BMC Medical Genetics

BioMed Central

Research article

Molecular epidemiology of DFNB1 deafness in France

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Published: 06 March 2004

BMC Medical Genetics 2004, **5**:5

Received: 10 December 2003
Accepted: 06 March 2004

This article is available from: http://www.biomedcentral.com/1471-2350/5/5

Abstract

Background: Mutations in the *GJB2* gene have been established as a major cause of inherited non syndromic deafness in different populations. A high number of sequence variations have been described in the *GJB2* gene and the associated pathogenic effects are not always clearly established. The prevalence of a number of mutations is known to be population specific, and therefore population specific testing should be a prerequisite step when molecular diagnosis is offered. Moreover, population studies are needed to determine the contribution of *GJB2* variants to deafness. We present our findings from the molecular diagnostic screening of the *GJB2* and *GJB6* genes over a three year period, together with a population-based study of *GJB2* variants.

Methods and results: Molecular studies were performed using denaturing High Performance Liquid Chromatograghy (DHPLC) and sequencing of the *GJB2* gene. Over the last 3 years we have studied 159 families presenting sensorineural hearing loss, including 84 with non syndromic, stable, bilateral deafness. Thirty families were genotyped with causative mutations. In parallel, we have performed a molecular epidemiology study on more than 3000 dried blood spots and established the frequency of the *GJB2* variants in our population. Finally, we have compared the prevalence of the variants in the hearing impaired population with the general population.

Conclusion: Although a high heterogeneity of sequence variation was observed in patients and controls, the 35delG mutation remains the most common pathogenic mutation in our population. Genetic counseling is dependent on the knowledge of the pathogenicity of the mutations and remains difficult in a number of cases. By comparing the sequence variations observed in hearing impaired patients with those sequence variants observed in general population, from the same ethnic background, we show that the M34T, V37I and R127H variants can not be responsible for profound or severe deafness.

Background

The genetic origin of deafness is suspected in more than half of the congenital hearing loss cases. More than 400 syndromes can include hearing loss or deficient hearing functions as a component. However, non syndromic expression of deafness is observed in more than 70 % of cases. In the non syndromic forms of hearing loss (NSHL), familial or sporadic cases are observed and the transmission is predominantly autosomal recessive. Genetic heterogeneity has been established by linkage studies: more that 50 loci associated with NSHL (including dominant and recessive autosomal, and X-linked types of transmission) have been localized, making possible the identification of a number of causative deafness genes http://dnalab-www.uia.ac.be/dnalab/hhh/. Despite the extreme genetic heterogeneity, the recessive DFNB1 locus, mapping to chromosome 13q12, is by far the most prevalent. This locus contains the two Gap junction genes *GJB2* and *GJB6*, encoding, respectively connexin 26 (CX26) and connexin 30 (CX30). These proteins associate in hexamers to form homo- and hetero-connexons [1,2]. Two connexons from adjacent cells dock to form a functional channel that will allow, among other small molecules, the diffusion of potassium ions critical for the normal sensory hair cell excitation [3].

The contribution of the *GJB2* gene in NSHL varies from 0 to 40 % in diverse populations [4] and this genetic heterogeneity is also emphasized by the variation in frequency of specific mutations among different populations. More than 70 mutations in the *GJB2* gene have been reported [5], and although the majority are rare or private, the prevalence of four mutations define specific ethnic origins. The 35delG mutation accounts for approximately 70 % of *GJB2* mutant alleles in Northern and Southern European, as well as American Caucasian populations, with a carrier frequency of 2.3 % to 4 % [6-9]. The three other mutations, 167delT, 235delC or R143W represent the most common pathogenic alleles in Ashkenazi Jews [10], Asian [11-14] and Ghanian populations [15,16], respectively.

