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Report

Prevalence and Evolutionary Origins of the del(*GJB6*-D13S1830) Mutation in the *DFNB1* Locus in Hearing-Impaired Subjects: a Multicenter Study

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Mutations in *GJB2*, the gene encoding connexin-26 at the *DFNB1* locus on 13q12, are found in as many as 50% of subjects with autosomal recessive, nonsyndromic prelingual hearing impairment. However, genetic diagnosis is complicated by the fact that 10%–50% of affected subjects with *GJB2* mutations carry only one mutant allele. Recently, a deletion truncating the *GJB6* gene (encoding connexin-30), near *GJB2* on 13q12, was shown to be the accompanying mutation in ~50% of these deaf *GJB2* heterozygotes in a cohort of Spanish patients, thus becoming second only to 35delG at *GJB2* as the most frequent mutation causing prelingual hearing impairment in Spain. Here, we present data from a multicenter study in nine countries that shows that the deletion is present in most of the screened populations, with higher frequencies in France, Spain, and Israel, where the percentages of unexplained *GJB2* heterozygotes fell to 16.0%–20.9% after screening for the del(*GJB6*-D13S1830) mutation. Our results also suggest that additional mutations remain to be identified, either in DFNB1 or in other unlinked genes involved in epistatic interactions with *GJB2*. Analysis of haplotypes associated with the deletion revealed a founder effect in Ashkenazi Jews and also suggested a common founder for countries in Western Europe. These results have important implications for the diagnosis and counseling of families with DFNB1 deafness.

Hearing impairment is the most common sensory disorder. In developed countries, >60% of the cases are due to genetic causes (Petit et al. 2001). Nonsyndromic forms, in which the hearing deficit is the only clinical sign, are highly heterogeneous, with >80 loci already reported and 30 genes identified so far (Hereditary Hearing Loss Homepage). Hearing impairment that manifests before speech acquisition (i.e., with prelingual onset) is mainly inherited in an autosomal recessive pattern, with 31 different loci and 16 currently identified genes (Hereditary Hearing Loss Homepage).

The *DFNB1* locus for nonsyndromic, autosomal recessive, prelingual hearing impairment (MIM 220290) was mapped to the 13q12 region (Guilford et al. 1994). This locus contains the *GJB2* gene (MIM 121011), encoding connexin-26 (Cx26), a transmembrane protein

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subunit of intercellular gap junctions (Kelsell et al. 1997). Six monomers of connexin bind together to form a hexamer (connexon), which, in turn, docks with another connexon on the surface of an adjacent cell to form an intercellular gap-junction channel (Goodenough et al. 1996; Kumar and Gilula 1996). In the cochlea, there are two networks of gap junctions, the epithelial cell system and the connective tissue cell system, which are thought to be involved in the recycling of potassium back into the cochlear endolymph, where it plays an essential role in sound transduction (Kikuchi et al. 2000). In fact, targeted inactivation of the *GJB2* gene in the inner-ear epithelial network led to hearing impairment in mice, demonstrating the key role of this network in cochlear function and cell survival (Cohen-Salmon et al. 2002). Several different connexins have been shown to participate in these gap junction systems. To date, mutations in the genes encoding three of these connexins (*GJB2* for Cx26, *GJB6* for Cx30 [MIM 604418], and *GJB3* for Cx31 [MIM 603324]) are known to result in hearing impairment (Kelsell et al. 1997; Xia et al. 1998; Grifa et al. 1999). Among them, *GJB2* stands out, because mutations in this gene account for as many as 50% of all cases of prelingual hearing impairment in many populations (Rabionet et al. 2000). More than 80 different mutations in *GJB2* have been described in subjects with hearing impairment (Connexin-Deafness Homepage). Molecular testing for *GJB2* mutations has rapidly become the standard of care for the diagnosis and counseling of patients with nonsyndromic hearing impairment of unknown cause.

