



Non-syndromic hearing impairment in a multi-ethnic population of Northeastern Brazil

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ABSTRACT

Objective: There are many hearing impaired individuals in Monte Santo, a rural municipality in the state of Bahia, Brazil, including multiple familial cases strongly suggestive of a genetic aetiology.

Methods: The present study investigated 81 subjects with hearing impairment (HI) recruited from 36 families. Mutations often associated with HI, i.e. the DFNB1 mutations c.35delG in *GJB2*, deletions del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854), and A1555G in the mitochondrial gene *MTRNR1* were initially analyzed, with additional mutations in *GJB2* identified by sequencing the coding region of the gene.

Results: Seven different mutations were present in *GJB2* with mutations c.35delG and p.Arg75Gln, which are known to be pathogenic, identified in 37.0% of the subjects. Individuals homozygous for the c.35delG mutation were diagnosed in eight families, corresponding to 24.7% of unrelated individuals with nonsyndromic hearing impairment (NSHI), and an additional heterozygote for this mutation was present in a single family. Ten individuals (12.4%) in another family were heterozygous for the mutation p.Arg75Gln.

Conclusions: Significant heterogeneity was observed in the alleles and patterns of NSHI inheritance among the subjects studied, probably due to the extensive inter-ethnic admixture that characterizes the peoples of Brazil, together with a high prevalence of community endogamy and consanguineous marriage.

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1. Introduction

Hearing impairment (HI) can result from genetic and/or environmental factors. In developed countries approximately 60% of cases are hereditary, while 30% are acquired and 10% have an undefined aetiology [1,2]. Of the hereditary forms of HI, nonsyndromic and syndromic disorders account for an estimated 70% and 30% of cases respectively [3,4]. Although no nationally representative data are available on the prevalence or aetiology of HI in Brazil, most causes appear to be environmental in nature [5,6]. Several studies investigating the genetic basis of HI have,

however, demonstrated the presence of a range of mostly recessive mutations [7–11].

Hereditary HI is highly heterogeneous, and to date mutations associated with nonsyndromic hearing impairment (NSHI) have been described in more than 70 genes exhibiting different modes of inheritance [12,13]. Despite the large numbers of genes and mutations implicated the DFNB1 locus appears to be principally involved [14–17], with the genes *GJB2* and *GJB6* present at this locus encoding the proteins connexin 26 (Cx26) and connexin 30 (Cx30) [18–20]. More than 100 mutations associated with NSHI have been described in *GJB2*, with both autosomal recessive and autosomal dominant modes of inheritance [17]. The most common AR mutation associated with NSHI in Caucasian populations is c.35delG(p.Gly12fx) [21].

A high frequency of c.35delG mutation carriers has been reported in many European countries [21]. In São Paulo, Brazil this mutation was detected in 2.2% of 223 newborns studied [22], while

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another screening programme on 620 newborns also conducted in São Paulo indicated a carrier frequency for the mutation of 1.0% [23]. No previous molecular studies in individuals with HI have been conducted in northeastern Brazil, although the c.35delG mutation was identified in individuals of African ancestry with normal hearing ability in Salvador, the state capital of Bahia, where the overall carrier frequency of the mutation was estimated to be 1.0% [24].

The 309 kb deletion in *GJB6*, del(*GJB6*-D13S1830), was the second most frequent connexin mutation reported for NSHI in Spain, France, Israel, Great Britain and Brazil [20,25]. Individuals who are either homozygous for del(*GJB6*-D13S1830) or compound heterozygotes of del(*GJB6*-D13S1830) and a mutant allele of *GJB2* develop NSHI, resulting in profound to severe HI [20]. The 232 kb deletion del(*GJB6*-D13S1854) also leads to partial loss of *GJB6* and has frequently been described in individuals with NSHI in Italy, England and Brazil [26]. A number of studies have indicated that the pathogenic effect of the 309 kb and 232 kb deletions is due to the deletion of a cis-acting enhancer of *GJB2* expression [27–29]. Mutations in mitochondrial DNA have been associated with both syndromic and nonsyndromic HI [13,30]. The A1555G mutation in mitochondrial gene *MTRNR1* has been identified in hereditary NSHI and in aminoglycoside-induced HI in families from several countries [31–35]. The A1555G mutation also has been reported in cases of NSHI in Brazil [8,9,36].