Recently, we and other groups have identified a large 309 kb deletion that includes the 5' region of the *GJB6* gene and most of its coding region [17]. It is unclear whether this deletion removes regulatory elements common to *GJB6* and *GJB2* resulting, in addition to the deletion of *GJB6*, in reduced expression of the wild type *GJB2* gene [17-20]. This deletion also appears to have an ethnic specific origin as it is absent from the Siberian (manuscript in preparation), Chinese [21], Austrian and Italian populations [17,22]. In this report, when we refer to *GJB6* mutations we will consider only this particular mutation Δ(*GJB6*-D13S1830).

Molecular diagnostic testing of non syndromic deafness was initiated in Montpellier in early 2000. This testing was carried out in parallel with a molecular epidemiology study of *GJB2* variants in the Languedoc Roussillon region. Although many reports have estimated the carrier frequency in French and Mediterranean populations (mostly of the 35delG mutation), differences of frequency between samples are observed (0.0 to 2.7 % in France). This is essentially due to the size and composition of control samples [23]; for review see [4]. Assessment of *GJB2* variant sequence distribution in Languedoc Roussillon was necessary, as we had observed significant differences in the distribution of *CFTR* mutations between several French regions [24].

In this study, we present our results from three years of molecular diagnostic testing of *GJB2/GJB6* including the clinical and associated audiologic findings and also determine the prevalence and spectrum of DFNB1 mutations in the southern France population. In addition, we report the first screening of the most frequent *GJB2* variants, on several thousand dried bloodspots (Guthrie cards) from newborns and thus re-evaluate the pathogenic status of some *GJB2* variants.

Methods

Patients

A total of 159 unrelated families, comprised of 184 patients with sensorineural hearing loss, were referred from the Genetic Counseling Department and/or the Ear, Nose and Throat specialized clinics (Centre Hospitalier Universitaire of Montpellier and Lyon). All patients had permanent hearing loss not caused by infections, exposure to drugs or other prenatal or perinatal etiology of deafness. Informed consent was obtained for each individual. The hearing loss could be moderate to profound, bilateral or unilateral, symmetrical or asymmetrical, stable or progressive, pre or post-lingual, syndromic or non syndromic. From family histories, 79 patients were classified as sporadic cases, 54 defined as familial cases, including 33 with autosomal recessive, 19 with autosomal dominant, one with X-linked and one ambiguous modes of inheritance. Seven families were consanguineous. For 26 patients, family histories were unknown.

Among these 159 families, 84 were affected with a non syndromic, stable, bilateral, congenital, mild to profound deafness: 55 families had a single deaf child and the others, considered as familial cases, showed an autosomal recessive (24 cases), dominant (4 cases) or unclear (1) mode of inheritance. Hearing loss was moderate in 4 families, mild in 17, severe to profound in 60 and observed with variable expression in 3 families.

Audiological assessment

Pure-tone audiometry (PTA) was performed on every affected family member. PTA was performed for air conduction on each ear using an Interacoustics audiometer. Air-conduction thresholds were obtained at 0.5, 1, 2, 3, 4, 6 and 8 kHz. Severity of deafness was defined as mild (20–40 dB HL), moderate (41–70 dB HL), severe (71–90 dB HL) or profound hearing loss (above 90 dB HL). In young children, behavioral audiometry was used to determine the auditory thresholds in free field conditions. All subjects had an auditory brainstem response (ABR) assessment to determine the hearing loss level for high frequencies.

DNA extraction from patient WBC and mutation analysis

Blood samples were obtained from deaf patients, and their parents and sibs (when possible) and DNA was extracted with the Nucleon BACC3 DNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ).

