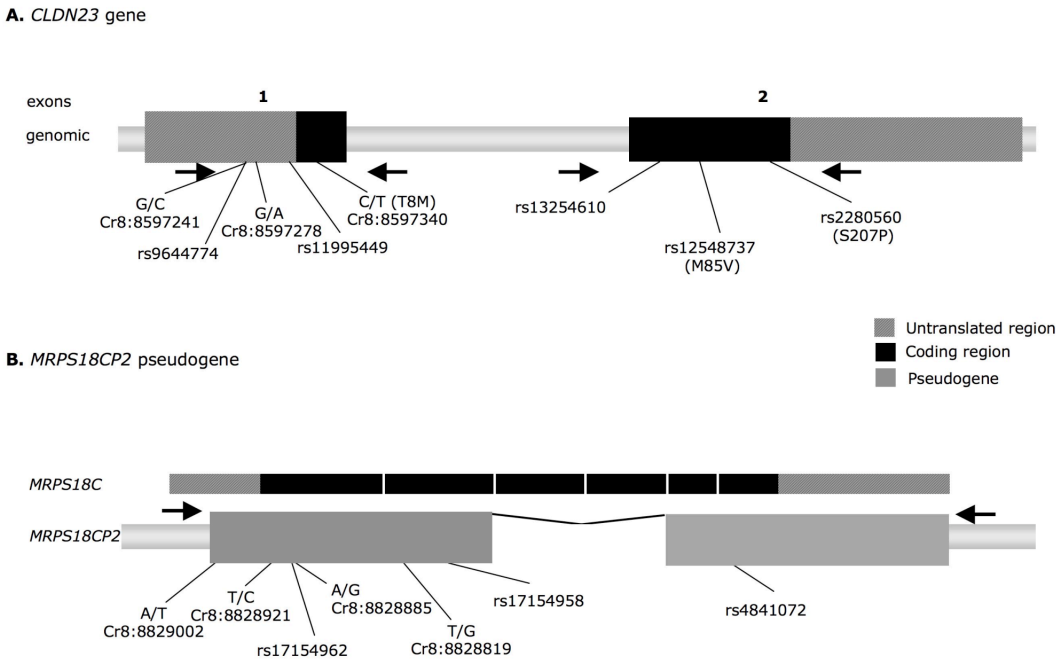


Figure 2. Genetic variants identified in *CLDN23* gene **(A)** and *MRPS18CP2* pseudogene **(B)**. The rs entry for the previously described SNPs or the nucleotide positions for the new ones are given. Arrows represent the position of the primers used for the PCR amplification of the corresponding genomic fragments.

Although none of the variants segregated with the deafness phenotype in the analysed families, to completely rule out the involvement of *CLDN23* gene as a

modifying factor for A1555G mutation, a family based association test was performed (Table 3). The test could be only performed for two of the variants, as the others were found in a small number of samples. No significant association was found for any of the SNPs comparing the expected vs. observed transmission of each possible genotype (Table 3).

Table 3. Family based association study of *CLDN23* and *MRPS18CP2* SNPs in A1555G families.

Gene	Marker	Genotype	Freq	Fam#	S	E(S)	Var(S)	Z	P
CLDN23	rs9644774	GG	0.38	5	4.00	4.98	1.32	-0.82	0.41
		GA	0.38	10	7.00	7.40	2.93	-0.24	0.81
		AA	0.24	8	7.00	5.65	2.21	0.91	0.36
	rs11995449	GG	0.56	6	7.00	6.17	2.06	0.58	0.56
		GA	0.36	7	5.00	6.83	2.44	-1.17	0.24
		AA	0.08	2	*****				
MRPS18CP2	rs4841072	AA	0.38	8	13.00	8.78	3.48	2.26	0.02*
		AC	0.31	9	6.00	9.28	4.03	-1.63	0.10
		CC	0.31	3	*****				
	rs17154962	CC	0.85	5	4.00	6.28	2.00	-1.60	0.10
		CT	0.15	5	7.00	4.55	2.17	1.67	0.09
		TT	0.00	2	*****				

SNPs with less than 5 informative families were excluded from the analysis.
[#] Number of informative families; S, observed transmission of genotype to affected offspring; E(S), expected transmission under Mendelian inheritance; Var(S), variance; P, two-tailed P value; *Significant p value <0.05.

A mitochondrial ribosome protein pseudogene located in 8p23.1 region is expressed in humans.

Pseudogenes, in the case of protein-coding genes, are gene copies that have lost the ability to code for a protein. A processed pseudogene, i.e. made through mRNA retrotransposition, derived from mitochondrial ribosomal protein S18C gene (*MRPS18C*) was identified 2 Mb centromerically from the D8S1819 marker in chromosome 8p23.1. The *MRPS18CP2* pseudogene on chromosome 8p23.1 expands 293 bp, corresponding to the whole coding region of exons 1, 2, 5 and 6 of *MRPS18C* gene, but lacking all introns and exons 3 and 4. *MRPS18CP2* pseudogene shares 96,9 % homology with *MRPS18C* nucleotide coding sequence. There are 13 nucleotide alterations and a 6 bp deletion compared to *MRPS18C* gene (Figure 3A).

Despite lacking the original promoter, a processed pseudogene can occasionally be transcribed [39]. In the public databases, neither mRNAs nor ESTs are annotated for *MRPS18CP2* pseudogene in chromosome 8p23.1. To check whether *MRPS18CP2* is transcribed, its expression was assessed by RT-PCR experiments using total RNA from different human tissues, human lymphoblastoid cell lines and mouse brain. A transcript containing *MRPS18CP2* was found to be expressed in all tested tissues, except for human kidney and skeletal muscle and mouse brain (Figure 3B).

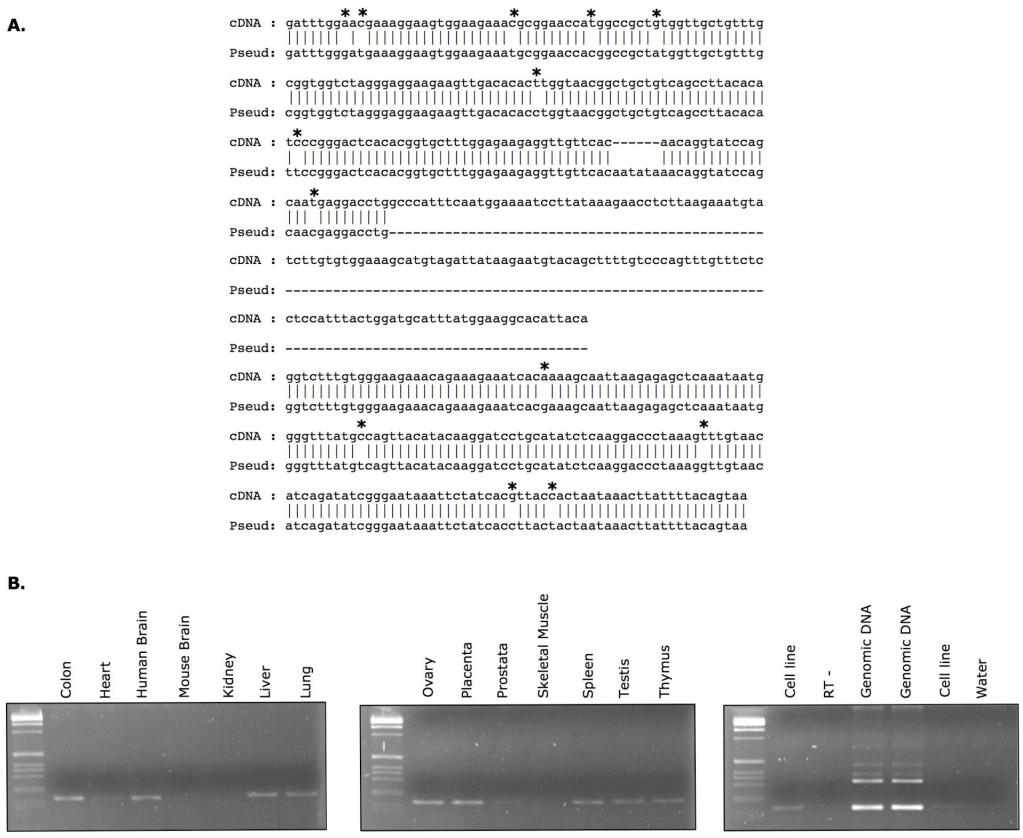


Figure 3. A. Alignment of *MRPS18CP2* pseudogene with *MRPS18C* mRNA (GenBank accession number NM_016067). Asterisks indicate sequence changes between the gene mRNA and the chromosome 8p23.1 pseudogene. **B.** RT-PCR experiments showing expression of a transcript containing MRPS18CP2 pseudogene in different tissues.

Based on the physical localization of *MRPS18CP2*, its expression pattern and the function of its corresponding coding gene, *MRPS18CP2* was selected for a genetic screening as a candidate to be involved in the phenotypic manifestation of A1555G mutation. The mutational screening of *MRPS18CP2* pseudogene in A1555G pedigrees resulted in the identification of seven polymorphisms, three of them already reported in public databases (Figure 2B). None of the SNPs segregate with the deafness phenotype in any of the A1555G pedigrees analysed. A family based association analysis was also performed for the two informative SNPs identified (Table 3). In the case of SNP rs4841072, an overtransmission of the AA genotype ($Z = 2.26$; $p = 0.02$) was found associated to the disease, although after Bonferroni correction statistical significance was no longer supported (Table 3).

DISCUSSION

Large-scale chromosomal rearrangements, such as duplications, deletions and inversions, are now known to be common in the human genome [40]. The substrates for these common rearrangements are generally highly homologous sequences, known as segmental duplications or LCRs, which flank the rearranged genomic segment [41]. To take into account genomic structural variation is crucial in linkage studies of human diseases for different reasons. First, when a fixed marker order is assumed for all individuals in an inverted region, one tends to see spurious recombination events among inversion carriers and/or to find genotyping contradictions, which may lead to discard some observations. In addition, the polymorphic genomic structure of the rearranged regions, which apart from large-scale genomic rearrangements can include sequences that vary in copy number, might difficult the mapping of putative disease genes. Chromosome 8p23.1 is such a region where a common neutral inversion mediated by clusters of olfactory-receptor genes, is present in a variable proportion of subjects, depending on the population [28-30]. The position of a major nuclear modifier gene for the deafness phenotype linked to A1555G mtDNA mutation has been localized in chromosome 8p23.1 [27], but the identification of this gene has remained elusive. This lack of progress may be partially explained because of 8p23.1 genomic organization.

In an attempt to further study the putative genetic modifying factors for A1555G mutation, including those derived from the presence of segmental duplications, we have performed a detailed analysis of three 8p23.1 candidate genetic features: *CLDN23* gene, *MRPS18CP2* pseudogene and *DEFA3* gene absence. *CLDN23* gene and *MRPS18CP2* pseudogene were selected based on their putative biological role in the inner ear, whereas *DEFA3* gene absence was tested due to its close location to the marker with a higher lodscore.