Due to widespread inter-ethnic admixture between people of European, African and Amerindian ancestry in the 500 years since European colonization, and more recent admixture with Middle Eastern and East Asian migrants, the Brazilian population has been described as one of the most heterogeneous in the world [37]. With a total population approaching 200 million, ethnicity in different regions of Brazil reflects both historical patterns of settlement and internal migration related to economic dynamics. Within Bahia 76.3% of residents, mainly living in the coastal regions, self-identified as being of African ancestry in the 2010 Census [38]. By comparison, an increasing proportion of European ancestry is seen in communities resident in inland regions of the state [39].

The aim of the present study was to investigate the genetic aetiology of HI by analyzing mutations in genes *GJB2*, *GJB6* and *MTRNR1* in individuals with hearing impairment from Monte Santo, a multi-ethnic inland community in Bahia.

2. Subjects and methods

2.1. Subjects

According to the 2010 Census of Brazil, the predominantly rural municipality of Monte Santo had a population of 52,338 inhabitants resident in some 200 villages [38]. Eighty-one patients with HI recruited from 36 families resident in Monte Santo were investigated between 2008 and 2010. The study initially included individuals with both sporadic and familial histories of HI, and with prelingual and postlingual HI. However, individuals who presented with evidence of syndromic or postlingual HI but with no familial recurrence subsequently were excluded.

Audiological evaluations using a tympanometry test, estimations of acoustic reflex thresholds (0.5, 1, 2 and 4 kHz), pure tone audiometry thresholds and evoked auditory brainstem response (ABR) were performed in the School of Audiology Clinic of the Metropolitan Union of Education and Culture College (UNIME), located in Lauro de Freitas, Bahia, Brazil. Institutional Review Board (IRB) approval was obtained from Research Ethics Committee of the Gonçalves Moniz Research Center (CPqGM), Oswaldo Cruz Foundation (FIOCRUZ), Salvador, Bahia, Brazil (Case No. 182/2008, Protocol No. 274). Informed written consent was obtained from all

individuals with HI, their legal representatives, or adult members of their immediate families.

2.2. Molecular analysis

Molecular analyses were performed at the Laboratory for Advanced Public Health at CPqGM/FIOCRUZ in Salvador, Bahia, Brazil. Genomic DNA was extracted from peripheral blood leucocytes [40,41], with mutations in *GJB2*, the deletions del(*GJB6*-13S1830) and del(*GJB6*-D13S1854) in *GJB6*, and the mitochondrial mutation A1555G in *MTRNR1* investigated. All c.35delG/*GJB2* mutations were first analyzed by PCR-RFLP using the *Bst*NI enzyme [42,43]. The genotypes of all individuals with c.35delG mutations were confirmed by sequencing, and the mutations del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) were investigated by multiplex assays [26]. The A1555G/*MTRNR1* mutation was investigated using a protocol described by Kupka et al. (2002) [44], and analyses of the other mutations, including c.35delG were performed by direct sequencing of the *GJB2* coding region [45].

3. Results

3.1. Family backgrounds of the study population

Preparatory genealogical investigations identified a total of 1479 individuals belonging to 36 large extended families, 31 of which reported recurrence of HI. Pedigree analysis indicated that 163 subjects with HI in the 36 extended families were of probable genetic origin, with 152 of these affected individuals resident in Monte Santo. Of the 81 people with HI recruited into the present study, 95.1% reported a family history of hearing defect. No specific information on consanguinity was available for 11 subjects, but 36/70 (52.2%) of the remaining cases were born to parents related as second cousins or closer ($F \geq 0.0156$) (Table 1). The average age of participants in the study was 32 years (range: 2–70 years), 56.0% were male, 33.3% self-identified as White and 66.7% as *Pardo* (mixed ethnic ancestry).

