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Mutation in gap and tight junctions in patients with non-syndromic hearing loss

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ABSTRACT

Biallelic mutations in the *GJB2*, *GJB3*, *GJB6* and *CLDN14* genes have been implicated in autosomal recessive non-syndromic hearing impairment (ARNSHI). Moreover, a large number of *GJB2* heterozygous patients was reported. The phenotype was in partly justified by the occurrence of two deletions including *GJB6*. We analysed *GJB2*, *GJB6*, *GJB3* and *CLDN14* in 102 Tunisian patients with ARNSHI. The deletions del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) were also screened. The c.35delG in *GJB2* was the most frequent mutation (21.57%). It was detected at heterozygous state in 2 patients. The del(*GJB6*-D13S1830) was identified in one case at heterozygous state. No other mutation in studied *gap junction* genes was detected in heterozygous patients. Several polymorphisms were identified in *GJB3*, *GJB6* and *CLDN14*. Our study confirms the importance of *GJB2* screening in ARNSHI and suggests that in consanguineous populations, a single DFNB1 mutant allele in individuals with HI is likely due to a coincidental carrier state.

Introduction

Hearing impairment (HI) is the most common sensory disorder worldwide. The prevalence of profound congenital HI is approximately 1 in 1000 births. More than 60% of cases of congenital HI are of genetic origin, of which approximately 70% are non-syndromic. Most frequently (80%) the affection is of autosomal recessive inheritance [1]. To date, over 27 different genes implicated in autosomal recessive non-syndromic HI (ARNSHI) have been identified [The Hereditary Hearing Loss Homepage: http://webh01.ua.ac.be/hhh/]. Among the identified genes *GJB2*, *GJB3*, *GJB6* and *CLDN14* encode intercellular gap and tight junctions.

Despite the important number of genes that have been characterized for ARNSHI, *GJB2* gene is the predominant cause in most populations worldwide [2,3]. More than 100 mutations in the *GJB2* gene have been reported. Three mutations of *GJB2* are of particular importance because of their unique ethnically based frequency. The c.35delG mutation accounts for the majority of *GJB2* mutant alleles in North American, European and Mediterranean populations [2–4]. The c.167delT mutation represents the most common pathogenic alleles in Ashkenazi Jews [5] and the c.235delC mutation is the most common in Asians [6,7].

Two large deletions del(*GJB6-D13S1830*) and del(*GJB6-D13S1854*) of 309 and 232 kb, respectively have been identified in *GJB6* gene [8,9]. These deletions result in truncation of an extensive segment of the open reading frame of *GJB6*, which is located upstream of *GJB2* on chromosome 13. They segregate with HI when present in a homozygous state or in heterozygous state with each other or in *trans* with a recessive *GJB2* mutation. Loss of appropriate regulation of *GJB2* from chromosomes bearing these deletions may underlie the HI in these individuals [9].

Mutations in the *GJB3* gene were initially linked to autosomal dominant NSHI [10]. Subsequently, mutation screening of 25 Chinese families with recessive deafness identified two small families with compound heterozygosity for the same two *GJB3* mutations [11]. Later several heterozygous *GJB3* variations were identified in familial and sporadic cases with ARNSHI [12–14]. Recently, Liu et al. provided evidence that mutations in *GJB2* and *GJB3* genes cause HI in a digenic mode in humans. They showed that in three

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unrelated families, two different *GJB3* mutations (c.497A>G and c.194A>T) occurred in heterozygosity with the c.235delC and c.299delAT of *GJB2* (c.235delC/c.497A>G, c.235delC/c.194A>T and c.299delAT/c.194A>T) [15].

Mutations in *CLDN14* were identified in two families among 100 Pakistani families with profound congenital HI [16]. No *CLDN14* mutation was found in 60 Turkish families with ARNSHI [13].

To determine the spectrum and the frequencies of gap junctions and tight junction mutations in Tunisia, we screened 102 families with ARNSHI for mutations in the *GJB2*, *GJB3*, *GJB*6, and *CLDN14* genes. The 35delG mutation was found at homozygous state in 21 families and at heterozygous state in two families. The del(*GJB6*-D13S1830) was detected at heterozygous state in one family. There was no evidence of a second mutation in *GJB2*, *GJB6* or *GJB3* segregating with the HI in these three heterozygous families. Despite the important contribution of *GJB2* gene to HI, our data confirms the complexity of the molecular diagnosis of such a disorder.

Materials and methods

Subjects. We analysed 102 Tunisian families in which severe to profound NSHI is transmitted as an autosomal recessive trait. The medical history was obtained and all individuals were evaluated by clinical examination including otoscopic exploration and pure-tone audiometry. Syndromic forms and patients with suspected environmental cause of HI were excluded. As Controls, we used 99 unrelated Tunisian individuals. Appropriate informed consent was obtained from all subjects.