Claudins are essential components of tight junctions [42] and therefore, they play important roles in the physiological function of the inner ear. Tight junctions are well developed in the epithelial cell layers that delineate the inner ear compartments containing perilymph and endolymph, to prevent intercellular leakage of solutes and ions [43]. In fact, mutation of the *Claudin-14* gene was reported to cause human hereditary deafness [38] and *Claudin-11* null mice exhibit severe deafness associated with low endocochlear potential [37]. In addition, at least 10 species of claudins are expressed in the inner ear [44].

Pseudogenes are nonfunctional sequences of genomic DNA originally derived from functional genes [45]. The human genome encodes at least 79 mitochondrial ribosomal proteins from which more than 100 pseudogenes have been identified

[46]. Located on chromosome 8p23.1, there is *MRPS18CP2*, a processed pseudogene of mitochondrial ribosomal protein S18C (MRPS18C). Five other pseudogenes derived from *MRPS18C* gene are located in the human genome on chromosomes 3q26.1, 8p21.3, 12p13.31, 15q11.2 and 22q13.31 respectively [46]. Interestingly, the *MRPS18C* pseudogene on chromosome 15q11.2 is located only 1-Mb apart from a microsatellite marker, which gave a positive linkage score in the analysis performed by Bykovskaya and colleagues [26]. It has been postulated that pseudogenes may play regulatory roles for the genes which they have been derived, such as serving as a source of antisense RNA [45]. Taking all these evidences into account and regardless that the functional role of pseudogenes is not clear, *MRPS18CP2* was considered a good candidate.

None of the identified SNPs in either *CLDN23* or *MRPS18CP2* segregate with the phenotype in A1555G families, but as modifying factors are likely to be multiple [25, 26], this observation did not provide enough evidence to completely discard their contribution in the A1555G deafness phenotype. Thus, a family-based association test was used to analyse the genotype data from *CLDN23* gene and *MRPS18CP2* pseudogene. Family-based association designs are particularly attractive, since they test for linkage as well as association, avoid spurious associations caused by admixture of populations, and are convenient for investigators interested in refining linkage findings in family samples [35]. With this approach, a weak positive association with a single SNP in *MRPS18CP2* pseudogene was found.

These results, although have to be taken with caution, are of great interest as they may suggest a possible role for *MRPS18CP2* pseudogene. Three sequence variants have been found for MRPS18 protein of the small mitochondrial ribosome subunit. In analogy to bacterial ribosomes, it is likely that each mitochondrial ribosome contains a single copy of MRPS18. Therefore, the presence of three different isoforms suggests that there is a heterogeneous population of mitochondrial ribosomes, which may have different decoding properties and may be subjected to a precise regulation of its expression [47]. The existence of MRPS18 pseudogenes could play a role in the regulation of each isoform expression, for example by blocking the expression of the corresponding gene. If this is demonstrated, it could explain the tissue specificity of A1555G homoplasmic mtDNA mutation, leading to a clinical phenotype confined in the cochlea. Thus, additional studies involving typing of additional SNPs in gene-coding and regulatory regions in additional A1555G families are needed, especially in the case of pseudogenes, whose putative biological function is still unclear.

CNVs have been proposed to have an important role in the pathological variation in the human population [48]. The *DEFA1A3* CNV is located within the region previously described to contain a major modifying locus for mutation A1555G [32, 34]. On the premise that the presence of a gene in multiple copies could have a dosage effect and therefore, contribute to genetic basis of some complex disorders, it is feasible that the copy number polymorphism of α-defensin cluster could be involved in the pathogenesis associated to the A1555G mutation. When a family based association was performed, an overrepresentation of *DEFA3* gene absence was found in deaf A1555G carriers. It is difficult to establish a direct relationship between defensin function and A1555G deaf phenotype, as defensins are small cationic peptides that form an important part of the innate immune system. However, as the distinction between *DEFA1* and *DEFA3* is based on the typing of a single SNP (C3400A), the differences in the rate of *DEFA3* gene absence observed between deaf and hearing carriers of A1555G mutation could also be considered as a positive association signal that confirms the localization of a modifier factor.

In summary, both positive results found in *MRPS18CP2* pseudogene and *DEFA3* gene absence within the deaf group of A1555G carriers are weak associations, that do not demonstrate a role in the phenotype linked to A1555G mtDNA mutation. However, they give further evidences of the involvement of 8p23.1 region as a modifying factor for A1555G mutation. Further analyses in additional families as well as functional studies, which should shed light on the function of these genetic features, are needed in order to confirm or discard the associations found.

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Inter-population variability of DEFA3 gene absence: correlation with haplotype structure and population variability

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The aim of this work was to analyse *DEFA3* gene absence on human chromosome 8p23.1, in different human populations. The α-defensin cluster on human chromosome 8p23.1 is one of the better-characterized CNVs, in which high copy number variability affecting the *DEFA1* and *DEFA3* genes has been reported.

We tested 697 samples from HapMap populations and Spanish general population subjects and found that the proportion of subjects lacking *DEFA3* vary from 10% to 37%, depending on the population tested. These results suggest differences in innate immune function between populations. Absence of *DEFA3* was correlated with the region's haplotype block structure. African samples showed a higher intra-populational variability together with the highest proportion of subjects without *DEFA3* (37%). Association analysis of *DEFA3* gene absence with 136 SNPs from a 100-kb region identified a conserved haplotype in the Caucasian population, extending for the whole region.

CNVs involving immune genes, such as α-defensins, are possibly contributing to innate immune differences observed between individuals and influencing predisposition and susceptibility to disease. The identification of population differences in subjects lacking the *DEFA3* gene may be suggestive of population-specific selective pressures with potential impact on human health.

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Inter-population variability of DEFA3 gene absence: correlation with haplotype structure and population variability

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Abstract

Background: Copy number variants (CNVs) account for a significant proportion of normal phenotypic variation and may have an important role in human pathological variation. The α-defensin cluster on human chromosome 8p23.1 is one of the better-characterized CNVs, in which high copy number variability affecting the *DEFA1* and *DEFA3* genes has been reported. Moreover, the *DEFA3* gene has been found to be absent in a significant proportion of control population subjects. CNVs involving immune genes, such as α-defensins, are possibly contributing to innate immunity differences observed between individuals and influence predisposition and susceptibility to disease.

Results: We have tested the *DEFA3* absence in 697 samples from different human populations. The proportion of subjects lacking *DEFA3* has been found to vary from 10% to 37%, depending on the population tested, suggesting differences in innate immune function between populations. Absence of *DEFA3* was correlated with the region's haplotype block structure. African samples showed a higher intra-populational variability together with the highest proportion of subjects without *DEFA3* (37%). Association analysis of *DEFA3* absence with 136 SNPs from a 100-kb region identified a conserved haplotype in the Caucasian population, extending for the whole region.

Conclusion: Complexity and variability are essential genomic features of the α-defensin cluster at the 8p23.1 region. The identification of population differences in subjects lacking the *DEFA3* gene may be suggestive of population-specific selective pressures with potential impact on human health.

Background

Defensin genes encode a family of small cationic peptides that act as antimicrobial mediators of the innate immune system [1]. Defensins are arginine-rich peptides and invariably contain disulfide-linked cysteine residues, whose positions are conserved [2]. The two main defensin subfamilies, α- and β-defensins, differ in the length of the peptide segments between cysteine residues and in the arrangement of disulphide bonds that link them. β-defensins have been found in most vertebrate species, whereas α-defensins are specific to mammals [3]. Based on their adjacent chromosomal location, similar precursor peptides and gene structures, it has been postulated that all vertebrate defensins arose from a common gene precursor [4]. While the efficacy of individual defensins against specific infectious agents varies, they have shown

antimicrobial activity against gram-negative and gram-positive bacteria, fungi and enveloped viruses [1,5]. At high concentrations, some defensins are also cytotoxic to mammalian cells, as cells exposed to high amounts of defensins in inflamed tissues generate pro-inflammatory signals that can contribute to tissue injury [1]. In humans, most of the genes encoding α- and β-defensins are located in clusters on chromosome 8p23.1 [6,7]. Within the region, two different defensin clusters can be distinguished: a telomeric cluster mostly containing α-defensin genes (*DEFB1*, *DEFA6*, *DEFA4*, *DEFA1*, *DEFT1*, *DEFA3* and *DEFA5*) and at least two centromeric clusters of β-defensin genes (*DEFB109p*, *DEFB108*, *DEFB4*, *DEFB103*, *DEFB104*, *DEFB106*, *DEFB105* and *DEFB107*) [7].

Chromosome band 8p23.1 is known to be a frequent site of chromosomal rearrangements mediated by low copy repeats (LCRs) or segmental duplications (SDs). It has been described that as many as one in four individuals from the general population carry a 4.7 Megabase (Mb) inversion of the region [8-10]. In addition, copy number variability involving both α-defensin (*DEFA1* and *DEFA3*) and β-defensin (*DEFB4*, *DEFB103* and *DEFB104)* genes in chromosome 8p23.1 has been well detected and characterized [11-14]. The number of *DEFA1* and *DEFA3* gene copies has been reported to range from 4 to 11 in a sample of 111 subjects, the *DEFA3* allele being completely absent in 10% of them [12]. Gene nomenclature for *DEFA1*, *DEFT1* and *DEFA3* has been replaced by *DEFA1A3*, following recommendations of Aldred et al, since these genes have been considered as being part of a copy number variant (CNV) region [14]. In another study, Linzmeier and colleagues determined copy numbers of the *DEFA1* and *DEFA3* alleles in 27 subjects and found between 5 and 14 copies per diploid genome, with *DEFA3* being absent in 26% of them [14].

Despite *DEFA1* and *DEFA3* being considered as members of the same CNV *(DEFA1A3)*, they encode different peptides, HNP-1 and HNP-3, respectively. The mature HNP-1 and HNP-3 peptides differ only in their N-terminal amino acid, due to a single nucleotide difference, C3400A, between the *DEFA1* and the *DEFA3* genes [15]. This C3400A is a paralogous sequence variant (PSV) that allows discrimination between the two gene copies. The HNP-2 peptide is identical to the last 29 amino acids of both the HNP-1 and the HNP-3 peptides. HNP-2 is presumably produced from proHNP-1 and/or proHNP-3 by post-translational proteolytic cleavage [1]. It is likely that one or both genes, or another member of the *DEFA1A3* CNV cluster encode the HNP-2 peptide. The three peptides are constitutively produced by neutrophil cell precursors and packaged in granules before mature neutrophils are released into the blood. During phagocytosis, the defensin-containing granules fuse to phagocytic vacuoles where defensins act as antimicrobial agents [15].