Mutation screening of *GJB2* in subjects with autosomal recessive hearing impairment has, however, revealed an unexpected problem—namely, a high number of patients carrying only one mutant allele. Exhaustive screening of the coding region (fully contained in exon 2), exon 1, and splice sites did not reveal any mutation in the second allele. These cases accounted for 10%–50% of all deaf subjects with at least one *GJB2* mutation. These findings could be attributed to intrinsic limitations in the techniques used for mutation screening, to the high frequency of carriers for some *GJB2* mutations, or to the existence of mutations in other noncoding parts of the gene that have not yet been identified. However, it was also suspected that other mutations might exist, in the *DFNB1* locus but not in the *GJB2* gene, that could provide an explanation for the high proportion of heterozygous affected subjects. Recently, this hypothesis received experimental support from the finding of a novel class of mutations in the *DFNB1* locus, which were deletions not affecting *GJB2* but truncating the neighboring *GJB6* gene, which encodes Cx30. These deletions were found accompanying in *trans* the only *GJB2* mutant allele in heterozygous affected subjects (double heterozygosity) (Lerer et al. 2001; del Castillo et al. 2002; Pallares-Ruiz et al. 2002) and were also found to be the cause of deafness in three unrelated affected subjects who were homozygous for the deletion (del Castillo et al. 2002; Pallares-Ruiz et al. 2002). In one study, the deletion breakpoint junction was isolated and sequenced, revealing the loss of a DNA segment of ~342 kb, with one breakpoint inside the *GJB6* coding region (del Castillo et al. 2002). This deletion, named "del(*GJB6*-D13S1830)," was the accompanying mutation in ~50% of the deaf *GJB2* heterozygotes (del Castillo et al. 2002). It remained to be determined whether the deletions detected by other groups (Lerer et al. 2001; Pallares-Ruiz et al. 2002) were also del(*GJB6*-D13S1830).

These findings may indicate a digenic pattern of inheritance of hearing impairment from mutations involving *GJB2* and *GJB6*. This hypothesis is supported by several facts: (i) both Cx26 and Cx30 are expressed in the same inner-ear structures (Lautermann et al. 1998, 1999), (ii) connexons composed of Cx26 can bind connexons composed of Cx30 to form heterotypic gap-junction channels (Dahl et al. 1996), (iii) a mutation in *GJB6* was reported in a case of autosomal dominant hearing impairment (Grifa et al. 1999), and (iv) Cx30-deficient mice exhibit a severe constitutive hearing impairment and lack an endocochlear potential (Teubner et al. 2003). However, the fact that point mutations in *GJB6* have not yet been found in cases of autosomal recessive hearing impairment argues against this hypothesis. An alternative explanation is that the deletion may eliminate an upstream regulatory element for *GJB2* that is essential for the normal expression of this gene in the inner ear. So far, such an element has not been found.

Given the association previously found between *GJB2* monoallelic mutations and the del(*GJB6*-D13S1830) mutation in several studies (Lerer et al. 2001; del Castillo et al. 2002; Pallares-Ruiz et al. 2002), we investigated the contribution of this deletion to hearing impairment in nine countries. A genetic analysis of five microsatellite markers flanking the deletion breakpoints was also conducted, to determine the haplotypes associated with the del(*GJB6*-D13S1830) mutation and to explore its evolutionary origins. The implications of our results for molecular diagnosis of *DFNB1* mutations are presented.