Mutation analysis. Genomic DNA was extracted from whole blood following a standard phenol–chloroform method. Mutation screening was performed by direct sequencing of the coding regions of *GJB2*, *GJB3*, *GJB6* and *CLDN14* genes. Primers used for PCR and sequencing are listed in Table 1. The coding exons in each DNA sample were first amplified by PCR. In brief, PCR was conducted in a volume of 25 μl containing 100 ng genomic DNA, 200 μM dNTP, 1 U of Taq DNA polymerase and 20 μM forward and reverse primers. The non-coding exon 1 and the relevant splice sites of *GJB2* were PCR-amplified as described by Denoyelle et al. [17]. The promoter region of the *GJB2* was amplified as described by Matos et al. [18]. Once purified, the PCR products were directly sequenced with one of the primers used for the amplification using an ABI 3100-Avant automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequence data were then compared with published sequences of the correspondent genes.

Table 1
Gene primers used for screening.

Gene	Name	Oligonucleotide (5′–3′)
CLDN14	CLDN1A	TTTCCTTTCTCTCCCTGCTC
CLDN14	CLDN1M	AGGATGGCAAAGGTGGTCTT
CLDN14	CLDN2A	CAGATCTACCGATCCCTGCT
CLDN14	CLDN2M	GACATTTCCTCGCATTCACA
GJB3	GJB3-1A	AGAGGGTCGTTGTGAGTATTG
GJB3	GJB3-1M	AGAGGCGGATGTTGGAGATG
GJB3	GJB3-2A	TGCTACGACAACTACTTCCCC
GJB3	GJB3-2M	GGCGCCCACCATGAAGTAG
GJB3	GJB3-3A	GCCCGACCTACCGAGAAGAA
GJB3	GJB3-3M	ACCTCTCCACCTGCCACAC
GJB6	GJB6-1A	GACTAGCAGGGCAGGGAGTT
GJB6	GJB6-1M	CTCTTCTCTCCTCGCCTGAA
GJB6	GJB6-2A	GACCACTTTTTCCCGGTGT
GJB6	GJB6-2M	AGGTTGGTATTGCCTTCTGG
GJB2	*GJB2*A	ACACGTTCAAGAGGGTTTGG
GJB2	*GJB2*M	GGGAAATGCTAGCGACTGAG

The oligonucleotide primers and the conditions used for PCR amplification of del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) have been previously described [9]. PCR products were then separated on 2% agarose gel. DNA from individuals with del(*GJB6*-D13S1830) or del(*GJB6*-D13S1854) were used as a positive controls for each amplification.

Results

Mutations in the *GJB2* gene were analyzed in 102 unrelated individuals with ARNSHI from Tunisia. The Tunisian population originated from a mixture of ethnicities (Phoenicians, Roman, Berber, Arab, Ottoman, etc.). This heterogeneity, along with the local customs and social traditions of the Tunisian population contributed to an increased level of endogamy and consanguineous marriages [19]. The c.35delG mutation was found at homozygous state in 21 families (20.58%) and at heterozygous state in 2 probands from two consanguineous families (Fig. 1). Analysis of these families showed no segregation between the c.35delG mutation and HI in family 2 (Fig. 1). Sequencing of the promoter, the non-coding exon of *GJB2* and the coding region of *GJB3* and *GJB6* revealed no mutation. In addition, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) were not detected in these patients.

All *GJB2*-wildtype affected individuals were sequenced for the coding region of *GJB6* gene. The del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) were also tested in all patients. The del(*GJB6*-D13S1830) mutation was identified at heterozygous state in only one family (1/102) with profound deafness. Analysis of the transmission of this deletion showed that only the affected individual II-4 carry the del(*GJB6*-D13S1830) (Fig. 2). Both of his parents with normal hearing were negative for this deletion suggesting that it might be a de novo mutation. No Mendelian transmission inconsistency was detected by analysis of several microsatellite markers in this family. No other mutation was found in *GJB2* or *GJB6* genes in this patient. The del(*GJB6*-D13S1830) was not detected in 99 unrelated control individuals.

Analysis of the entire coding region of the *GJB3* gene revealed the presence of two previously reported sequence variations at heterozygous state in five families. A C → T substitution at nucleotide position 94 (c.94C>T) in one family that leads to the substitution of a highly conserved Arginine residue in the first transmembrane domain (R) to Tryptophan (W) at amino acid position 32 (p.R32W). A silent alteration c.357C>T (p.D119D) in the cytoplasmatic loop was found in four familial cases.