Recent work has shown that CNVs are a major source of genetic variation [16]. Individual variability in resistance to infectious diseases has been extensively reported [17]. However, the causes of this diversity in immune function are poorly understood. CNVs involving immune genes could contribute to the differences in innate immunity between individuals and influence predisposition and susceptibility to diseases, as it has been shown for human immunodeficiency virus and AIDS [18]. Thus, it is important to analyze the impact of defensin gene CNVs on human health, both in healthy volunteers and in patients with disease [1,19]. In this report we have studied the presence of *DEFA3* in samples from different human populations. For this purpose, we used the International Haplotype Map (HapMap) Project collection and a cohort of Spanish healthy individuals.

Results

Differences in the proportion of DEFA3 *absence between populations*

We have analyzed 786 samples from four populations with ancestry in Europe, Africa or Asia (the HapMap collection), including Spanish healthy individuals. The source used for this study was the HapMap collection of 269 samples utilized by the International HapMap Consortium for the study of human genomic variation, initially through the investigation of SNPs and their associated haplotypes [20], and 180 additional HapMap samples. This collection comprises four populations: 30 parent-offspring trios (90 individuals) of the Yoruba from Ibadan, Nigeria (YRI), 30 parent-offspring trios (90 individuals) of European descent from Utah, USA (CEU), 45 unrelated Japanese from Tokyo, Japan (JPT) and 44 unrelated Han Chinese from Beijing, China (CHB). In addition, 30 Yoruban trios, 45 unrelated Japanese and 45 unrelated Chinese from the HapMap collection, but not genotyped in the HapMap project, were analyzed. The Spanish samples were 336 unrelated blood donor controls, all of Caucasian origin. Genomic DNA from EBV-transformed lymphoblastoid cell-lines was used. As Chinese and Japanese allele frequencies are found to be very similar [20], the analysis was performed combining both datasets, resulting in four different groups of samples tested: two Caucasian groups (CEU and Spanish general population subjects), Yoruba and Chinese/Japanese.

The coding sequence of *DEFA1* and *DEFA3* differs only by a single nucleotide (C3400A), which allows distinguishing between *DEFA1* and *DEFA3* by *Hae*III digestion, since a restriction site for this enzyme is absent in the *DEFA3* sequence. All samples had at least one *DEFA1* copy, but *DEFA3* was absent in several subjects of all populations.

DEFA3 was absent in different proportions depending on the population tested, ranging from 10% in the Chinese/Japanese dataset to 37% in the Yoruba samples (Table 1). There were statistically significant differences for the absence of *DEFA3* when comparing Yoruba samples with each of the other population groups (Table 1) or with the total of non-Yoruban unrelated subjects ($p < 0.001$). As both Caucasian and Yoruba samples are trios, inheritance of the *DEFA3* allele could also be assessed, showing no abnormal segregation in any of the trios analyzed (data not shown).

Segmental duplications and genomic organization of α-defensin cluster

The genomic organization of the α-defensin cluster was precisely defined by PipMaker analysis [21]. For this analysis, a region of 150 kb containing the whole α-defensin cluster on 8p23.1 was used (based on May 2004 human genome assembly). The alignment of the region against itself identified different sequences with high homology, which correspond to six α-defensin genes (*DEFA6*, *DEFA4*, *DEFA1*, *DEFA3* and *DEFA5*), six α-defensin pseudogenes (*DEFA8P*, *DEFA9P*, *DEFA10P*, *DEFA11P* and *DEFA7P*) and one θ-defensin pseudogene (*DEFT1P*) (Figure 1a). Such clustered organization of α-defensin genes is common in other species, suggesting that α-defensin have arisen from a common ancestor by gene duplication followed by diversification [3]. Phylogenetic analysis of all human α-defensin genes and pseudogenes showed that *DEFA5* and *DEFA6* seem to be the ancestral genes. All pseudogenes are clustered together with these two genes, with the exception of *DEFA10P* and *DEFT1P*, which are closely related with *DEFA1* and *DEFA3* (Figure 1b).

Three copies of a 19-kb repeat unit were identified within the α-defensin cluster, which correspond to the *DEFA1A3* CNV, previously reported to be variable in copy number between individuals (Figure 2) [12,14]. Each of the 19-kb repeats contained a copy of the *DEFA1* or *DEFA3* genes, together with a pseudogene, either *DEFA10P* or *DEFT1*. *DEFA10P* and *DEFT1P* have a high sequence identity and are closely related in the phylogenetic analysis, which is in accordance with the theory that primate specific θ-defensins evolved from α-defensins after divergence of the primates from other mammalian species [3] (Figure 1b). Variation in both number and position of *DEFA1* and *DEFA3* alleles has been reported, indicating that these genes are located in interchangeable variant cassettes within tandem gene arrays [12,14]. Thus, the existing diversity in *DEFA1*/*DEFA3* copy number and localization is probably the result of unequal crossing-over events between tandem arrays [12]. Interestingly, multiple copies of *DEFA1*, but not the *DEFA3* gene, can be *in silico* identified in chimpanzee by BLAST sequence similarity searches. On the other hand, in the case of Rhesus macaque the *DEFA5* gene is present in multiple copies, suggesting a different evolutionary pattern driven by the responses to specific microbial challenges [3].

HapMap samples have been tested for the presence of CNVs by two different techniques Affymetrix SNP array and BAC array [22]. *DEFA1A3* region was identified as a CNV in 23 subjects (3 Caucasian, 7 Yoruban, and 13 Chinese/Japanese), but only in four cases where a gain or loss was detected, *DEFA3* is absent. Copy number variation in the *DEFA1A3* region is reported to be much more common than the variation identified by Redon et al [22]. However, the small size of the *DEFA1A3* CNV makes it undetectable with BAC arrays. Moreover, the presence of segmental duplications in the region entails a bad SNP coverage of the region by the Affymetrix SNP array, which does not allow an accurate detection of the CNV. Thus, the study of this CNV for association purposes has to be performed by quantitative methods or by the analysis of paralogous sequence variants.

Patterns of linkage disequilibrium for **DEFA1A3** *in HapMap samples*

A region of 100 kb, spanning from 6,810,001 bp to 6,910,000 bp, which contains the *DEFA1A3* cluster and the single copy gene *DEFA5* was chosen for the linkage disequilibrium analysis (based on human genome assembly hg17) (Figure 2). The HapMap data for the *DEFA1A3* region included around 150 SNPs for each population (151 Caucasian, 169 Yoruba, 158 Japanese and 154 Chinese). However, only 136 of the SNPs had genotype data in all four populations. Interestingly, almost all genotyped SNPs are located outside the *DEFA1A3* cluster (Figure 2). The absence of genotyped SNPs in the *DEFA1A3* cluster is in agreement with the presence of segmental duplications that include the *DEFA1A3* genes. Thus, the non-homogeneous distribution of SNPs within the region could be at least partially explained by the presence of high homologous repeated sequences. Genotyping errors enhanced by the presence of *DEFA1*/*DEFA3* tandem gene arrays could have lead investigators to discard SNPs located within this region.

Of the 136 SNPs analyzed in all four populations, 55 were monomorphic in at least one of them (28 out of the 55 SNPs were monomorphic in all populations). Monomorphic SNPs can be used to measure genetic variability, by analyzing their distribution in the different populations. The Chinese and Japanese groups had the highest proportion of monomorphic SNPs (34%) which was very similar to that observed for Caucasian samples (31%), whereas the Yoruba samples had the smallest number of monomorphic SNPs (24%). This indicates that genetic variability is higher within Yoruba samples, while Chinese/Japanese and Caucasian populations show similar pro-

Table 1: Absence of *DEFA3* in Caucasian, Yoruba, Chinese/Japanese reference HapMap samples and in Spanish control samples

POPULATION*	*DEFA3*	NO *DEFA3*	Paired chi-square p-value		
			Yoruba	Caucasian	Asian
Yoruba (n = 120)	76 (63%)	44 (37%)	-	-	-
Caucasian (n = 60)	51 (85%)	9 (15%)	0.003	-	-
Japanese/Chinese (n = 181)	163 (90%)	18 (10%)	<0.001	0.344	-
Spanish (n = 336)	294 (87.5%)	42 (12.5%)	<0.001	0.538	0.472

*For the analysis of Caucasian and Yoruba samples only the parents of the trios are considered.

portions of genetic variability. This higher variability for Yoruba samples is similar to that detected in the HapMap analysis for the whole genome [20]. Interestingly, the proportion of monomorphic SNPs in this region is about 10% higher for each population group than the average reported for the HapMap data [20].

The patterns of linkage disequilibrium (LD) in each population are summarized in Figure 3. The Yoruba samples show the lowest LD, the greatest variability and smaller haploblocks compared to Caucasian or Chinese/Japanese samples, which have similar patterns of LD. The differences observed in LD patterns between populations are in accordance with *DEFA3* locus absence results; the Yoruba samples showing highest LD variability and also having the highest proportion of *DEFA3* absence.

DEFA1A3 *region haplotype association with* DEFA3 *absence in HapMap samples*

To assess whether *DEFA3* is inherited together with neighbor SNPs, an association study was performed using the HapMap data for the 100-kb region including the *DEFA1A3* cluster. All the SNPs of the region genotyped in the HapMap project were tested for association with the C3400A PSV, which defines the presence or absence of *DEFA3* gene, respectively. No association for any of the genotyped SNPs was found in the Yoruba or Japanese/Chinese populations. However, a significant association was found between absence of *DEFA3* and 18 SNPs in the Caucasian samples, under a recessive mode of inheritance (Figure 4a, Additional file 1). Association between estimated haplotypes within defined LD blocks and the C3400A PSV has also been tested. Again, the Caucasian group was the only one in which significant association was obtained (Figure 4b). Moreover, the associated haplotype spans nearly the whole 100-kb region, indicating a lack of recombination between the LD blocks when *DEFA3* gene is absent. The frequency of *DEFA3* lacking haplotype's would be similar to that estimated by the Haploview program, which varies from 16%–33% depending on the haplotype block (Figure 4b). This estimation correlates well with the observed frequency of *DEFA3* absence in Caucasians (15%).

