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

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SUMMARY

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 previously associated with HI were initially analyzed: c.35delG in gene *GJB2*, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) in gene *GJB6*, and A1555G in the mitochondrial gene *MTRNR1*, 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.R75Q, 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 24.6% 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.R75Q.

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, and a high prevalence of community endogamy and consanguineous marriage.

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 63 genes exhibiting different modes of inheritance [12,13]. Despite the large numbers of genes and mutations implicated locus 13q11-q12 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]. Some 111 mutations associated with NSHI have been described in *GJB2*, of which 92 have an autosomal recessive (AR) mode of inheritance, nine are inherited as autosomal dominant (AD) mutations, and 10 others have no clearly defined pattern of transmission [17]. The most common AR mutation in patients with NSHI is c.35delG [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 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 bp deletion del(*GJB6*-D13S1830) leads to a partial loss of *GJB6*. This deletion 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 [26]. The 232bp 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 [27].

Mutations in mitochondrial DNA have been associated with both syndromic and nonsyndromic HI [13,28]. The A1555G mutation in mitochondrial gene *MTRNR1* has been identified in hereditary NSHI and in aminoglycoside-induced HI in families from several countries [29-33]. The A1555G mutation also has been reported in cases of NSHI in Brazil [8,9,34].

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 [35]. 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 [36]. By comparison, an increasing proportion of European ancestry is seen in communities living in inland regions of the state [37].

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.

SUBJECTS AND METHODS

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 [36]. 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. The study was approved by the Research Ethics Committee of the Gonçalo 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.

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 [38,39], 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 [40,41]. 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 [27]. The A1555G/*MTRNR1* mutation was investigated using a protocol described by Kupka et al. (2002) and analyses of the other mutations, including c.35delG, were performed by direct sequencing of the *GJB2* coding region [43].

RESULTS

Family backgrounds of the study population

Preparatory genealogical investigations identified a total of 1,479 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 ($F \geq 0.0156$). 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).

Audiological assessment

In each of the study subjects the pattern of HI observed by imittance 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–12 years). In the five reported cases of postlingual HI the average age of HI 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.

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.R75Q are known to be pathogenic, a genetic aetiology for HI could therefore be confirmed in 30/81 (37.0%) of the subjects studied.

Table 1 *Summary of genotypes identified by analysis of the coding region of GJB2 in 81 individuals with HL from Monte Santo, Bahia, Brazil*

Genotype(s)	Frequency (%)
+/+	47.1
c.35delG/c.35delG**	24.7
p.R75Q/+ **	12.4
c.-22-12C>T/+	4.9
c.-22-12C>T/p.V27I	1.2
c.-15 C>T/+	1.2
c.35delG/+	1.2
p.V27I/+	4.9
p.K168R/+	1.2
c.*1C>T/+	1.2

+: wild-type allele; **genotypes known to be pathogenic

Individuals homozygous for the mutation c.35delG were identified in eight families, corresponding to 14/57 (24.6%) of the individuals analyzed who were known to be non-consanguineous or for whom information on consanguinity was unavailable. 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.R75Q mutation was responsible for NSHI in 12.4% of the cases analyzed. All of these individuals were heterozygous for the p.R75Q 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.V27I(p.Val27Ile or V27I) and c.*1C>T(682C>T) [17], and two were of undefined pathogenicity: c-22-12C>T(IVS1-12C>T) and p.K168R(K168R or p.Lys168Arg) [9,17,44].

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 3/1,000 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 1.7/1,000 and 1.8/1,000 respectively [36].

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 [14-17,45]. In the present study, 94.0% of the cases were

reported as prelingual, yet 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 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 [8,9,44,46-53]. 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 [49], Italy [46], and Bulgaria [54], where 25.4%, 30.2% and 39.2% respectively of affected individuals were homozygous for the c.35delG mutation.

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	Cordeiro-Silva et al. (2011)
São Paulo, Brazil	7.3	Batissoco et al. (2009)
Brazil (unstated)	6.5	Oliveira et al. (2007)
Mexico	2.6	Arenas-Sordo et al. (2012)
France	6.3	Roux et al. (2004)
Italy (northern)	16.9	Primignani et al. (2009)
Italy	30.2	Murgia et al. (1999)
Croatia	25.4	Medica et al. (2005)
Bulgaria (Bulgarian and Turkish)	39.2	Popova et al. (2012)
Altai Republic (all Russian)	9.2	Posukh et al. (2005)
Turkey	5.3	Baysal et al. (2008)
Iran (Azerbaijani)	15.3	Bonyadi et al. (2009)
Iran (Ardabil)	18.0	Davarnia et al. (2012)
Iran (not stated)	10.0	Bazazzadegan et al. (2012)
China (Han)	0.8	Xiao and Xie (2004)
China (Han and other ethnicities)**	0.1	Dai et al. (2009)
Saudi Arabia	6.4	Al-Qahtani et al. (2010)
South Africa (Limpopo)	0.0	Kabahuma et al. (2011)

**Han Chinese (n=1,640), Tibetan (n=122), Southwest region (n=119), Hui (n=79), Xinjiang (n=62), Mongolian (n=21), Maan (n=18) and Korean (n=2)

In part, the variation in global genotype frequencies of c.35delG may be explained by founder effect [55], 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 (1/40) and Spain (1/32) [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 [56], 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 (Figs. 2a, 2b). 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 [57].