The study was performed on probands with nonsyndromic prelingual hearing impairment from nine countries (table 1). In Spain, Israel, and the United States, two independent studies were performed. After getting written informed consent, blood samples were obtained, and DNA was extracted by standard procedures. Testing for the del(*GJB6*-D13S1830) mutation was performed using the previously reported primers *GJB6*-1R (forward) and BKR-1 (reverse) (del Castillo et al. 2002), as well as a modification of the method to positively detect a wild-type product by adding another reverse primer that is located in the deleted segment of *GJB6* (R2, 5′-TCATCGGGGGTGTCAACAAACA-3′). When these three primers were used together, two different PCR products were ob-

Table 1

Results from the Screenings for the del(*GJB6*-D13S1830) Mutation

Country/Laboratory	No. of (del[*GJB6*-D13S1830]+*GJB2*) Double Heterozygotes/ No. of *GJB2* Heterozygotes	No. of del(*GJB6*-D13S1830) Homozygotes/Total Screened
Spain/Madrid[a]	29/68 (42.6%)[b]	1/425 (.2%) + 2 heterozygotes[c]
Spain/Barcelona	5/35 (14.3%)	1/236 (.4%)
Italy	0/31 (.0%)	0/238 (.0%) + 1 heterozygote[c]
France	23/60 (38.3%)	0/208 (.0%) + 1 heterozygote[c]
Belgium	2/19 (10.5%)	0/151 (.0%)
United Kingdom	6/19 (31.6%)	Not performed
Israel/Tel Aviv	7/20 (35.0%)	1/191 (.5%)
Israel/Jerusalem	5/7 (71.4%)	Not performed
United States/Virginia	14/88 (15.9%)	1/486 (.2%) + 4 heterozygotes[c]
United States/Iowa	7/95 (7.4%)	Not performed
Brazil	2/9 (22.2%)	Not performed
Australia	2/29 (6.9%)	Not performed

[a] This work expands the results of a previous study (del Castillo et al. 2002).
[b] By performing haplotype analysis for genetic markers from 13q12, linkage to *DFNB1* was excluded in 11 cases. After correction, the figures are 29/57 (50.9%).
[c] Number of del(*GJB6*-D13S1830) heterozygotes with no mutation in *GJB2*.

tained—GJB6-1R→R2 (681 bp) and GJB6-1R→BKR-1 (460 bp)—allowing for discrimination between wild-type subjects (681-bp product), homozygotes for the deletion (460-bp product), and heterozygotes (both products) in a single test. In all subjects carrying the deletion, the PCR product that contains the breakpoint junction of the deletion was sequenced, to confirm that the breakpoint junction was identical in all cases. Thus, we confirmed that all the deletions reported by Lerer et al. (2001) were del(*GJB6*-D13S1830). In an independent study, it was also shown that the deletions reported by Pallares-Ruiz et al. (2002) were del(*GJB6*-D13S1830) (A. F. Roux, personal communication).

Two different screenings were performed, the first on deaf subjects carrying only one *GJB2* mutant allele, which had been found during routine testing for *GJB2* mutations (tables 1 and 2); and the second on subjects with nonsyndromic prelingual hearing impairment, carrying no mutation in *GJB2* (table 1). The frequency of the del(*GJB6*-D13S1830) allele among *DFNB1* alleles is shown in table 3.

The del(*GJB6*-D13S1830) allele is most frequent in Spain, France, the United Kingdom, Israel, and Brazil, accounting for 5.9%–9.7% of all the *DFNB1* alleles (table 3). Its frequency is lower in Belgium and Australia (1.3%–1.4%), and it has not been found among Italian *GJB2* unelucidated heterozygotes. In the United States, two different screenings yielded different results: a moderate frequency (4.5%, Virginia cohort) or a low frequency similar to those of Belgium and Australia (1.6%, Iowa cohort). A likely explanation for the higher frequency observed in the Virginia cohort is that it contains a higher proportion of individuals of Spanish descent than does the Iowa cohort, but the contribution of other factors cannot be excluded (for instance, differences in the proportion of probands who were the offspring of intermarriages among deaf people).