Discussion

Several studies have recently reported a previously unknown high prevalence of copy number variation in humans [16]. A recent study of CNVs in the HapMap samples has defined over 1400 CNV regions [22]. On average, each individual varies at over 100 CNVs, representing

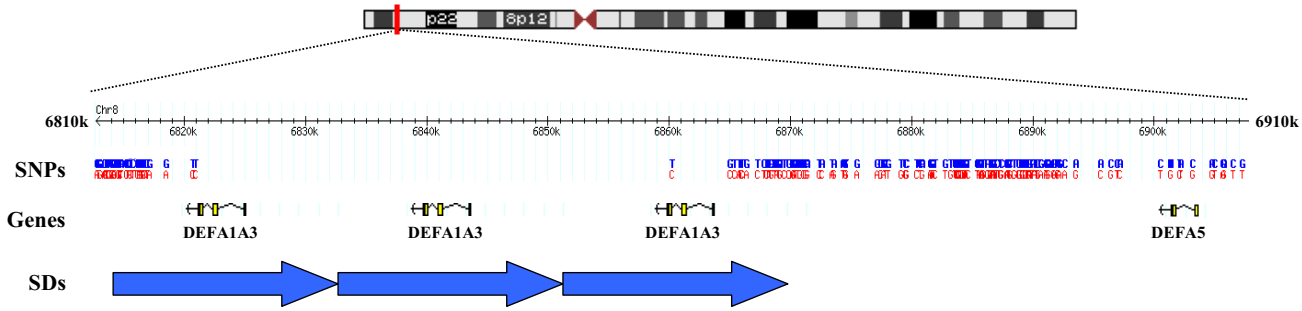

Figure 2
Schematic representation of a 100-kb region of human chromosome 8p23.1 containing the *DEFA1* and *DEFA3* genes. Gene and SNP positions are based on May 2004 genome assembly (hg17), in which three copies of the *DEFA1A3* are annotated. The non-homogeneous distribution of SNPs at the telomeric and centromeric regions of the *DEFA1A3* cluster is clearly seen.

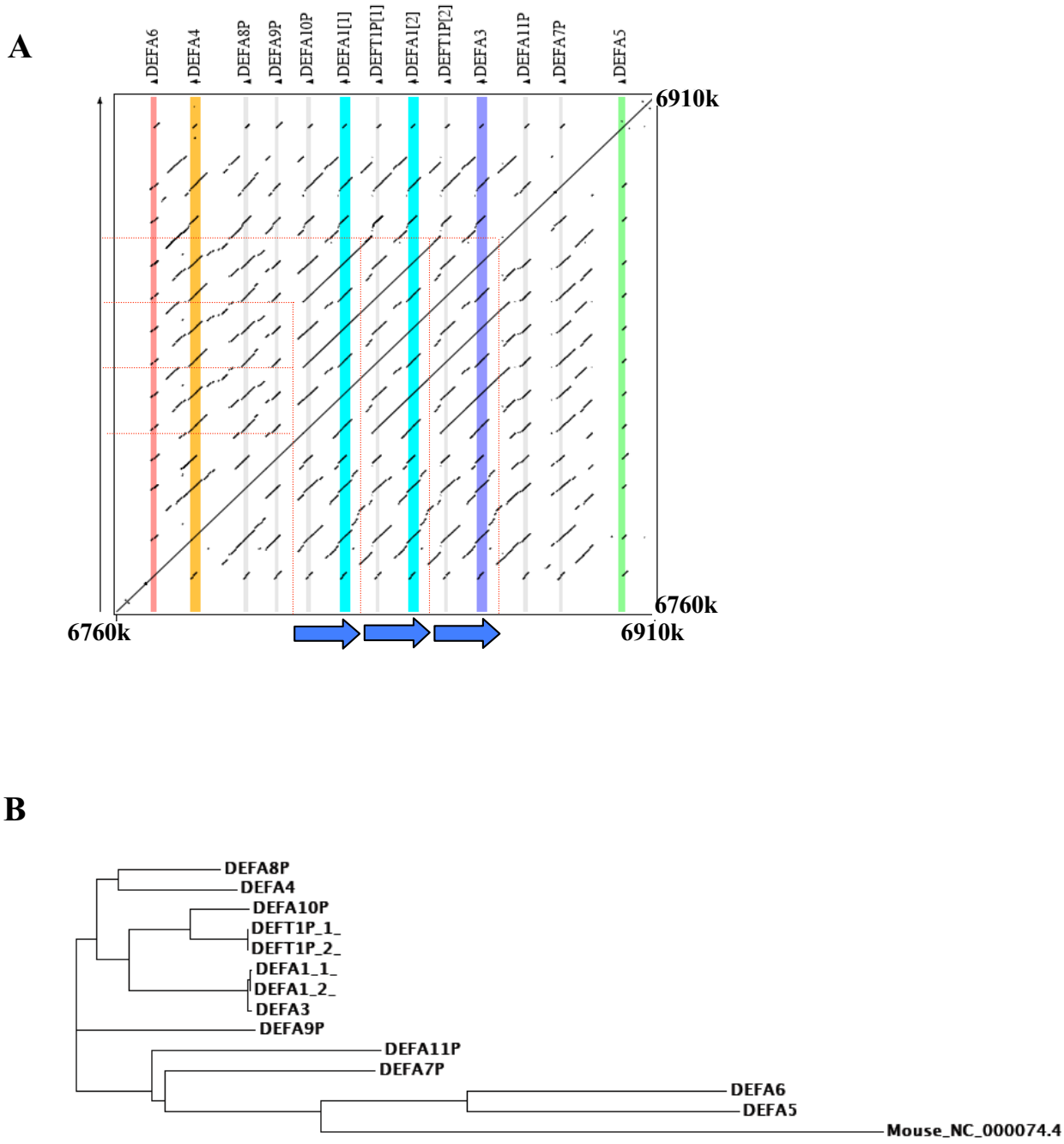

Figure 1
Genomic organization of α-defensin cluster at 8p23.1 region. **A**. Dot-plot of the PipMaker alignment of the 150 kb region containing the α-defensin cluster. The high density of segments showing alignment is due to the presence of defensin genes and pseudogenes, sharing a common genomic structure. Vertical coloured lines represent α-defensin genes and grey lines correspond to pseudogenes localizations. The 19 kb duplicons are indicated by arrows. Note that all human defensins are transcribed from the same direction. **B**. Phylogenetic tree of human α-defensins. Mouse ortholog of human *DEFA1* gene was included as a root.

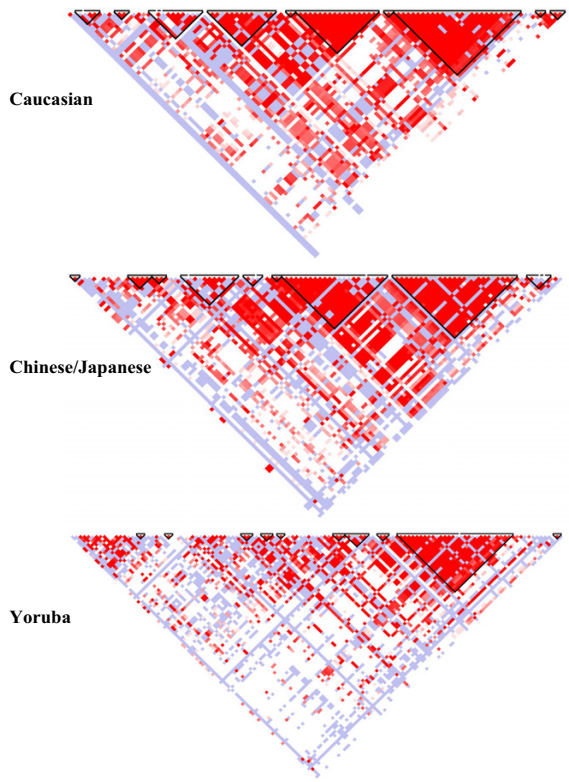

Figure 3
Haplotype blocks in the 100-kb region of *DEFA1A3* cluster on human chromosome 8p23.1 generated by Haploview in Caucasian, Japanese/Chinese and Yoruba populations.

about 20 Mb of genomic DNA difference. It has been suggested that CNVs account for a significant proportion of human normal phenotypic variation. It is thought that CNVs may also have an important role in the pathological variation in the human population [16,23]. Analyses of the functional attributes of currently known CNVs reveal a remarkable enrichment for genes that are relevant to molecular-environmental interactions and genes that influence response to specific environmental stimuli, such as genes involved in immune response and inflammation [16].

CNVs involving α- and β-defensin genes (*DEFA1A3* and *DEFB4/DEFB103A)* in the 8p23.1 region have been extensively characterized [12-14]. From a pathologic point of view, it is likely that α- and/or β-defensin CNVs affect the function and effectiveness of innate immunity. Such effects could be influenced by the frequent absence of the *DEFA3* allele. In the present work, we have tested the absence of the *DEFA3* allele in different human populations, finding significant differences between them, which could be indicative of differences in innate immune function between populations. This is not surprising since the different human population groups have been exposed to different environments regarding infectious agents and other factors. One obvious way by which CNVs result in human phenotypic diversity is by altering the transcriptional levels of the genes which vary in copy number [16]. In addition, it has been postulated that retention of duplicate genes, rather than mutation to pseudogenes or neofunctionalization, is due to the generation of increased amounts of a beneficial product [24]. This could be the case of *DEFA1A3* in which variation in *DEFA1* and *DEFA3* copy number, and *DEFA3* absence could underlie variable resistance to infection among individuals. Different selective pressures acting in each geographic region could likely explain population differences in DEFA3 absence.

Taudien and colleagues by manual clone-by-clone alignment significantly improved the assembly of defensin 8p23.1 locus, providing *in silico* evidences of the experimentally verified variability in defensin copy number and better representing the locus diversity [7]. The exceptional genomic complexity and heterogeneity of the human 8p23.1 locus and the prominent role of defensins in the innate immunity framework raise the question of whether individual patterns of haplotypes, together with the variability in defensin genes copy number, affect the functionality of the defensin system. To address this issue, Taudien et al provided a molecular approach for the determination of individual defensin gene repertoires limited to 8p23.1 β-defensin clusters and using data from a 500 bp fragment in 4 individuals [7]. In our case, we have characterized in detail the haplotype diversity and LD structure of a 100-kb region around α-defensin locus in 269 HapMap samples. The SNP distribution of the region is characteristic of the presence of segmental duplications, which result in a low-density of SNPs selected for genotyping. As previously reported for other genomic regions [25], the Yoruba samples present a higher variability than both the Chinese/Japanese and Caucasian samples. Additionally, in the Yoruban, the haploblock structures were smaller and the extent of LD between SNPs was lower, in accordance with the out-of-Africa theory for the origins of humans. The observation that the proportion of subjects lacking the *DEFA3* gene is greater in Yoruba samples together with the fact that *DEFA3* is thought to be human specific [12] may be an indication of the higher amount of original genetic variation among the first humans living in Africa, which afterwards migrated to other continents. The initial migration occurred as multiple, branching events and involved many founder effects in which certain haplotypes, SNPs and alleles appear to have increased in frequency in emigrant populations owing to genetic drift and different selection pressures [25]. In this sense, we observed a