All samples were tested for the 35delG mutation, the non coding and coding exons of *GJB2* (E1 and E2 respectively) and for the Δ(*GJB6*-D13S1830) mutation. The experimental protocols were as follows: 35delG mutation was screened by PCR-mediated site-directed mutagenesis (PSDM assay) as previously described [6,25]. *GJB2* was screened by DHPLC and sequencing analysis as previously described [25]. To obtain an optimal detection of mutations, some primers have been modified and are as follows: R1-F: AGTCTCCCTGTTCTGTCCTA, R3-F: TTCTCCATGCAGCGGCTGGT, R3-R: TGAGCACGGGTTGCCTCATC. The 309 kb deletion including most of the *GJB6* gene, Δ(*GJB6*-D13S1830), was screened by two different PCRs encompassing the deletion breakpoints using the following primers PCR1: CCACCATGCGTAGCCTTAACC /GCAGCAGGTAGCACAACTCT; PCR2: CACTGAAGTGGTTTCTTGTGC /TCTGTGCTCTCTTTGATCTC, revealing breakpoint-junction fragments of 390 and 335 bp, respectively.

DNA extraction from dried bloodspots and mutation analysis of GJB2

Guthrie cards were obtained from the GREPAM (Center for neonatal screening) of Montpellier. All samples were anonymized and no phenotypic data could therefore be available. Spots of 3 mm diameter, punched from the cards, were distributed in 96-well plates and DNA was extracted using methanol extraction [26]. All experiments were then set up using the robot BioMEK 2000 (Beckman). A first set of 2,777 spots were screened using the PSDM assay, specific for the 35delG mutation. When DHPLC technology became available in the laboratory, a further 3,516 spots were analyzed for the R1' fragment (covering from the ATG codon to position 230 of the coding sequence (CDS)). 528 of these 3 516 spots were also analyzed in the R2 fragment (position 190 to 500 of the CDS). Pools of 2 DNAs were systematically used for DHPLC screening, thus eliminating the risk of missing the detection of a homozygote sample. Any abnormal DHPLC profile was re-evaluated on individual DNA samples followed by sequencing analysis.

Statistical analysis

Statistical analyses were performed with the Rv.1.3.1 software (The free software Fundation, Inc). Proportion, chi-square and Fisher exact test were used to test differences between groups. All p-values were taken to be significant at <0.05. When observed or expected values were below 5, a Fisher exact test was performed.

Results

Patients

The analysis of the coding and non coding *GJB2* exons plus the Δ(*GJB6*-D13S1830) screening allowed us to genotype 30 unrelated individuals with biallelic *GJB2* and/or *GJB6* (Δ(*GJB6*-D13S1830)) mutations (Table 1). Clinical and audiological evaluation showed that 27 of these patients with biallelic DFNB1 mutations had bilateral congenital severe or profound NSHL with no evidence of progressive phenotype. No mutation was identified among families with an autosomal dominant mode of inheritance.

Sixteen additional patients carried 2 *GJB2* mutations/variants with a controversial pathogenic effect or a single *GJB2* sequence variation (Table 2).

A total of 21 different *GJB2* sequence variations (1 in frame deletion, 5 nonsense, 4 frameshift, 8 missense, 1 splicing, 1 in the 5' untranslated region (UTR) and 1 in the intron 1(IVS1)) were found in 46 unrelated subjects from the cohort presented here. All were previously reported with the exception of the C64X (c.192 C>A) mutation, the -34T>G and IVS1-12C>T sequence variations (Tables 1 and 2). Ten subjects were 35delG homozygotes, 2 were Δ(*GJB6*-D13S1830) homozygotes. All the other patient mutations were found in the compound heterozygous state, with the 35delG accounting for 35/60 (58.3 %) of the *GJB2*/*GJB6* mutated alleles (Table 1).

Δ(*GJB6*-D13S1830) is the second most frequent mutation (9/60) and accounts for 15 % of the mutated alleles: in addition to the 2 homozygotes patients, 5 patients were identified as compound heterozygotes Δ(*GJB6*-D13S1830) /*GJB2* mutation.