Before the screenings reported here, subjects with only one mutant *GJB2* allele accounted for 11.2%–51.5% of those with (one or two) mutations in *GJB2* in our cohorts of cases. The lowest percentage was observed in Italy (11.2%), a result that is consistent with the absence of the deletion in that screening. In screenings performed on large cohorts (>50 subjects) in other countries, data were, in general, rather homogeneous (22.1%–35.1%). Differences between the two screenings in Spain are probably due to regional bias in the Barcelona cohort (40% of cases are from the same Spanish region, Cantabria). It is noteworthy that screenings not including exon 1 and splice sites show percentages that are similar to those that do include them, a result that suggests a low frequency for mutations in these noncoding parts of the gene.

As expected, the observed frequencies for the del(*GJB6*-D13S1830) allele correlate with the percentage of cases with only one mutant *GJB2* allele that were elucidated by the finding of the deletion (table 1). The highest figures correspond to France, Spain, Israel, and the United Kingdom (31.6%–71.4%). Differences between the two Israeli studies, both performed on Ashkenazi Jews, are likely to be due to the small size of the cohorts that were analyzed. On the other end, Australia, the United States, and Belgium show much lower percentages of elucidated cases (6.9%–15.9%). A slightly higher percentage was found in an independent study performed in the United States (Stevenson et al. 2003), with the deletion accounting for 20% of *GJB2* deaf heterozygotes. After screening for the deletion, cases that remain not elucidated fell to 16.0%–

Table 2

Impact of Screening for del(*GJB6*-D13S1830) on the Elucidation of Cases with Only One Mutant *GJB2* Allele

Country/Laboratory	*GJB2* Testing[a]	No. of Monoallelic Subjects/No. of Monoallelic + No. of Biallelic Subjects[b]: Before Screening for the Deletion	No. of Monoallelic Subjects/No. of Monoallelic + No. of Biallelic Subjects[b]: After Screening for the Deletion
Spain/Madrid	Ex2-CR, Ex1, SpS	68/244 (27.9%)	39/244 (16.0%)[c]
Spain/Barcelona	Ex2-CR, Ex1, SpS	35/68 (51.5%)	30/68 (44.1%)
Italy	Ex2-CR	31/278 (11.2%)	31/278 (11.2%)
France	Ex2-CR, Ex1, SpS	60/177 (33.9%)	37/177 (20.9%)
Belgium	Ex2-CR	19/86 (22.1%)	17/86 (19.8%)
United Kingdom	Ex2-CR	19/64 (29.7%)	13/64 (20.3%)
Israel/Tel Aviv	Ex2-CR, Ex1, SpS	20/75 (26.7%)	13/75 (17.3%)
Israel/Jerusalem	Ex2-CR, Ex1, SpS	7/44 (15.9%)	2/44 (4.5%)
United States/Virginia	Ex2-CR, Ex1, SpS	88/251 (35.1%)	74/251 (29.5%)
United States/Iowa	Ex2-CR, Ex1, SpS	95/305 (31.1%)	88/305 (28.9%)
Brazil	Ex2-CR, Ex1, SpS	9/21 (42.9%)	7/21 (33.3%)
Australia	Ex2-CR, Ex1, SpS	29/102 (28.4%)	27/102 (26.5%)

[a] Ex2-CR = exon 2 coding region; Ex1 = entire exon 1; SpS = splice sites.
[b] "Monoallelic" refers to subjects carrying only one mutant *GJB2* allele; "biallelic" refers to subjects carrying two mutant *GJB2* alleles.
[c] After excluding cases not linked to *DFNB1* according to haplotype analysis, results were 28/244 (11.5%).

20.9% in France, Spain, and Israel (results from larger cohorts) and are now closer to data from Italy (11.2%) (table 2). Moreover, in the screening performed by the Madrid team in Spain, genotyping and haplotype analysis for genetic markers close to *GJB2* allowed us to exclude linkage to *DFNB1* in 11 of 39 unelucidated cases, with the percentage of unelucidated cases then decreasing to 11.5% (table 2). In contrast, these figures remain high in all the other countries (19.8%–33.3% in Belgium, the United Kingdom, Australia, the United States, and Brazil). It is remarkable that Spanish subjects carrying a single mutation in *GJB2* but without linkage to *DFNB1* (coincidental carriers) account for at least 4.5% (11 of 244; data from the Madrid laboratory) of cases with *GJB2* mutations. It must be taken into account that linkage to *DFNB1* cannot be tested in simplex (sporadic) cases, impeding the detection of additional coincidental carriers, and so this figure undoubtedly is higher. However, even if we assume a higher percentage of coincidental carriers, it clearly emerges that other *DFNB1* mutations remain to be identified in most countries.