3.2. Audiological assessment

In each of the study subjects the pattern of HI observed by immittance audiometry was suggestive of sensorineural HI, with all individuals presenting with bilateral HI and 94.0% reporting prelingual HI. The average age at initial diagnosis of HI in the prelingual cases was 2.5 years (range: 1 month to 12 years). In the five reported cases of postlingual HI the average age of HI

Table 1

Summary of genotypes identified by analysis of the coding region of *GJB2* and parental consanguinity in 81 individuals with HI from Monte Santo, Bahia, Brazil.

Genotype(s)	N (%)	Consanguinity		
		Yes (N)	No (N)	Unclear (N)
+/+	38 (46.9)	19	17	2
c.35delG/c.35delG ^a	20 (24.7)	11	7	2
p.Arg75Gln/+ ^a	10 (12.4)	0	7	3
c.-22-12C>T/+	4 (4.9)	3	1	0
c.-22-12C>T/p.V27I	1 (1.2)	1	0	0
c.-15 C>T/+	1 (1.2)	1	0	0
c.35delG/+	1 (1.2)	0	0	1
p.Val27Ilep/+	4 (4.9)	0	2	2
p.Lys168Arg/+	1 (1.2)	0	0	1
c.*1C>T/+	1 (1.2)	1	0	0
Total	81 (100.0)	36	34	11

+ wild-type allele.

^a Genotypes known to be pathogenic.

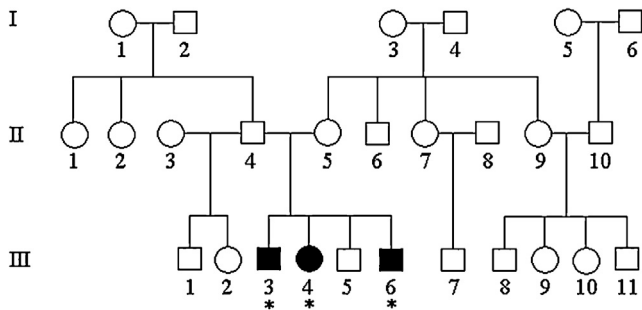


Fig. 1. Family pedigree with inherited HI caused by a c.35delG mutation in homozygous individuals. The three subjects recruited into the study are indicated by an asterisk.

awareness or diagnosis was 17 years (range: 5–38 years). Possible involvement of infectious diseases was reported in four postlingual cases: one with meningitis and three with measles. However, both of these diseases were common in the overall study population with 44.0% of all subjects reporting a history of measles, 1.5% with meningitis, and 1.5% with both diseases.

3.3. Molecular analysis

Seven mutations and 10 genotypes were identified by analysis of the coding region of *GJB2* (Table 1). As homozygous and heterozygous genotypes for the mutations c.35delG and p.Arg75Gln(p.R75Q or c.224G>A) are known to be pathogenic, a genetic aetiology for HI could therefore be confirmed in 30/81 (37.0%) of the subjects studied.

Individuals homozygous for the mutation c.35delG with a typical AR pattern of inheritance (Fig. 1) were identified in eight families, corresponding to 20/81 (24.7%) of the individuals investigated. Parental consanguinity ($F \geq 0.0156$) was, however, reported in 11/20 (55.0%) of HI subjects with the c.35delG mutation. A person heterozygous for c.35delG was found in one

family, corresponding to 1.2% of patients with NSHI (Table 1). Intra-familial genetic heterogeneity also was observed with, for example, a homozygous c35delG mutation identified in individual IV-13 (Fig. 2a), and individual IV-7 (Fig. 2b), but not in other persons with HI in their respective families.