We assessed the polymorphism 765C>T (referred to as SNP1 [27]) in 159 unrelated patients (see Table 3). The frequencies of the genotypes 765C>C, C>T, T>T in control samples (with no *GJB2* sequence variations, N = 113) are

Table 1: DFNB1 genotypes identified in 30 patients with deafness

Number of families	Genotype	Degree of deafness	Mode of inheritance**
10	35delG/35delG	10 profound	5 SC; 4 AR; 1cAR; 1un
2	35delG/E47X	2 profound	2AR
2	35delG/312del14	1 profound, 1 moderate	1AR
1	35delG/N206S	1 moderate	1AR
1	35delG/R184P	1 profound	1 SC
1	35delG/W24X	1 profound	1AR
1	35delG/C64X*	1 profound	1 SC
1	35delG/delE120	1 moderate	1AR
1	35delG/Q57X	1 profound	1SC
1	35delG/R143W	1 profound	1SC
1	35delG/W44X	1 profound	1SC
1	290insA/IVS1+1G>A	1 profound	1AR
3	35delG/Δ(GJB6/D13S1830)	3 profound	1 SC; 1AR; 1un
2	Δ(GJB6/D13S1830)/Δ(GJB6/D13S1830)	2 profound	1 SC; 1 AR
1	E47X/Δ(GJB6/D13S1830)	1 profound	1un
1	235delC/Δ(GJB6/D13S1830)	1 profound	1 AR

top: genotypes of the 23 familieswith GJB2 biallelic mutations; bottom: 7 families with DFNB1 mutations All cases presented bilateral, congenital hearing loss with no evidence of progression * novel mutation ** SC: Sporadic case; AR: autosomal recessive; cAR: autosomal recessive with consanguinity; un: unknown

Table 2: GJB2 genotypes with unknown consequences in 16 families

Number of families	Genotype**	Hearing loss****	Mode of inheritance
3	35delG/+	1P progressive; 1M; 1U	1SC,1AR, 1un
2	R127H/M34T	2P	1AR,1SC
1	R127H/W24X	P to M ***	1AR
1	M34T/V37I	1 mild	1SC
2	M34T/+	1 mild, 1M	2AD
1	(IVS1-12C>T)2*/-34T/G*	P	un
2	IVS1-12C>T*/+	1P progressive, 1P	1SC, 1un
2	V37I/+	1M, 1P	1AR, 1un
1	V153I/+	M	1SC
1	G160S/+	P	1SC

* novel sequence variant ** + designates the wild type allele *** variable phenotype within the family **** **P:** profound; M: moderate; U: unilateral; un: unknown

57.5 %, 39.8 % and 2.7 % respectively. However, all alleles carrying the 35delG mutation (n = 38) and the 309 kb *GJB6* deletion (n = 9) were associated with the 765T variant.

Parents of deaf individuals

No *de novo* mutations were detected in patients, as every parent of patients carrying bi-allelic *GJB2*/*GJB6* mutations was heterozygous for one of the mutations. In two unrelated families, 2 normal-hearing parents of children genotyped R127H/M34T or R127H/W24X, were found to be homozygous R127H. Interestingly, in each of these families, a normal-hearing sib also carried the genotype R127H/M34T or R127H/W24X. Thus, R127H may not be associated with deafness.

Analysis in the general population

Twenty-two different *GJB2* sequence variations (lying in R1' and R2) were identified in the general population and are listed in table , together with the calculated carrier frequencies. Ten of these sequence variations were detected more than once (35delG, M34T, V37I, V27I, W24X, E47X, Y68C, R127H, V153I, F83L), with relative frequencies

Table 3: Distribution of the C765T polymorphism among patients

35delG				
	C/C	**C/T**	**T/T**	**p-values**
Patients 35delG/35delG	0	0	10	$p < 10^{-6}$
Patients 35delG/other	0	13	5	
Controls	65	45	3	
Δ(GJB6/D13S1830)				
	C/C	**C/T**	**T/T**	**p-values**
Patients Δ(GJB6/D13S1830)/Δ(GJB6/D13S1830)	0	0	2	$p < 10^{-6}$
Patients Δ(GJB6/D13S1830)/other	0	3	2	
controls	65	45	3	

Controls: patients with no *GJB2* mutation. Chi-square test was used to test the independence between the genotype 765T/T and the control group.