The p.R75Q (OMIM, img 121011.0026) mutation has variously been described in subjects with HI in Turkey [58], France [59], a family of Russian origin [60], 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 [58, 59]. 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.K168R mutation remains to be resolved [8,9]. In São Paulo, Brazil, two of 300 individuals (0.7%) with NSHI were heterozygous for p.K168R. 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.K168R 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.

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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Legends to figures

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.

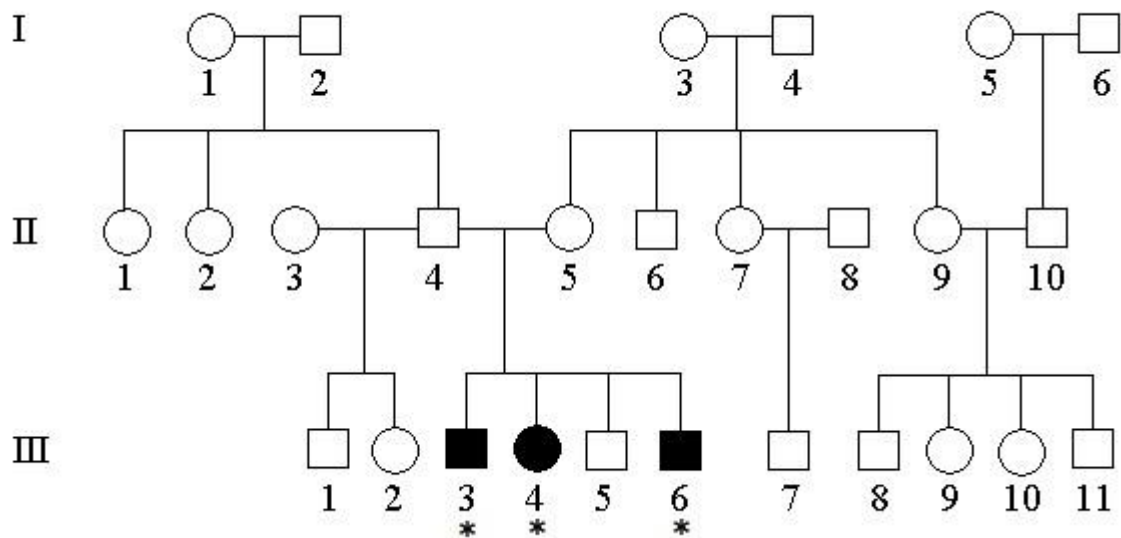


Fig. 2 Illustrative pedigrees of two families with HI, an AR pattern of inheritance (Fig. 2a), and a heterogeneous aetiology of hearing impairment (Fig. 2b). The four subjects in each family recruited into the study are indicated by asterisks. HI was caused by a homozygous c.35delG mutation in individual IV-13, Fig. 2(a) and individual IV-7, Fig. 2(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.

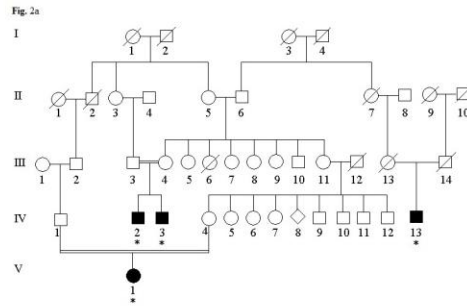


Fig. 2b

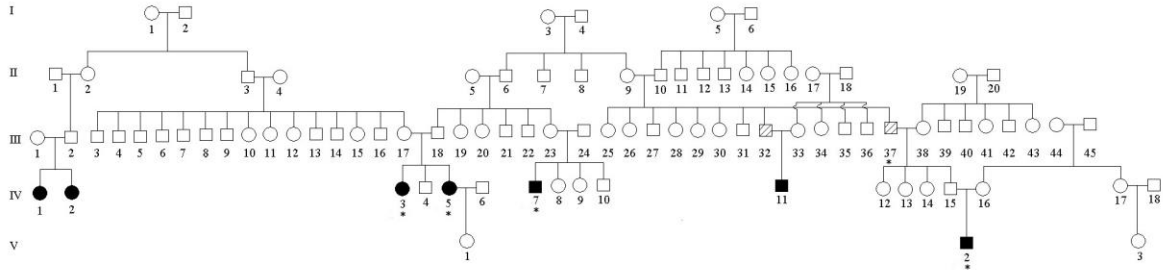


Fig. 3 Family pedigree with HI suggestive of an AD mode of inheritance, caused by a p.R75Q mutation in heterozygous individuals. The 10 subjects recruited into the study are indicated by asterisks; the three subjects with HI and palmoplantar keratoderma are circled. Information on other individuals with HL in this family but not included in the study was provided by relatives.