Screening of *CLDN14* in the Tunisian families with ARNSHI revealed six sequence variations (Table 2). The c.243C>T transition was the most prevalent sequence variation (13.86%). It is a silent alteration that does not disturb the amino acid sequence. The c.11C>T variation was the second most frequent (9.9%) and was found either at homozygous or heterozygous state in affected individuals. Three other single nucleotide substitutions c.63G>A, c.243C>T and c.372C>A do not affect the *CLDN14* amino acid sequence. We also identified a novel heterozygous G → A substitution at nucleotide position 58 (c.58G>A) in one family. The c.58G>A would lead to the substitution of Glycine (G) for Serine (S) at amino acid position 20 (p.G20S).

Discussion

Mutations in *GJB2* have been found to be the predominant cause of ARNSHI in most populations worldwide [2,3]. Less frequently encountered are mutations in other *gap junction* genes such as *GJB6* and *GJB3* [8–11,20]. Previously, two studies have been carried out to analyse mutations in *GJB2* gene using a total of 140 Tunisian families with ARNSHI. In the first report, c.35delG mutation was

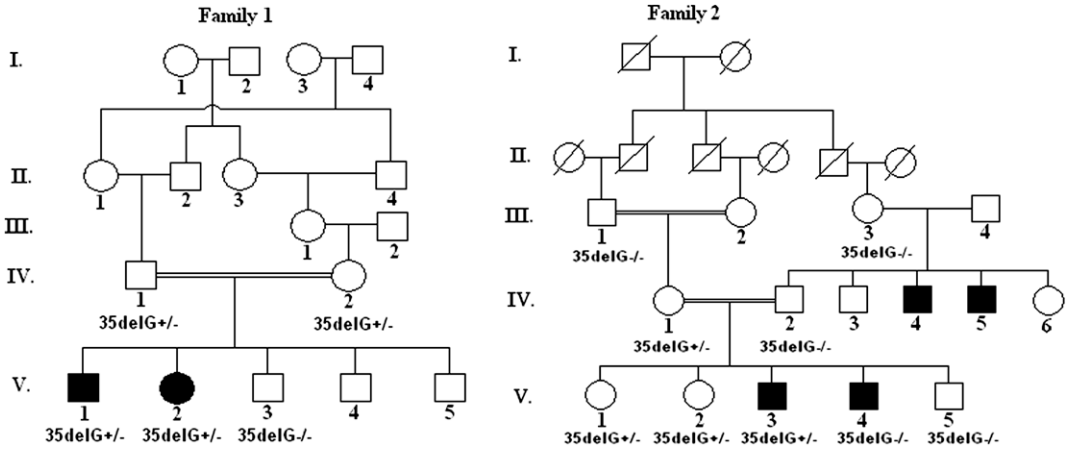

Fig. 1. Pedigrees of the two Tunisian families harbouring the 35delG mutation at heterozygous state.

found in 11 out of 70 families (15.71%) [21]. Whereas, it was detected in 17 out of 70 families (24.29%) in the second study [22]. In this study, we found that 22.55% of affected families have the c.35delG mutation. Combining these data, the prevalence of c.35delG in the Tunisian population was 21.07% (51/242). This result is in accordance with previous studies reporting that the c.35delG mutation is a major component of ARNSHI in Mediterranean population [4]. Evidence of Mediterranean ancestor for this mutation has been proposed previously which explain the high frequency of this mutation [22].

In this study, the c.35delG mutation was found in 2 consanguineous families at heterozygous state (Fig. 1). Previously, a large number of affected subjects with a single c.35delG mutation has been found in several populations. When compared to the c.35delG carrier frequency in normal-hearing controls, the number of deaf c.35delG carriers was greater than expected [23]. These results were explained by the existence of at least one other mutation outside the *GJB2* coding region that does not complement *GJB2* deafness-causing allele variants. Based on the c.35delG transmission in the pedigree of the family 2, no segregation was found between this variant and the phenotype of HI. Moreover, in these two families, no other mutation was detected in the non-coding exon and the promoter of *GJB2* and also in the coding exon of *GJB3* and *GJB6* genes. In addition, the two deletions del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) were not detected. In a previous study, analysis of 236 unrelated Tunisian controls from the general population showed three c.35delG heterozygotes, which gave a carrier prevalence of 1.3% [21]. The carrier frequency in this study (2/102, 1.96%) or in combined data (2/242, 0.82%) in the Tunisian deaf population showed no significant difference to those obtained in controls ($\chi^2 = 0.23$, 1 df, Fisher-exact test $p = 0.479$) and ($\chi^2 = 0.22$, 1 df, Fisher-exact test $p = 0.49$), respectively. We then assumed that 35delG mutation heterozygous individuals in our study were simple carriers for this mutation. Results generated in some populations are not applicable to other populations and the hypothesis of double heterozygosity should be considered with prudence in population with high level of consanguinity.