Table 4: Frequencies of the sequence variations identified in the general population from Languedoc-Roussillon.

Name	Nucleotide change	Number of alleles	Number of chromosomes tested	Allele frequencies in % (95 % CI)	Carrier frequencies in % (95 % CI)
M34T	101T>C	81	7 032	1.15 (0.92–1.44)	2.3 (1.83–2.86)
35delG	c.35delG	96	12 586	0.76 (0.62–0.93)	1.53 (1.24–1.85)
R127H	380G>A	7	1 056	0.66 (0.29–1.42)	1.33 (0.58–2.82)
V37I	109G>A	30	7 032	0.43 (0.29–0.62)	0.85 (0.58–1.24)
V153I	457G>A	4	1 056	0.38 (0.12–1.04)	0.76 (0.14–1.79)
F83L	249C>G	3	1 056	0.28 (0.07–0.90)	0.57 (0.14–1.79)
V27I	79G>A	10	7 032	0.14 (0.07–0.27)	0.28 (0.14–0.58)
E114G	341A>G	1	1 056	0.09 (0.005–0.61)	0.19 (0.009–1.22)
DelE120	358-360delGAG	1	1 056	0.09 (0.005–0.61)	0.19 (0.009–1.22)
G160S	478G>A	1	1 056	0.09 (0.005–0.61)	0.19 (0.009–1.22)
D159D*	477C>T	1	1 056	0.09 (0.005–0.61)	0.19 (0.009–1.22)
W24X	71G>A	5	7 032	0.07 (0.03–0.18)	0.14 (0.06–0.36)
E47X	139G>T	4	7 032	0.06 (0.02–0.16)	0.11 (0.04–0.32)
Y68C*	203A>G	3	7 032	0.04 (0.01–0.14)	0.09 (0.02–0.28)
W44X	132G>A	1	7 032	0.014 (0.007–0.092)	0.03 (0.01–0.18)
R32H	95G>A	1	7 032	0.014 (0.007–0.092)	0.03 (0.01–0.18)
S19T	56G>C	1	7 032	0.014 (0.007–0.092)	0.03 (0.01–0.18)
IVS1-7G>A*		1	7 032	0.014 (0.007–0.092)	0.03 (0.01–0.18)
G4D*	11G>A	1	7 032	0.014 (0.007–0.092)	0.03 (0.01–0.18)
Q7Q*	21G>A	1	7 032	0.014 (0.007–0.092)	0.03 (0.01–0.18)
T26T*	78C>T	1	7 032	0.014 (0.007–0.092)	0.03 (0.01–0.18)
H67R*	200A>G	1	7 032	0.014 (0.007–0.092)	0.03 (0.01–0.18)

*: novel sequence changes.

ranging between 2,3 % to 0,09 % and 7 of these were not previously reported (Y68C, IVS1-7G/A, G4D, Q7Q, T26T, H67R, D159D).

The M34T variant is the most frequent, with a carrier frequency of 1/43 (2.3 %). In addition, among the 3 516 dried bloodspots screened, one homozygote M34T/M34T was detected.

Table 5: Comparison of the allelic frequencies of GJB2 sequence variations in unrelated french patients with NSHL and in the general population

GJB2 Sequences variations	Number of unrelated chromosomes				Significance (p-values)
	patients (n = 318)		general population		
35delG	38	11.9 %	96/12586	0.76 %	S ($p < 10^{-6}$)
M34T	5	1.6 %	81/7034	1.15 %	NS (p = 0.66)
R127H	3	0.94 %	7/1056	0.66 %	NS (p = 0.89)
V37I	3	0.94 %	30/7034	0.43 %	NS (p= 0.36)

-: not studied; S: significant difference; NS: no significant difference. P-values significant at <0.05

The 35delG mutation was screened in a total of 6,293 newborns (2,777 were analyzed by PSDM and 3,516 by PCR-DHPLC). Ninety-two 35delG heterozygotes and 2 35delG homozygotes were identified, resulting in a carrier frequency of 1/66 (1.53 %) for 35delG.