The frequency of the del(*GJB6*-D13S1830) mutation in all populations is not high enough to result in a large number of homozygous subjects. They represent <0.5% of all cases of prelingual hearing impairment without mutations in *GJB2* (table 1; none were born from consanguineous parents). It is noteworthy that four screenings detected del(*GJB6*-D13S1830) heterozygotes without accompanying *DFNB1* mutation (table 1), which are to be added to the cases lacking elucidation.

We investigated the evolutionary origins of the deletion by studying haplotypes associated with this mutation. Five microsatellite markers closely flanking the deletion breakpoints were selected for this study. Their relative order and physical distances were as follows: D13S1835–117 kb–$(TG)_n$–42 kb–D13S141–68 kb–$(GAAA)_n$–4 kb–deletion proximal breakpoint–309 kb–deletion distal breakpoint–233 kb–D13S1831. Note that the deletion size, according to the latest sequencing data, is 309 kb (National Center for Biotechnology Information database, *Homo sapiens* genome view, build

Table 3

Frequency of the del(*GJB6*-D13S1830) Allele among the *DFNB1* Alleles

Country/Laboratory	No. of del(*GJB6*-D13S1830) Alleles/Total No. of *DFNB1* alleles[a]
Spain/Madrid	31/408 (7.6%)
Spain/Barcelona	7/72 (9.7%)
Italy	0/494 (0%)
France	23/280 (8.2%)
Belgium	2/138 (1.4%)
United Kingdom	6/102 (5.9%)
Israel/Tel Aviv	9/126 (7.1%)
Israel/Jerusalem	5/84 (6.0%)
USA/Virginia	16/356 (4.5%)
USA/Iowa	7/434 (1.6%)
Brazil	2/28 (7.1%)
Australia	2/150 (1.3%)

[a] Only cases in which both *DFNB1* alleles were identified contribute to this total.

Table 4

Haplotypes Associated with the del(*GJB6*-D13S1830) Mutation

Marker[a]	Heterozygosity[b] (%)	Allele in Haplotype															Genotype for CEPH Individual 134702
		A1	A2	A3	A4	B1	B2	B3	B4	B5	B6	C1	D1	E1	E2	F1	
D13S1835	78	134	134	134	136	132	132	134	136	136	138	136	134	138	165	138	134/136
$(TG)_n$	65	204	204	204	204	208	208	208	208	208	208	210	204	208	208	208	206/208
D13S141	55	124	124	124	124	124	124	124	124	124	124	124	126	126	126	124	126/126
$(GAAA)_n$	79	209	209	209	209	209	209	209	209	209	209	209	209	209	209	205	209/216
D13S1831	84	96	103	105	105	87	105	105	87	105	105	105	105	105	105	101	99/101

[a] Relative order and physical distances are as follows: D13S1835–117 kb–$(TG)_n$–42 kb–D13S141–68 kb–$(GAAA)_n$–4 kb–deletion proximal breakpoint–309 kb–deletion distal breakpoint–233 kb–D13S1831.

[b] Calculated from 100 Spanish control chromosomes.