The p.Arg75Gln mutation was responsible for NSHI in 12.4% of the cases analyzed. All of these individuals were heterozygous for the p.Arg75Gln mutation and they belonged to a single large non-consanguineous family. Exceptionally, in this family HI segregated in a pattern typical of AD inheritance (Fig. 3). Five other mutations in the coding region of gene *GJB2* were identified in 12/81 (14.6%) of the individuals studied (Table 1). Three of these mutations have been described as non-pathogenic polymorphisms: c.15C>T(-15C>T), p.Val27Ile(c.79G>A) and c.*1C>T(682C>T) [17], and two were of undefined pathogenicity: c-22-12C>T(IVS1-12C>T) and p.Lys168Arg(c.503A>G) [8,9,17,45,46].

4. Discussion

The 152 people diagnosed with NSHI represented 0.29% of the total population of the municipality, and so it has been estimated that at least 3/1000 individuals living in the region have hereditary HI. HI therefore appears to be more common in Monte Santo than elsewhere in the state of Bahia or in Brazil, where the prevalence of people with hearing defects has been estimated to be 1.7/1000 and 1.8/1000 respectively [38].

Mutations in the *DFBN1* locus where *GJB2* and *GBJ6* are located have been implicated as one of the leading causes of hereditary HI, particularly in prelingual cases with an AR mode of inheritance [14–17,47]. In the present study, 94.0% of the cases were reported as prelingual but neither deletion *del(GJB6-D13S1830)* nor *del(GJB6-D13S1854)* involving a partial loss of *GJB6* was present in subjects from Monte Santo.

Although there was no evidence to suggest maternal inheritance of HI in the families studied, the mitochondrial A1555G mutation in *MTRNR1* was specifically investigated because of its prior identification in other Brazilian studies, including individuals

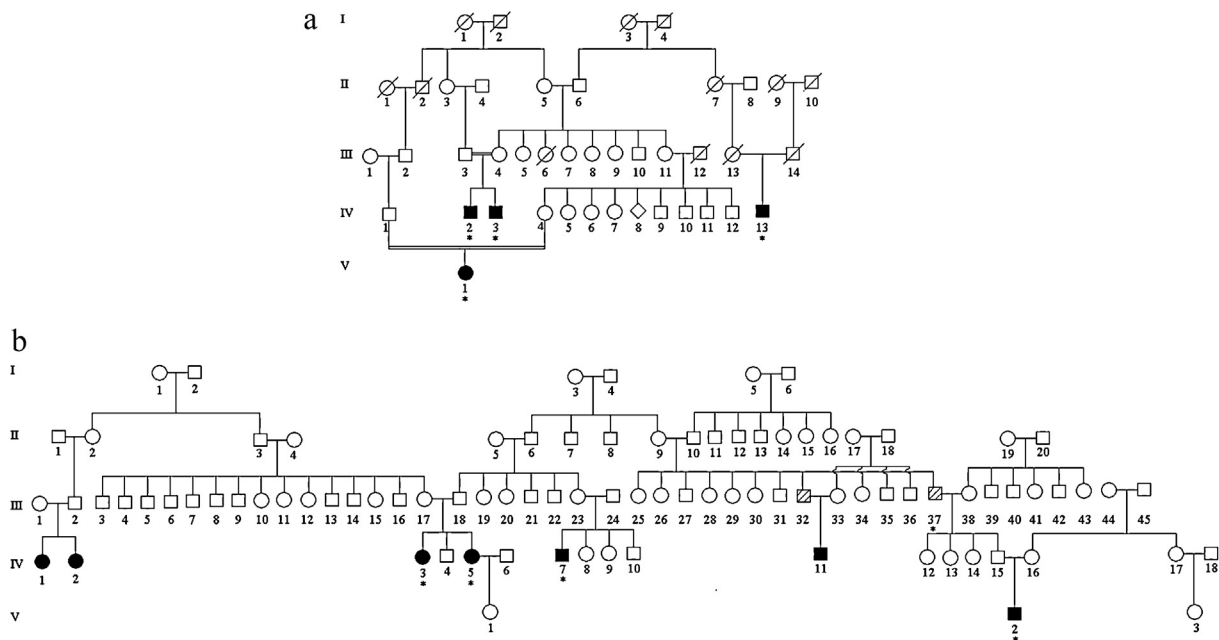


Fig. 2. Illustrative pedigrees of two families with HI, an AR pattern of inheritance (a), and a heterogeneous aetiology of hearing impairment (b). The four subjects in each family recruited into the study are indicated by asterisks. HI was caused by a homozygous c.35delG mutation in individual IV-13 (a) and individual IV-7, (b). Information on other individuals with HI in this family but not included in the study was provided by relatives. Subjects who reported presbycusis (age-related HI) are indicated by hatched shading.