In addition, 3 individuals were compound heterozygotes (M34T/35delG, V37I/G160S and V27I/E114G) for *GJB2* sequence variants.

We also present the allelic frequencies of some *GJB2* mutations in individuals referred for NSHL with respect to the frequency in the general population (Table 5). We could not detect any significant difference between the two groups for the V37I, R127H and M34T sequence variations.

Discussion

The 35delG mutation in patients and in general population

Thirty families were clearly genotyped with causative mutations in *GJB2*/*GJB6*. Homozygosity for 35delG was found in 33.3 % (10/30) of the genotyped unrelated deaf patients, 50 % were carrying this deletion in a compound heterozygous state and 16.7 % had other mutations. The 35delG accounts for 58.3 % (35/60) of the DFNB1 mutated alleles in these families. As in previous studies, this study shows an important implication of *GJB2* in non syndromic prelingual hearing loss. However, we have observed a lower frequency of the 35delG mutant allele and a higher heterogeneity of other mutations than in previous studies [28-30].

Our study region (Languedoc Roussillon) shows a significantly higher carrier rate of the 35delG (1.53 % – 1/66), compared to the North-East part of Europe (0.9 % – 1/110 among 1,212 controls) [31] and a lower carrier rate compared to other south European areas such as Spain (2.31 % – 1/43) [7], Italy (3.45 % – 1/32) [23] and Greece (3.54 % – 1/28) [32]. The epidemiologic study presented here is based on the largest number of random samples describing an unbiased general population screen and once more supports the heterogeneous composition of the Languedoc Roussillon population. This situation has direct implications for genetic counseling as well as on the development of potential diagnostic kits (as it was in the case for the design of the CF neonatal screening kit [24]). The explanation for such a different carrier rate lies in the heterogeneity of the migrations "landing" in Languedoc Roussillon, historically and still today. A recent study, carried out on the prevalence of Hemochromatosis gene (HFE) mutations in the Languedoc Roussillon population has annotated the origins of the four grand-parents for each newborn. It was observed that the population originated from various regions in France and also from other European or African countries [33].

Other sequence variations in the GJB2 gene

35delG remains by far the most frequent mutation in the hearing impaired population, although its frequency is lower than was expected in comparison with other Mediterranean areas. The second most common mutation in our region is the Δ(*GJB6*-D13S1830) with 15 % of the mutated alleles, and the E47X mutation represents 5 %. The other mutations were identified twice or only once.

This genetic heterogeneity is emphasized by the number of sequence variations observed in both the patient and general population. Ninety variations have been reported [5]. In this study, besides 35delG and Δ(*GJB6*-D13S1830), we describe 33 sequence variations, 10 for the first time (-34T>G, IVS-12C>T, IVS1-7G>A, G4D, Q7Q, T26T, C64X, H67R, Y68C, D159D tables 1, 2, 4). The pathogenicity of 11 of these has been previously well established (E47X, 312del14, 290insA, IVS1+1G>A, W24X, W44X, delE120, Q57X, R143W, W44X, 235delC). As well, the novel mutation C64X described in this study results in a truncated protein with pathogenic consequences. Of the 21 other sequence variations observed, four were previously reported as recessive mutations (R32H, S19T, N206S,

R184P), five are known non pathogenic variants (V27I, F83L, E114G, V153I, G160S), three of them are silent (D159D, Q7Q, T26T), six have unknown consequences (-34T>G, IVS1-12C>T, IVS1-7G>A, G4D, H67R, Y68C) and three are still controversial (M34T, V37I, R127H and see below). Three of the newly identified sequence variations are in the noncoding region. According to splicing prediction programs, both IVS variations should be silent. Interestingly, the IVS1-12C>T was found in a homozygous state in a patient originating from Guadeloupe with one IVS1-12C>T variation allelic to the -34T>C variation. This T to C transition has yet unknown consequences on the regulation of the *GJB2* gene.