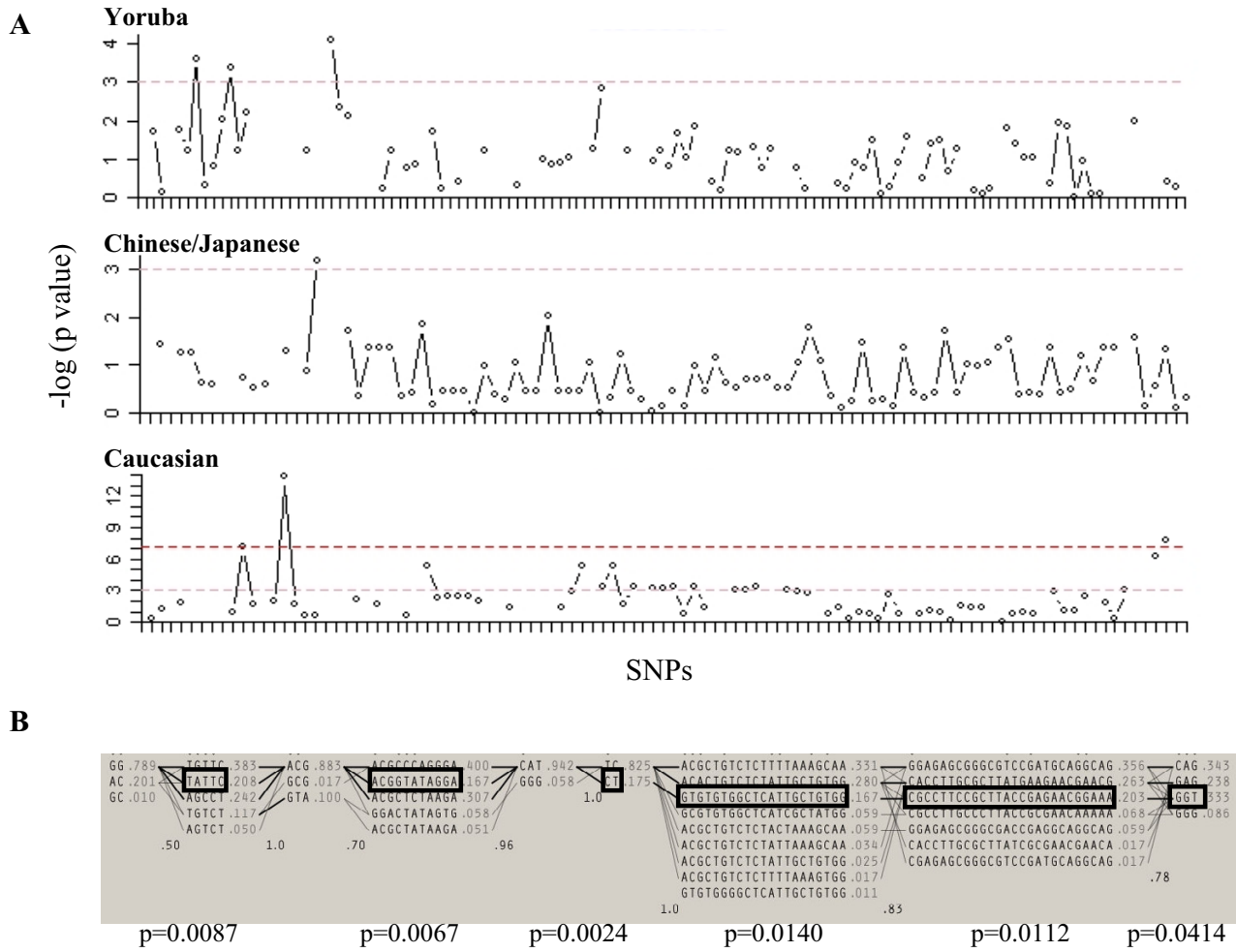

Figure 4
Association of SNPs in the 100-kb region of *DEFA1A3* cluster with the absence of the *DEFA3* gene. **A**. Diagram with the association results testing each SNP with *DEFA3* absence under a recessive mode of inheritance. The red line indicates significance after Bonferroni correction. Positive association was only detected in Caucasian population. **B**. Haplotype blocks defined by the haploview program in the Caucasian population. The estimated frequency of each haplotype is depicted on its right hand side. The boxes represent the haplotype found associated to *DEFA3* absence and the corresponding p-value is shown.

diminished frequency of subjects without *DEFA3* in Caucasian and Asian samples.

When association with *DEFA3* absence was tested, SNPs and haplotypes in the Caucasian population were the only ones to be significant. The association observed in the Caucasian samples could be the result of strong founder effect. Founder effects and, particularly, the decrease in genetic diversity resulting from continental migrations, are associated with an increased haplotype length [25]. This is observed when comparing the haplotype block patterns of the different populations analyzed, in which the Caucasian samples set has the longest haplotype blocks. Alternatively, Aldred and colleagues demonstrated that *DEFA3* has arisen at the 5' end repeat position and has transferred to other positions within the array through unequal recombination between alleles [12], suggesting that recombination has been active in shaping diversity in the *DEFA1A3* locus. However, our results indicate that, at least in the Caucasian samples, there has been little recombination between chromosomes with and without *DEFA3*, as we are able to find a haplotype associ-

ated with *DEFA3* absence extending for nearly 100-kb. Moreover, as for *DEFA3* absence, other haplotypes are likely to be associated with other patterns of CNV polymorphisms. However, other situations cannot be rule out without analyzing large pedigrees to determine unambiguously each chromosome structure at DEFA1A3 CNV.

The impact on human health of this qualitative variation in the presence of the *DEFA3* gene product deserves to be explored in epidemiologic studies. Different studies have described differences in the function and specificity of *DEFA1* and *DEFA3* gene products, HNP1 and HNP3 [1,19]. In general, HNP3 is thought to be less active than HNP1 against both gram-positive and gram-negative bacteria [26], but it is expressed at about twice the level of HNP1 [12]. On the other hand, *DEFA3* but not *DEFA1*, has been found upregulated in patients with systemic lupus erythematosus, idiopathic thrombocytopenic purpura or rheumatoid arthritis, suggesting that *DEFA3* upregulation might be a general feature of autoimmune diseases [27,28]. Therefore, the observed differences in *DEFA3* absence may partially explain the different population incidences of infectious and/or autoimmune diseases in which *DEFA3* plays an important role. Future studies are needed to establish whether patterns of *DEFA3* absence correlate with certain population microbial exposures or different prevalence of autoimmune disorders. This could also be important in determining the exact nature of *DEFA3* function and its specificity of action, if any, against certain antigens. Last, but not least, further studies focused on the determination of the total copy number of *DEFA1A3* units will be crucial to build the complete picture of *DEFA1A3* CNVs' impact on human health.

Conclusion

Complexity and variability are essential genomic features of the α-defensin cluster at 8p23.1 region. The present work gains insight into the existent variability in human populations in this specific region. The identification of population differences in the proportion of subjects lacking the *DEFA3* gene may be suggestive of population-specific selective pressures, which should be studied in further inter-population epidemiological studies.

Methods

Patients and samples

The analysis was performed on 450 HapMap samples and 336 Spanish controls. Unless otherwise noted, all samples were obtained from the Coriell Institute for Medical Research. A detailed description of HapMap populations samples can be found elsewhere [20]. Written informed consent for the Spanish controls was obtained with the approval of the Institute Review Board and Ethics Committee.

DEFA3 determination

A PCR amplification assay followed by restriction enzyme digestion (PCR-RFLP) has been used to discriminate *DEFA1* (GenBank accession number L12690) and *DEFA3* (GenBank accession number L12691) genes differing by a single nucleotide. A fragment of 304 bp around C3400A SNP was PCR amplified with fluorescently labelled primers (Forward 5'-TGAGAGCAAAGGAGAATGAG-3', Reverse 5'-GCAGAATGCCCAGAGTCTTC-3') and digested with HaeIII enzyme. In order to accomplish complete digestion, we used saturating conditions (2.5 U/25 μl reaction) of the enzyme to digest a short DNA fragment containing only one cutting site. In addition, in all the runs, a *DEFA3* negative sample was included, as a positive control of the assay. About 2 μl of digestion product was added to 10 μl HiDi formamide containing ROX500 marker (Applied Biosystems) and run on an ABI 3100 capillary system (Applied Biosystems). Peaks were analysed using Genemapper software (Applied Biosystems).

Characterization of the segmental duplications

The UCSC Genome Browser [29] served as the main source of genomic sequence, using the human genome assembly hg17. The region analysed was a 150 kb contig from 6,760,001 bp to 6,910,000 bp of chromosome 8p23.1 (based on human genome assembly hg17). Sequences were repeat-masked and aligned against itself using PipMaker [21]. The size, orientation and structure of segmental duplications can be interpreted by using the PIP and Dot-Plot output generated by PipMaker. Multiple sequence alignments and phylogenetic tree construction were carried out by using the ClustalW program [30].

Statistical analysis

Between groups chi-square test was performed to compare the proportion of *DEFA3* absence in different human populations. Genotyping data from HapMap public database [31] was used to test the hypothesis of association between geneticpolymorphisms and *DEFA3* absence using logistic regression models. Odds ratios (OR)and 95% confidence intervals (95% CI) were calculated for eachgenotype compared with the homozygous for the major allele (theallele with greater frequency among individuals lacking the *DEFA3* allele). Analyses were initially done under a codominant inheritance model (three genotypes separated). Then, simplified models were fitted: a dominant model (heterozygous grouped with the homozygous for the minor allele), a recessive model (heterozygous grouped with the homozygous for the major allele), an overdominant model (homozygous grouped) and a log-additive model (a score was assigned counting the number of minor alleles: the homozygote for the major allele was given score 0, the heterozygote score 1, and the homozygote for the minor allele score 2). The model with lowest Akaike information criteria was the

recessive one (minus twice the log likelihood of the model plus the number of variables in the model) and it was selected for an easy summary of the results. P values were derived from likelihood ratio tests, and a significance level of 5% (two sided) was used for the analyses. All these analyses were performed using the SNPassoc R package [32].

Haploblocks were constructed using Haploview program [33]. Haplotypes were reconstructed using the expectation maximization (EM) algorithm implemented in the haplo.stats R package [34]. The OR and 95% CI were estimated using a generalized linear-regression framework that incorporates haplotype phase uncertainty by inferring a probability matrix of haplotype likelihoods also implemented in haplo.stats library.

Authors' contributions

EB carried out the genetic molecular studies, the bioinformatics work and drafted the manuscript. JRG carried out the statistical analysis. NB participated in the bioinformatics work and design of the study. XE conceived the study and participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

Results of the association study for the three population groups. The p-values under all inheritance modes tested are shown.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-8-14-S1.pdf]

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Discussion

Discussion

In this section, the previously described results are examined in detail, reasoning which are the main contributions of the present doctoral thesis to the knowledge of mitochondrial deafness, but also considering their weaknesses. Therefore, it provides the basis of the conclusions of the work, presented in the next section.

Following the initial discovery that mutations in the connexin 26 gene are the cause of deafness in a significant proportion of cases in many populations worldwide, it became possible to etiologically identify many deaf cases and give them a more accurate genetic counselling. To date, many other genes have been identified to be involved in the development of non-syndromic hearing impairment. Between them, a mitochondrial mutation in the 12S rRNA gene, the A1555G mutation, has been reported to account for a high proportion of familial cases of late-onset sensorineural hearing loss and aminoglycoside-induced deafness. This is an example of a growing list of genes that determine response to a drug and for which routine genotyping would be recommended prior to certain drug treatment.