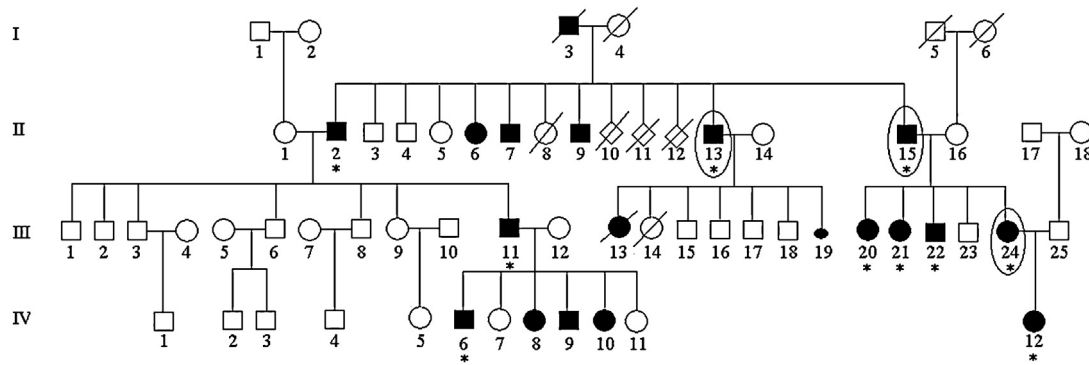


Fig. 3. Family pedigree with HI suggestive of an AD mode of inheritance, caused by a p.Arg75Gln mutation in heterozygous individuals. The 10 subjects recruited into the study are indicated by asterisks; the three subjects with HI and palmoplantar keratoderma are circled. Information on other individuals with HL in this family but not included in the study was provided by relatives.

with no indication of familial recurrence [7]. The mutation was also reported in Brazilians with profound HI who had been treated with aminoglycosides [7,8]. No subjects in the present study reported prior treatment with aminoglycosides, and contrary to previous Brazilian studies the A1555G mutation was not detected in any cases of HI from Monte Santo.

Investigations into the genetic aetiology of HI have mainly focused on unrelated individuals [8,9,46,48–55]. The data in the present study are derived from a comprehensive community-based medical genetics survey established as a service for the inhabitants of the study region, with related individuals therefore included. The genotypic frequency of mutation c.35delG was calculated both for all individuals and on the basis of a single affected individual per nuclear family. Across all subjects with genotypically confirmed NSHI the frequency of homozygotes was 24.6% and 1.2% for heterozygotes, while for unrelated individuals only the corresponding frequencies were 24.1% for homozygotes and 1.7% for heterozygotes. Equal numbers of c.35delG homozygous individuals self-identified as White or as *Pardo*.

As shown in Table 2, the homozygote frequency for c.35delG in Monte Santo (24.1%) was significantly higher than reported elsewhere in Brazil, where the corresponding frequencies were 3.7–7.3%, and in diverse world populations with homozygote frequencies ranging from 0–18.0%. However, it was lower than in

Croatia [51], Italy [48], and Bulgaria [56], where 25.4%, 30.2% and 39.2% respectively of affected individuals were homozygous for the c.35delG mutation.

In part, the variation in global genotype frequencies of c.35delG may be explained by founder effect [57], with evidence of a common founder mutation originating in Europe or the Middle East responsible for the high frequency of the c.35delG/GJB2 allele in Caucasians [21]. A population-based study reported high carrier frequencies of c.35delG allele in southern European countries, e.g. Portugal (1/45), Italy (1/32) and Spain (1/40) [21]. As the c.35delG homozygous individuals in Monte Santo self-identified either as White or *Pardo* (mixed ancestry including White), it seems probable that the c.35delG mutation in the region was originally introduced during the successive waves of colonization from southern Europe that commenced in the early 16th century.