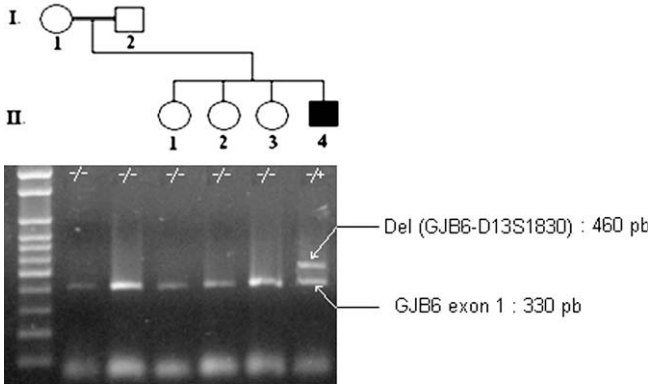

Fig. 2. Pedigree of the Tunisian family harbouring del(*GJB6*-D13S1830) in *GJB6* gene at heterozygous state. Separation of PCR products by electrophoresis in a 2% agarose gel. The fragment sizes corresponding to the deletion and to *GJB6* exon 1 are shown on the right. I1, I2, II2, II2 and II3: Wild type (wt); II4: del(*GJB6*-D13S1830)/wt heterozygote.

Our analysis detected del(*GJB6*-D13S1830) in one family at heterozygous state whereas the del(*GJB6*-D13S1854) was not found. The del(*GJB6*-D13S1830) was found only in the patient although his parents were normal suggesting that it may be a de novo mutation. The paternity tie was confirmed by analysis of several microsatellite markers. In a multicenter study in nine countries, Del Castillo et al. showed that del(*GJB6*-D13S1830) is present in most of the screening populations, with higher frequencies in France, Spain, and Israel. Moreover, analysis of haplotypes associated with the *GJB6* deletion revealed a founder effect for countries in Western Europe [24,25]. Absence of the del(*GJB6*-D13S1854) deletion in Tunisian (this study), Turkish, Austrian, Chinese and Greek Cypriot

Table 2
Sequence alterations in *CLDN14* in patients with NSHI.

Nucleotide change	Amino acid change	Frequency	Genotype state	References
c.C11T	p.T4M	10/101 (9.9%)	2 heterozygous 8 homozygous	Uyguner et al. [13]
c.G58A	p.G20S	1/101 (0.99%)	1 heterozygous	This study
c.G63A	p.T21T	1/101 (0.99%)	1 homozygous	Uyguner et al. [13]
c.C243T	p.R81R	14/101 (13.86%)	5 heterozygous 9 homozygous	rs219780
c.C372A	p.L120L	3/101 (2.97%)	3 heterozygous	This study
c.G787A	p.T229T	6/101 (5.94%)	6 heterozygous	rs219779

hearing loss patients [11,13,24,26] indicated that this mutation is restricted to certain populations.

The screening of *GJB3* gene identified 2 sequence variations in 2 patients. The c.C94T transition results in the substitution of a positively charged amino acid arginine to a neutral amino acid tryptophan at codon 32 (p.R32W). The altered arginine residue is conserved across species and across other members of the connexin family. This variant was previously identified in patients with NSHI, but it was also detected in control cases indicating that p.R32W is a coding polymorphism [14,27,28]. Kelsell et al. speculated that p.R32W may contribute to the severity of hearing impairment based on its segregation within a small family harbouring the p.D66H mutation in *GJB2* [29]. The second alteration c.357C>T was detected in four families. This variation represents single nucleotide polymorphism (SNP) that does not alter the translated polypeptide. This substitution was also detected in heterozygous pattern in 8.8% and 1% of patients from Austrian and Moroccan population, respectively [28,30]. This result would lead to conclude that alterations in *GJB3* gene have no genetic relevance in the Tunisian hearing-impaired population with or without *GJB2* alterations.

In this study, sequencing of *CLDN14* gene showed 6 sequence variations in 27 affected families. Four of the six sequence variations represent silent SNP that do not alter the polypeptide sequence. Two novel *CLDN14* polymorphisms were observed: c.58G>A (p.G20S) and c.372C>A (p.L120L). No mutation was found in the *CLDN14* gene confirming that alterations in this gene are not common cause of ARNSHI in Tunisia [13].

In summary, we have assessed the relative contribution of *GJB2*, *GJB3*, *GJB6* and *CLDN14* mutations in ARNSHI in the consanguineous Tunisian population. The data presented in this study indicate that mutations in the *GJB2* gene are prevalent in Tunisian patients. Interestingly, individuals with a single DFNB1 mutant allele (c.35delG or del(*GJB6*-D13S1830)) were detected, but we concluded that they are likely to be coincidental carriers. Finally, we confirmed that mutations in other gap and tight junction genes are unlikely to be of epidemiological importance. These results strengthen the difficulty of genetic diagnosis of ARNSHI if no biallelic *GJB2* mutations were detected.

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