Founder effects and sequence variations

A founder effect for the 35delG mutation has recently been described [27], which explains the variable frequency of this mutation in different populations rather than resulting from a mutational hot spot. Similarly, the 235delC mutation, frequent in the Japanese population, is derived from a common ancestor [34]. Founder effects may account for a number of other *GJB2* sequence variations, whose frequencies depend on ethnic background (such as M34T [4]). The polymorphism 765C>T (referred to as SNP1 [27]) has been systematically included in our series. The 765T allele showed complete association with the 35delG as well as the Δ(*GJB6*-D13S1830). The genotype comparison between *GJB2* and/or Δ(*GJB6*-D13S1830) patients with *GJB2* negative subjects is significant with P-values < 10^{-6} (Table 3). These results are in accordance with the fact that Δ(*GJB6*-D13S1830) is absent in some populations and strongly suggest the existence of a founder effect as confirmed by a recent multicenter study [17].

Controversial effects of sequence variations

Among the sequence variations that have controversial consequences, a few variants have been extensively discussed based on families with deafness and on studies using *in vitro* expression systems. Since we have performed a population based study, in parallel with the analysis of a number of hearing impaired patients, we show that M34T, V37I and R127H represent common variants that are not responsible for severe or profound deafness.

The M34T variation was first described as a dominant mutation [35] and the dominant negative effect was supported by *in vitro* functional studies [36,37]. However, the description of normal hearing carriers abolished this hypothesis and furthermore, normal hearing patients were found to be compound heterozygotes, M34T/167delT or M34T/35delG [29,38]. Since the two alleles *in trans* of the M34T corresponded to a null allele, Griffith et al. (2000) suggested that the M34T was functional *in vivo* and therefore, the phenotypic consequences of the M34T allele would depend on the opposing CX26 allele variant [39]. The possibility of considering the M34T as a non pathogenic variant was also raised [40,41].

M34T represents the highest carrier rate in our population. We do not deal with a carrier rate of 1/116 as estimated from a small sample in Paris [29,41] but with 1/43 (2.3 %). We identified one M34T homozygote in the general population, as expected from the carrier frequency (1/4,444). The M34T is more frequent than the 35delG and, in contrast to the 35delG mutation, no M34T homozygote or in compound heterozygosity with a deleterious mutation was observed in our cohort of patients. This carrier rate is similar to the one observed by Green et al. [9]. The allele frequency of 1.15 %, based on 3,516 individuals, shows no significant discrepancy with the M34T allele frequency in the deaf population (1.6 %). Although a study based on the general population does not rule out the possibility of hearing deficiency in few individuals, these data eliminate the possibility of considering the M34T as a dominant or recessive mutation associated with severe or profound deafness.

The R127H, first described by Estivill [7] is also contentious and functional studies of this variant are inconsistent [42,43]. The frequency of carrier rate of in our region is 1/75 (1.33 %), not significantly different from that of the deaf population. Moreover, two normal-hearing parents were genotyped R127H/R127H.

Finally two normal-hearing sibs were compound heterozygotes R127H/M34T or R127H/W24X emphasizing the non pathogenic nature of this sequence variation. However, the genotype R127H/M34T was identified twice in our patient cohort (2/159 1.25 %) but never in the general population (odds to be associated randomly of 1.5×10^{-4}). The observed frequency in the patients is significant (p = 1.3 %), and therefore we still cannot rule out that the combined genotype R127H/M34T can act as a variant that, under certain circumstances (associated with other modifiers such as alterations in other deafness genes), would contribute to the phenotype.