Moving mutation detection from the research to the service laboratory remains a challenging task in genetic deafness, as in many other genetic areas. However, this could not be accomplished without an in-depth knowledge of the factors that determine the phenotypic consequences of a given genetic defect, providing at its turn, a much accurate genetic counselling.

Prevalence of mutation A1555G in Spanish population

Mutations in the 12S rRNA gene have been shown to be a common cause of hearing impairment in different populations. The A1555G mutation in the mitochondrial 12S rRNA gene is one of the most common causes of sensorineural hearing loss and

aminoglycoside-induced deafness. This mutation was first discovered in a large Arab-Israeli family and subsequently found in various ethnic groups from Europe, Asia, and Africa, with a variable prevalence[141,152,162,167,168,173,174].

The frequency of A1555G mutation has been found to differ greatly in different populations, from 1% to 20% of hearing loss cases[152,173]. We have assessed the prevalence of A1555G mutation in the Spanish population using two different approaches. First, a screening of the presence of A1555G mutation was performed in all available families and sporadic cases of our cohort. Among the 313 families analysed, we identified 69 unrelated nonsyndromic hearing loss cases, positive for mutation A1555G (22% of the total of the cohort, 61% of all cases compatible with maternal inheritance), corresponding to 290 samples carrying mutation A1555G, 183 affected (63%) and 107 asymptomatic individuals[229].

In another study, the prevalence for A1555G mutation was assessed in a group of adult patients recruited in a single Otolaryngology department. In this case, the A1555G mutation was found in 9 out of 54 patients (17%) affected of nonsyndromic hearing loss; when only familial cases were considered, the prevalence raised to 21%[230]. These data suggest, as previously reported, that A1555G is a major factor in the onset of deafness, explaining around 20% of all deaf cases, without taking into account the inheritance pattern. The prevalence of A1555G mutation rises over 60%, considering only familial cases with an inheritance pattern compatible with maternal inheritance. These prevalence figures are likely to represent the most accurate estimate of A1555G prevalence in Spanish population, as we obtained very similar results with two different approaches and the ascertainment of cases was not biased in any of the two studies.

In our cohort, we have observed that less than 2/3 of all A1555G carriers developed hearing loss, suggesting that other factors must contribute to the development of the hearing loss phenotype[229]. It is well established that aminoglycosides trigger the deafness onset in conjunction with mutation A1555G[187]. Thus, in both prevalence studies, 22% of deaf patients with mutation A1555G reported a previous aminoglycoside exposure[229,230]. This gives further evidences of the strong relationship between this mutation and an increased susceptibility to aminoglycoside antibiotics. However, a large proportion of deaf subjects did not receive aminoglycosides, indicating the involvement of other environmental and/or genetic factors in the phenotype linked to A1555G mutation.

Compared to other populations, our results indicate a higher prevalence of mutation A1555G in Spain, demonstrating that this mutation is an important contributor to sensorineural deafness in our population. However, no obvious explanation can be found for such high prevalence of A1555G mutation in Spain,

compared to other European populations[168]. One possibility is that Spanish population is much more exposed to exacerbating environmental factors, including aminoglycoside treatment. Different from other countries, where aminoglycoside use has been more restricted, especially streptomycin was extensively used in Spain during the 60s and 70s to treat upper airways infections. This is a plausible reason that may contribute to detect high number of families in our country, as most of affected subjects that have not received aminoglycoside treatment do have relatives with aminoglycoside-induced hearing loss. However, there is no evidence of any other major difference in diet, living conditions or medical treatments between Spain and most other western European countries.

From the point of view of clinical practice, our results stress the need for mutation detection before the administration of aminoglycoside antibiotics. The early detection of the mutation would enable prevention of the onset of hearing loss (such as avoiding exposure to aminoglycosides) and/or mitigation of the progression of hearing impairment.

Phenotypic heterogeneity in A1555G carriers: implications for genetic studies

The phenotype associated to mutation A1555G is extremely variable by means of the severity of hearing loss and age of onset, even in the absence of aminoglycoside exposure. Moreover, as demonstrated in the prevalence studies, a significant proportion of individuals (37%) have normal hearing for their entire life. Thus, it is needed to clinically characterize, both deaf and hearing carriers of mutation A1555G, to gain knowledge on the reasons of the differential auditory function.

A detailed clinical evaluation of the auditory function in A1555G deaf and hearing carriers was performed, taking especially into consideration the characterization of normal hearing carriers of the mutation.

Audiological evaluation of hearing loss in affected carriers of A1555G mutation indicated that they suffer from bilateral sensorineural hearing loss, more severe at high frequencies, which is in accordance with previously reported audiometric features[231-233]. The more severe phenotype observed at high frequencies indicates a topographic effect in the cochlea, being the basal turn the most damaged one. The absence of DPOAE together with the results obtained in the ABR recordings indicates that the hearing loss phenotype linked to A1555G mutation is due to a cochlear defect.

Interestingly, the evaluation of normal hearing carriers of mutation A1555G indicated the presence of subclinical alterations. Tinnitus was present in two normal hearing subjects. It has previously been reported that approximately 50% of patients who initially experienced tinnitus alone developed hearing loss at a later time[234], indicating that tinnitus is a clinically significant warning sign for future hearing loss. Absence of acoustic reflexes at 4.0 kHz was also noted in two hearing carriers, suggesting a subclinical alteration. In addition, recording of DPOAE in the non-hearing impaired group showed lower amplitude at high frequencies, compared to normal reference values. These findings indicate again a deficit in cochlear physiology of all A1555G carriers, but not severe enough to lead to hearing impairment. Thus, the identification of subclinical alterations supports the recommendation of preventive measures against environmental hearing loss factors (i.e., avoiding loud noises and ototoxic drugs and undergoing periodic hearing tests) for A1555G hearing carriers. Moreover, the observation that there are cochlear alterations in asymptomatic subjects is consistent with data showing that there was severe mitochondrial dysfunction in cells derived from asymptomatic subjects in the Arab-Israeli pedigree carrying the A1555G mutation[178].

In summary, our findings indicate that hearing loss associated with the A1555G mutation is a cochlear form of deafness resulting from hair cell dysfunction. These results agree with the proposed mechanism of action for the A1555G mutation in the cochlea, where hair cells are damaged as a consequence of a mitochondrial translational defect of ribosomes with the A1555G mutation. This defect results in a decline in ATP production and an increase in the generation of reactive oxygen species, which leads to hair cell apoptosis. Nevertheless, it is not clear why the A1555G mutation commonly causes hearing loss, affecting only cells in the cochlea, without additional abnormalities commonly affected by other mitochondrial mutations such as vestibular dysfunction, even though the mutation is present in all tissues.

Identification of variants in mitochondrial DNA: mutation or polymorphism?

Since mutations in mtDNA account for a high number of familial and sporadic sensorineural hearing loss cases, we considered the possibility that other mutations in the 12S rRNA gene could also be causing deafness. The search for additional mutations in the 12S rRNA gene led to the identification of two changes in hearing impaired subjects: T1243C and T1291C. These findings suggest that the

mitochondrial 12S rRNA gene is a hot-spot for deafness associated mutations, as it has been already reported in other cohorts[162].

Based on segregation of the variants with the deafness phenotype, the absence in control general population subjects and the predicted effects on the secondary structure of 12S rRNA, variant T1291C was considered responsible of the deafness phenotype, whereas the role of variant T1243C could not be determined. However, Abreu-Silva and colleagues gave evidences in favour of being T1291C a rare mitochondrial polymorphism of African origin, based on a genetic screening for mtDNA mutations in a Brazilian cohort of patients[228]. They found mutation T1291C in five unrelated deafness cases and two control samples, most of them belonging to the L1/L2 African mitochondrial macrohaplogroup, to which the family we reported also belongs[228].

The question is, are there enough evidences to consider this mtDNA variant a mutation or a polymorphism? It is well known that phenotypic variability is a hallmark of mitochondrial disorders, presumably resulting from different nuclear backgrounds[179]. Mutation T1291C is located in the mitochondrial 12S rRNA gene, where several variants have been identified associated with aminoglycoside-induced and non-syndromic hearing loss. In our pedigree, it segregates with the disease in maternal relatives and the clinical phenotype was similar to that associated with other mtDNA deafness causing mutations, giving evidences in favour of being a disease-related variant. Similarly, mutation A1555G, the most common and well-studied mtDNA variant associated to hearing loss, gives rise to different clinical phenotypes, ranging from severe deafness to moderate progressive hearing loss or even completely normal hearing[125,152]. Thus, pathogenicity of mutation T1291C cannot be excluded only based on the phenotypic variability found in T1291C carriers. Incomplete penetrance and variable expressivity of hearing loss associated with mutation A1555G seem to require the contribution of additional genetic factors, such as nuclear modifier genes or mitochondrial haplotype, and/or environmental factors, such as aminoglycoside antibiotics. A similar scenario could explain the phenotypic variability observed regarding the T1291C substitution, being an indication of the contribution of environmental factors or additional genetic factors to the final phenotype observed.

In conclusion, it is not an easy issue to completely understand the pathogenic nature and/or mechanism of mtDNA variants. On one hand, detailed clinical examinations are essential to shed light on the phenotypic variability, especially regarding exposure to environmental factors that may modulate the penetrance or expressivity of the mtDNA variants. Mutational data from different populations, including the mitochondrial haplogroup of the samples, would help to

determine the exact contribution of the mtDNA variants in the onset and development of the disease in each population. Finally, only after performing functional studies with cell lines derived from patients resulting in an impaired mitochondrial function, the nature of a mtDNA genetic variant could be clearly state.

Modifying factors for the phenotype linked to mtDNA mutations

As discussed above, mutation A1555G alone is not sufficient to produce the clinical phenotype. Different hypotheses have been proposed to explain the role of A1555G as a deafness predisposing mutation. Mitochondrial DNA may explain some of the differences between families and ethnic groups[169,170], but different A1555G penetrance is probably the result of a combined action of other susceptibility genes and environmental factors. Biochemical and genetic data suggest that the nuclear background modulate the phenotypic expression of the mutation[179,188]. Extensive genomewide search 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[190-192].

The search for A1555G phenotype modifying factors has been focused in two main aspects:

- Mitochondrial DNA factors: heteroplasmy and other mtDNA variants
- Nuclear DNA factors: chromosome 8p23.1 region

Mitochondrial factors and phenotypic differences in A1555G carriers: the case of heteroplasmy

Mitochondrial DNA factors, such as mitochondrial haplogroup and other mtDNA variants inherited together with A1555G mutation, have been proposed to act as modifiers of A1555G deafness phenotype[235,236]. The large number of A1555G families detected in Spain, relative to the few identified in other European populations[168], suggested the presence of particular features of mtDNA variation in Spanish population, due to a particular history/origin of mutation A1555G in this population, which could have a role in the expression of the disease. Although an excess of Spanish A1555G families harbour mitochondrial haplogroup H, it has been demonstrated that mitochondrial haplogroup has no effect increasing the penetrance or expressivity of A1555G mutation[152,169,170]. However, the contribution of other mtDNA factors has not been assessed.