The high frequency of homozygotes for the c.35delG mutation in Monte Santo can be ascribed to small effective population sizes and drift, and to parental consanguinity which was identified in 52.2% of the subjects analyzed, and was present in 55.0% of individuals with HI caused by the c.35delG mutation. However, as previously observed in a consanguineous Brazilian family [58], genetic heterogeneity was observed in familial cases of HI with an AR mode of inheritance, with the c.35delG mutation present in only some affected subjects (Fig. 2a and b). Rather than being confined to a small number of specific families and to a restricted number of inherited disorders, the phenomenon of disease heterogeneity within closely related families has been increasingly reported across a variety of recessive disorders [59].

The p.Arg75Gln mutation (OMIM, img 121011.0026) has variously been described in subjects with HI in Turkey [60], France [61], a family of Russian origin [62], Taiwan [63] and in 1/207 patients in Brazil [8]. In these cases an AD mode of inheritance was observed, and in some subjects the HI was associated with palmoplantar keratoderma [60,61]. An AD mode of inheritance appeared probable in the present study in which 10 members of a family with no reported consanguinity had HI, three of whom also had palmoplantar keratoderma.

As yet the pathogenicity of the p.Lys168Arg mutation remains to be resolved [8,9]. In São Paulo, Brazil, two of 300 individuals (0.7%) with NSHI were heterozygous for p.Lys168Arg. There were, however, no individuals with the mutation in an analysis of 100 Brazilians, 50 each of European and African ancestry, and investigation of the mutation phenotype concluded that it probably was a non-pathogenic variant [9]. In Monte Santo the p.Lys168Arg mutation was present in a single individual who reported a family history of NSHI, so further studies comparing individuals with and without HI in this family could contribute to clarification of the phenotype.

Table 2

Comparative frequencies of homozygotes for c.35delG in individuals with HL from Monte Santo, Brazil and other reference populations.

Location	Frequency of mutant homozygotes (%)	Reference
Monte Santo, Brazil	24.1	Present study
Espírito Santo, Brazil	3.9	[10]
São Paulo, Brazil	7.3	[9]
Brazil (unstated)	6.5	[8]
Mexico	2.6	[64]
France	6.3	[46]
Italy (northern)	16.9	[65]
Italy	30.2	[48]
Croatia	25.4	[51]
Bulgaria (Bulgarian and Turkish)	39.2	[56]
Altai Republic (all Russian)	9.2	[62]
Turkey	5.3	[52]
Iran (Azerbaijani)	15.3	[53]
Iran (Ardabil)	18.0	[66]
Iran (not stated)	10.0	[67]
China (Han)	0.8	[49]
China (Han and other ethnicities) ^a	0.1	[54]
Saudi Arabia	6.4	[55]
South Africa (Limpopo)	0.0	[68]

^a Han Chinese ($n=1640$), Tibetan ($n=122$), Southwest region ($n=119$), Hui ($n=79$), Xinjiang ($n=62$), Mongolian ($n=21$), Maan ($n=18$) and Korean ($n=2$).

Given the extensive family histories of NSHI (95.1%) in Monte Santo, it appears highly probable that many other cases of HI which are still to be investigated are genetic in origin, with complementary studies therefore merited to identify the causative mutations and characterize the disease phenotypes. Similarities in their history of colonization and in the resultant ethnic profiles of other northeastern states of Brazil suggest that the mutations found in Bahia also will be important causes of hereditary HI in the populations of neighbouring states. By comparison, with the exception of the apparent high prevalence of *GJB2* mutations throughout Brazil, it seems likely that the mutation profiles of HI in other regions of the country will be population-specific and reflect their particular demographic and genetic histories, and marital practices.

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