Similarly, the V37I variation does not show any significant difference in frequency between the general and deaf populations. This is consistent with the fact that it was originally identified as a non pathogenic polymorphism because of its occurrence in a heterozygous state in the general population [40,44]. However, homozygosity for V37I and compound heterozygosity of V37I were often described in patients with NSHL suggesting that V37I acts as a recessive mutation. Recent studies [11,28,45,46] clearly indicated its pathogenicity when associated with another mutated *GJB2* allele. Functional analyses [47]

also showed that V37I is devoid of functional activity and thus may be pathogically significant.

The likelihood of M34T to be associated with V37I is 1×10^{-4}, as calculated from the allele frequency, and is significantly different from the observed frequency in patients (p = 0.63 %), and once more can not rule out that the genotype M34T/V37I is not associated with mild or moderate deafness.

These data demonstrate the challenge of interpretating the association of two sequence variations. Moreover, combined genotypes with variants such as M34T, V37I, or R127H could have a phenotypic expression modulated by environmental factors or modifier genes. Conclusions from *in vitro* transfection assays can not be taken as complete because one sequence variant supposedly acting as a recessive mutation should be at least co-transfected with a second recessive mutation; but above all, one wonders whether the different cell types used for these *in vitro* experiments really reflect the molecular context existing *in vivo*. Complementary assays considering co-transfection of two different mutations need to be performed. These, together with compilation of observed combined genotypes correlated with phenotypes, should help in the interpretation of the molecular tests.

Phenotypes associated with GJB2/GJB6 *mutations*

Although a recent report recommended *GJB2* screening in cases of progressive and recurrent sudden HL [31]), no *GJB2* and/or *GJB6* biallelic mutations were identified in patients with progressive, postlingual, asymmetrical hearing loss in this study. The contribution of the *GJB2/GJB6* genes, in our cohort is exclusively found in non syndromic, prelingual, bilateral, stable deafness and is about 33 % (28/84). This is similar to the proportion found in Spain (31.6 %) [28], in France (39.8 %) [48] and in Greece (33.3 %) [30]. However the degree of implication of *GJB2/GJB6* genes in deafness depends on the composition in degree of severity of the patient group. In this study, the rate of DFNB1-associated deafness would be 41.6 % (25/60) if only congenital profound NSHL was considered.

Conclusions

The data presented here demonstrate that genetic counseling has been greatly improved with the identification of the Δ(*GJB6*-D13S1830) mutation and a total of 30 families could benefit from a *GJB2* and/or *GJB6* unambiguous molecular diagnosis. The genetic counseling was more difficult for 16 families (1/3) because of the compound heterozygosity of poorly-defined variations or the presence of a single *GJB2* alteration. Additional data certainly need to be collected to evaluate if two sequence variations considered as non-disease causing stay neutral when associated in trans.

The number of families (129) for which no genetic counseling could be provided, based on the mutation screening of the DFNB1 genes, remains very high and demonstrates that criteria for genetic testing must be very well defined and set according to the provided test (CX26). Genetic heterogeneity is still observed when prelingual, non syndromic, stable deafness is present, and the possibility for screening mutations in other genes should be offered.

Finally, neonatal screening programs bring a great improvement in the management of deafness; however, the option to offer molecular based screening remains very uncertain and if offered should be based on specific population studies because of the genetic heterogeneity of deafness.

Competing interests

There are no competing interests

Authors' contributions

FA, PB, GL and MD referred patients; NPR, AV, VF, CT and DL carried out the molecular studies; NM performed the statistical analyses; AFR supervised the whole study in the laboratory of MC.

Acknowledgements

We are grateful to the families, patients and control individuals who permitted us to perform this study. We also thank the GREPAM for efficient sample referral, Dr. Marie-Christine Picot for helpful comments, Sabine Low Hong for technical assistance, and Gail Billingsley for critical reading of the manuscript. This work was, in part, supported by a grant "PHRC 2001" from "la Recherche Clinique", CHU Montpellier.

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Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2350/5/5/prepub