Heteroplasmy, i. e. the presence of a mixture of mutant and wild-type mtDNA, may partially explain the variation in clinical phenotypes in mtDNA disorders, because of the tissue threshold and the distribution of mutant mtDNA among different tissues[166]. Thus, to predict a patient's clinical outcome, it is important to determine the extent of mutation load, and therefore, accurate and reliable methods for detecting heteroplasmy are needed.

In contrast with most pathogenic mtDNA mutations almost always found in heteroplasmy, A1555G is usually homoplasmic[166]. Different possibilities could explain why is A1555G mutation homoplasmic, differently from other mtDNA mutations. In general, it is believed that the homoplasmic state for most mtDNA mutations would be lethal, being then, the functional effects of A1555G are much less dramatic and limited[88]. This is in accordance with its clinical variability and exquisitely tissue specific phenotype limited to the cochlea. Alternatively, heteroplasmy for A1555G could have been underestimated due to the lack of assays enough sensitive to detect low-level heteroplasmy.

The development of DHPLC and Pyrosequencing assays for reliable measuring heteroplasmy allowed the identification of a three-generation pedigree segregating low-level mtDNA heteroplasmy and the confirmation that A1555G heteroplasmy is more frequent than expected. In this sense, two subjects previously considered homoplasmic for A1555G mutation were 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 A1555G heteroplasmic subjects[237].

Apart from the cases we detected, heteroplasmy for mutation A1555G has only been described in seven additional families[167,176]. Similarly to our results, only in a few of the previously described A1555G heteroplasmic cases the estimated mutation load correlated with the severity of hearing loss[176]. Thus, the contribution of heteroplasmy in the phenotypic differences associated with A1555G mutation, if any, seems to be small in most of the cases. Other genetic factors may determine the differences in the phenotypic expression of mutation A1555G.

As a result of the search for additional mtDNA factors influencing the phenotype associated with A1555G mutation, a novel mtDNA variant in the mitochondria-encoded *MTCYB* gene, T15287C, was found. Variant T15287C co-segregates with mutation A1555G in the three-generation heteroplasmic pedigree. The most likely explanation for this co-segregation is that the two sequence changes occurred simultaneously, or nearly so, within the same mtDNA molecule in a recent maternal ancestor and were then co-segregated in the matrilineal progeny.

Cytochrome *b* plays a central role as a catalytic subunit in complex III of the respiratory chain[238] and several point mutations have been associated to different diseases, all characterized by impaired complex III activity[103,104]. The role of T15287C variant identified is unclear: position 15287 is conserved both at DNA and protein levels, but none of the patients identified presented evidence of respiratory complex III deficiency. In addition, T15287C variant was identified in a control subject, thus, indicating that it might be a polymorphism with no pathogenic effect. However, as discussed above for 12S rRNA mutations, a specific role in the inner ear could not be ruled out, when found together with other mtDNA mutations. Further studies are needed to determine the role of this T15287C variant, especially in conjunction with 12S rRNA mutations.

It has been reported that mtDNA mutations can segregate and accumulate in certain tissues, being the level of heteroplasmy frequently lower in blood leukocytes compared to affected tissues[239]. The ideal situation for heteroplasmic mtDNA disorders would be to measure mutation load in the affected tissue. In cases where it is not possible to obtain DNA from the affected tissue/s, an alternative approach could be to measure heteroplasmy in other tissues. For the three-generation A1555G heteroplasmic pedigree, mtDNA was obtained from blood and saliva, identifying no differences in mutation load between tissues. This may give a clue of the situation in the cochlea and suggests that heteroplasmy is established early in development.

In summary, neither heteroplasmy nor other mtDNA variants have been identified as principal contributors to the phenotype associated to A1555G mutation. The presence of heteroplasmy, the threshold effect and differential tissue distribution present a diagnostic challenge for clinicians dealing with patients harbouring mtDNA mutations[93]. The first step for a better management of mtDNA diseases is the reliably detection of heteroplasmy, an issue which has been solved with the development of the DHPLC and Pyrosequencing assays. 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.

Mitochondrial DNA segregation and inheritance

The study of pedigrees segregating mtDNA heteroplasmic mutations provides insight into the complex process of mtDNA transmission. The three-generation pedigree segregating two distinct mtDNA mutations in heteroplasmy, represents a model for the study of mtDNA segregation and transmission in

humans. In this family, rapid shifts in the proportions of heteroplasmy are observed in the descendants of a single heteroplasmic woman. These observations are in accordance with the bottleneck theory of mtDNA inheritance[92]. According to this theory, mtDNA from a few mitochondria would be selectively amplified during oogenesis and thus, a genotype can become predominant and fixed in future generations[93]. In humans, studies of heteroplasmic mtDNA segregation have accumulated considerable data to support the existence of a developmental bottleneck, although its size is disputed and may vary among different human mtDNA lineages[240-245].

In the case of the studied pedigree, the inheritance pattern and mtDNA segregation is compatible with the effects of random genetic drift operating through a small developmental bottleneck, which has been calculated to contain between 2-46 segregating units, depending on the mathematical approach used. Similar reports of rapid shifts in mitochondrial haplotypes within a single generation suggested also a small bottleneck, whereas other estimations of bottleneck size concluded that it might not always be small[240-245]. These discrepancies regarding mtDNA transmission and bottleneck size could be partially explained by the nature and functional consequences of the particular mutation studied. Studies at cellular level of pathogenic mtDNA point mutations suggest that only those that allow survival of the oocyte will be passed on, either because they are present at low-levels or lead to little biochemical defects[245]. Mutation A1555G has been shown not to have severe functional effects, compromising cell survival only in cochlear hair cells[125]. This could explain the small bottleneck size calculated for this pedigree, in comparison with other mutations showing more severe functional effects[90,91].

From an evolutionary point of view, rapid genetic drift due to small mitochondrial bottlenecks would lead to the direct loss of the mutation or the formation of individuals that are homoplasmic for the new mutant species. The new mutation would be then exposed to the forces of natural selection operating at the level of the individual organism[93]. A germ-line bottleneck consequently would lead to the loss of deleterious mutations before they can accumulate within the population. In consequence, is A1555G mutation favoured by natural selection? The answer derived from our observations is no. Half of the third generation offspring is effectively fixed for the wild-type allele, and half for the mutant, showing no effect of natural selection in this pedigree. However, whether other factors (such as the identified cytochrome b mutation) drive the fixation of one or the other allele remains to be proved.

But, is such a small bottleneck plausible, if we take into account recent findings regarding nucleoid organization and dynamics of mitochondria? In the past

few years, cytological, biochemical and genetic studies have provided evidences of the organization of mtDNA into nucleoids[77]. In humans, nucleoids contain between 2 and 10 molecules of the circular mitochondrial genome[77]. Thus, if the estimation of a minimum bottleneck size of 2 is correct, it may represent either an indirect evidence of the dynamic nature of nucleoids, which would need to divide in as many parts as mtDNA molecules they carry, or alternatively, it may be an underestimation of the effective number of segregating units.

Little is known regarding mitochondrial nucleoid division and transmission, and most of the advances in understanding such processes come from yeast. It is still not known whether similar processes take place in humans, but understanding how nucleoid are organized and segregated has clinical relevance, as the ratio of heteroplasmy is determined by how mtDNA segregates during development. As we have shown, bottlenecks in mtDNA segregation during development can lead to dramatic shifts in the extent of heteroplasmy. Defects in the organization and inheritance of mt-nucleoids could directly alter these bottlenecks. On the other hand, nucleoids are composed by proteins, which have a dual function in both nucleoid biogenesis and mitochondrial metabolism[77]. This would potentially allow the coupling between the processes of mtDNA packaging, nucleoid division and nucleoid inheritance and mitochondrial metabolism[77]. Taking all these into account, an interesting possibility arise: mutations in genes encoding for nucleoid proteins could contribute to human disease, especially ageing-related mtDNA disorders, due to a compromised mtDNA metabolism. A1555G phenotype is normally characterized by a late-onset hearing impairment (related to ageing?) and it is thought to be the consequence of a decrease in ATP production in cochlear hair cells. Could then be possible that defects in mtDNA segregation could influence A1555G phenotype?

In any case, the calculation of the bottleneck size along with the knowledge of the functional consequences of mtDNA mutations can be used to better predict the risk of transmitting heteroplasmic mutations and developing the disease. In addition, the study of human pedigrees segregating heteroplasmic mtDNA mutations may provide insights into the biological mechanisms that drive mtDNA inheritance.

Nuclear factors and phenotypic differences in A1555G carriers: the case of chromosome 8p23.1

Chromosome 8p23.1 is thought to contribute to susceptibility to hearing impairment in patients with the A1555G mutation in mtDNA. However, there are no obvious candidate genes in this locus, and although an in depth mutational screening of genes in the region has been performed, the putative modifier nuclear

gene remains to be identified. In addition, the involvement of chromosome 8p23.1 as a modifying locus could not be confirmed when additional family samples were included in the analysis of a linked Finnish family[193]. A reason of this lack of progress could be the genomic organization of 8p23.1 region.

Chromosome band 8p23.1 is known to be a frequent site of chromosomal rearrangements mediated by low copy repeats (LCRs) or segmental duplications (SDs). It has been described that as many as one in four individuals from the general population carry a 4.7 Megabase (Mb) inversion of the region[215,217,218]. To take into account genomic structural variation is crucial in linkage studies of human diseases for different reasons. First, when a fixed marker order is assumed for all individuals in an inverted region, one tends to see spurious recombination events among inversion carriers and/or to find genotyping contradictions, which may lead to discard some observations. In addition, the polymorphic genomic structure of the rearranged regions, which apart from large-scale genomic rearrangements can include sequences that vary in copy number, might difficult the mapping of putative disease genes.

Thus, novel approaches for the study of 8p23.1 region have to be taken, considering a wider range of genetic factors that may be involved in the pathogenesis of A1555G mutation. Three candidate genetic features were selected to study: claudin-23 gene (*CLDN23)*, mitochondrial ribosomal protein S18C pseudogene-2 (*MRPS18CP2)* and α-defensin-3 (*DEFA3)* gene absence in DEFA1A3 copy number variant (CNV). *CLDN23* gene and *MRPS18CP2* pseudogene were selected based on their putative biological role in the inner ear, whereas *DEFA3* gene absence was tested due to its close location to the marker with a higher lodscore.

Claudins are essential components of tight junctions[246] and therefore, they play important roles in the physiological function of the inner ear. Pseudogenes are nonfunctional sequences of genomic DNA originally derived from functional genes[247]. It has been postulated that pseudogenes may play regulatory roles for the genes which they have been derived, such as serving as a source of antisense RNA[247].

CNVs have been proposed to have an important role in the pathological variation in the human population[209]. On the premise that the presence of a gene in multiple copies could have a dosage effect and therefore, contribute to genetic basis of some complex disorders, it is feasible that the copy number polymorphism of α-defensin cluster could be involved in the pathogenesis associated to the A1555G mutation.

Family-based association test was used to analyse the genotype data from *DEFA3* gene absence, *CLDN23* gene and *MRPS18CP2* pseudogene. Family-based association designs are particularly attractive, since they test for linkage as well as association, avoid spurious associations caused by admixture of populations, and are convenient for investigators interested in refining linkage findings in family samples. With this approach, a weak positive association with a single SNP in *MRPS18CP2* pseudogene and *DEFA3* gene absence was found.

It is difficult to establish a direct relationship between defensin function and A1555G deaf phenotype. However, as the distinction between *DEFA1* and *DEFA3* is based on the typing of a single SNP (C3400A), the differences in the rate of *DEFA3* gene absence observed between deaf and hearing carriers of A1555G mutation could be considered as a positive association signal that confirms the localization of a modifier factor.

More promising are the results obtained with *MRPS18CP2* pseudogene. The human genome encodes at least 79 mitochondrial ribosomal proteins from which more than 100 pseudogenes have been identified[248]. Apart from *MRPS18CP2,* five other pseudogenes derived from *MRPS18C* gene are located in the human genome on chromosomes 3q26.1, 8p21.3, 12p13.31, 15q11.2 and 22q13.31 respectively[248]. Interestingly, the *MRPS18C* pseudogene on chromosome 15q11.2 is located only 1-Mb apart from a microsatellite marker, which gave a positive linkage score in the analysis performed by Bykovskaya and colleagues[191]. Three sequence variants have been found in the human genome for MRPS18 protein of the small mitochondrial ribosome subunit. In analogy to bacterial ribosomes, it is likely that each mitochondrial ribosome contains a single copy of MRPS18. Therefore, the presence of three different isoforms suggests that there is a heterogeneous population of mitochondrial ribosomes, which may have different decoding properties and may be subjected to a precise regulation of its expression[83]. The existence of MRPS18 pseudogenes could play a role in the regulation of each isoform expression, for example by blocking the expression of the corresponding gene. If this is demonstrated, it could explain the tissue specificity of A1555G homoplasmic mtDNA mutation, leading to a cochlea-specific phenotype.

In summary, both positive results found in *MRPS18CP2* pseudogene and *DEFA3* gene absence within the deaf group of A1555G carriers are weak associations, that do not demonstrate a role in the phenotype linked to A1555G mtDNA mutation. However, they give further evidences of the involvement of 8p23.1 region as a modifying factor for A1555G mutation. Further analyses in additional families as well as functional studies, which should shed light on the

function of these genetic features, are needed in order to confirm or discard the associations found.

Population variability of α-defensin gene cluster on chromosome 8p23.1: implications for disease susceptibility

Not long ago, the high prevalence of copy number variation in humans was totally unexpected. With the definition of over 1400 CNV regions in HapMap samples[209], suggesting that CNVs account for a significant proportion of human normal phenotypic variation, an impressive new field of research on CNVs as factors that determine normal phenotypic and pathological human variation, is emerging[207,208]. Thus, it is interesting to perform large inter-populational studies of copy number variation, which will provide a more precise picture of the extent of variation explained by CNVs.

Moreover, analyses of the functional attributes of currently known CNVs reveal a remarkable enrichment for genes that are relevant to molecular-environmental interactions and genes that influence response to specific environmental stimuli, such as genes involved in immune response and inflammation[208].

Copy number variants involving α- and β-defensin genes (*DEFA1A3* and *DEFB4/DEFB103A)* in chromosome 8p23.1 have been extensively characterized[223-225]. Defensins are small cationic peptides, which play an important role in innate immunity. Thus, from a functional point of view, α- and/or β-defensin CNVs could affect the function and effectiveness of innate immunity[223,225].

As *DEFA3* gene absence was studied in relation with A1555G phenotype with promising data regarding inter-individual differences on rate of absence, a broader study using HapMap samples was planned. Significant differences in the *DEFA3* gene absence were found between human populations, which could be indicative of differences in innate immune function between populations. This is not surprising since the different human population groups have been exposed to different environments regarding infectious agents and other factors. CNVs could determine human phenotypic diversity by altering the transcriptional levels of the genes that vary in copy number[208]. It has also been postulated that retention of duplicate genes is due to the generation of increased amounts of a beneficial product[249]. This could be the case of *DEFA1A3* in which variation in *DEFA1* and *DEFA3* copy number, and *DEFA3* absence could underlie variable resistance to infection among

individuals. Different selective pressures acting in each geographic region could likely explain population differences in *DEFA3* absence.

The exceptional genomic complexity and heterogeneity of the human 8p23.1 locus and the prominent role of defensins in the innate immunity framework raise the question of whether individual patterns of haplotypes, together with the variability in defensin genes copy number, affect the functionality of the defensin system. The haplotype diversity and linkage disequilibrium (LD) structure of a 100-kb region around α-defensin locus, showed similar results as what has been observed for other genomic regions. The SNP distribution of the region is characteristic of the presence of segmental duplications, which result in a low-density of SNPs selected for genotyping. The Yoruba samples present a higher variability than both the Chinese/Japanese and Caucasian samples[250]. Additionally, in the Yoruban, the haploblock structures were smaller and the extent of LD between SNPs was lower, in accordance with the out-of-Africa theory for the origins of humans. The observation that the proportion of subjects lacking the *DEFA3* gene is greater in Yoruba samples together with the fact that *DEFA3* is thought to be human specific[223] may be an indication of the higher amount of original genetic variation among the first humans living in Africa, which afterwards migrated to other continents. The initial migration occurred as multiple, branching events and involved many founder effects in which certain haplotypes, SNPs and alleles appear to have increased in frequency in emigrant populations owing to genetic drift and different selection pressures[250]. In this sense, we observed a diminished frequency of subjects without *DEFA3* in Caucasian and Asian samples.

When association with *DEFA3* absence was tested, SNPs and haplotypes in the Caucasian population were the only ones to be significant. The association observed in the Caucasian samples could be the result of strong founder effect. Founder effects and, particularly, the decrease in genetic diversity resulting from continental migrations, are associated with an increased haplotype length[250]. This is observed when comparing the haplotype block patterns of the different populations analyzed, in which the Caucasian samples set has the longest haplotype blocks. Alternatively, Aldred and colleagues demonstrated that *DEFA3* has arisen at the 5' end repeat position and has transferred to other positions within the array through unequal recombination between alleles[223], suggesting that recombination has been active in shaping diversity in the *DEFA1A3* locus. However, our results indicate that, at least in the Caucasian samples, there has been little recombination between chromosomes with and without *DEFA3*, as we are able to find a haplotype associated with *DEFA3* absence extending for nearly 100-kb.

The impact on human health of this qualitative variation in the presence of the *DEFA3* gene product deserves to be explored in epidemiologic studies. Different studies have described differences in the function and specificity of *DEFA1* and *DEFA3* gene products, HNP1 and HNP3[219,251]. In general, HNP3 is thought to be less active than HNP1 against both gram-positive and gram-negative bacteria[252], but it is expressed at about twice the level of HNP1[223]. On the other hand, *DEFA3* but not *DEFA1*, has been found upregulated in patients with systemic lupus erythematosus, idiopathic thrombocytopenic purpura or rheumatoid arthritis, suggesting that *DEFA3* upregulation might be a general feature of autoimmune diseases[253,254]. Therefore, the observed differences in *DEFA3* absence may partially explain the different population incidences of infectious and/or autoimmune diseases in which *DEFA3* plays an important role. Future studies are needed to establish whether patterns of *DEFA3* absence correlate with certain population microbial exposures or different prevalence of autoimmune disorders. This could also be important in determining the exact nature of *DEFA3* function and its specificity of action, if any, against certain antigens.

Conclusions

Conclusions

Briefly, the contributions of this work can be summarised in:

1. A total of 69 unrelated cases of nonsyndromic hearing loss carrying mutation A1555G in the mitochondrial 12S rRNA gene have been identified. The prevalence for mutation A1555G is of 22% in the total of our cohort of cases of deafness, but it raises to 61% when only familial cases with a pattern compatible with maternal inheritance are considered.

2. Only 63% of A1555G mutation carriers are hearing impaired, suggesting the contribution of other factors to the development of the disease. Aminoglycoside antibiotics have been confirmed to trigger the onset of hearing loss in the presence of mutation A1555G, but only 22% of the cases reported previous aminoglycoside exposure.

3. The clinical characterization of A1555G carriers indicates that this mutation would lead to a cochlear form of deafness without affectation of the auditory nerve. Audiometric alterations are more severe at high frequencies, indicating a topographic affectation of the cochlea from base to apex. In addition, all A1555G mutation carriers, both deaf and hearing subjects, present alterations in cochlear physiology.

4. The search for additional deafness-related mutations in the 12S rRNA gene led to the identification of two new mitochondrial DNA variants: T1243C and T1291C. Their role as deafness-causing variants could not be clearly determined, although T1291C mutation fulfils most of the criteria of a disease-related mutation.

5. Two assays for accurately detecting and quantifying mitochondrial DNA heteroplasmy have been developed and applied to the analysis of heteroplasmy for A1555G mutation.

6. A three-generation pedigree, segregating in heteroplasmy A1555G mutation together with T15287C, a novel variant in mitochondrial cytochrome *b* gene has been identified. No differences in mutation load were detected between tissues in the heteroplasmic pedigree, suggesting that heteroplasmy is established early in development. The mitochondrial DNA inheritance in the heteroplasmic pedigree is compatible with the effects of random genetic drift operating through a small developmental bottleneck.

7. Chromosome 8p23.1 has been analysed as a putative modifying locus for the phenotype associated to A1555G mutation. Weak associations with a polymorphism in *MRPS18CP2* pseudogene and *DEFA3* gene absence have been identified, providing new evidences of the involvement of chromosome 8p23.1 in the pathogenesis of A1555G mutation.

8. The frequency of *DEFA3* gene absence varies between human populations, being more frequent in the Yoruban. The identification of such population differences in subjects lacking the *DEFA3* gene may be suggestive of population-specific selective pressures with potential impact on human health.

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Annex I

Original article

Electronic version:

Abreu-Silva RS, Batissoco AC, Lezirovitz K, Romanos J, Rincon D, Auricchio MT, Otto PA, Mingroni-Netto RC.

Correspondence regarding Ballana et al., "Mitochondrial 12S rRNA gene mutations affect RNA secondary structure and lead to variable penetrance in hearing impairment"

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