33, contig NT_009799). Conditions for the PCR amplification of these markers have been reported elsewhere (Hudson et al. 1992; Kibar et al. 1999; Lerer et al. 2001). Cases suitable for haplotype analysis were genotyped for these markers, but we report here only those cases in which the haplotype associated with the deletion could be determined unambiguously. These included 52 nonrelated chromosomes: 28 from Spain (including 1 of Russian origin), 9 from Israel, 5 from France, 3 from the United Kingdom, 3 from the United States, 2 from Brazil, and 2 from Australia (tables 4 and 5). Allele sizes were determined by DNA sequencing of a control sample, which was shared by all the laboratories in this multicenter study and was used as a standard in genotyping assays. To allow other laboratories to compare their data with those reported in this work, we provide allele sizes for individual 134702, available from CEPH (Dib et al. 1996) (table 4).

In 51 of 52 chromosomes carrying the deletion, we observed association with allele 209 from marker $(GAAA)_n$, which is at a distance of only 4 kb from the deletion proximal breakpoint (frequency of this allele: 0.415 in Spain and 0.364 in Ashkenazi Jews). When considering a core haplotype constituted by markers $(TG)_n$, D13S141, and $(GAAA)_n$, we could define six different haplogroups, A–F (table 4). Finally, an expanded haplotype with all the five markers revealed 15 variants associated with the deletion (table 4). When examining the geographic distribution of haplotypes (table 5), it is remarkable that all nine Israeli chromosomes belong to

Table 5

Distribution of Haplotypes Associated with the Deletion in Different Populations

Haplotype	*N*	Distribution
Group A:		
A1	1	Spain (Russian origin)
A2	1	Australia
A3	21	11 Spain (10 Madrid, 1 Barcelona) 8 Israel (6 Tel Aviv, 2 Jerusalem), 2 France
A4	1	Israel (Jerusalem)
Total	24	
Group B:		
B1	7	Spain (Madrid)
B2	2	Spain (Madrid)
B3	1	Spain (Madrid)
B4	1	Spain (Madrid)
B5	3	2 Spain (Madrid), 1 United Kingdom
B6	9	2 Spain (Madrid), 2 France, 1 United Kingdom, 1 Australia 1 United States (Virginia), 2 Brazil (Portuguese origin)
Total	23	
Group C (C1)	1	France
Group D (D1)	1	United States (Virginia)
Group E:		
E1	1	United Kingdom
E2	1	Spain (Madrid)
Total	2	
Group F (F1)	1	United States (Virginia)
Total	52	

group A (eight A3 and one A4; these two haplotypes differ only in D13S1835). Spanish haplotypes are mainly concentrated in groups A and B (11 A3, 15 B, and 1 E2), and the two Brazilian chromosomes are B6. Most chromosomes from other countries (France, the United Kingdom, and Australia) also belong to groups A and B. It is noteworthy that two of three chromosomes from the United States belong to groups D and F.

Our results show a clear founder effect for the del(*GJB6*-D13S1830) mutation in Ashkenazi Jews in Israel. Although the size of the sample should be increased to reach firmer conclusions in most of the analyzed populations, our data also suggest a common founder for the deletion in some countries in western Europe, since it is very scarce in Italy and has a low frequency in Belgium, whereas it is quite frequent in Spain and France, in Brazilian subjects of Portuguese origin, and—although to a lesser extent—in the United Kingdom (in all of these cases, the deletion is mainly associated with haplogroups A and B). The fact that the Ashkenazi affected subjects share a common haplotype along 464 kb contrasts with the diversity of haplotypes found in populations from western Europe, suggesting an older origin for the deletion in these countries. The existence of haplogroup F also suggests that the deletion could have other independent origins.

Inherited hearing impairment shows almost unparalleled genetic heterogeneity, not only in terms of numbers of genes and mutations, but also in prevalence of specific mutations among different populations. Therefore, data from genetic epidemiological studies are essential for the design of molecular diagnostic protocols well suited for each population. Our study provides some conclusions that should serve to improve the molecular diagnosis of *DFNB1* cases. Given the simplicity of the test for the detection of the del(*GJB6*-D13S1830) mutation, it should be performed for all subjects with prelingual hearing impairment, at least in populations having higher frequencies. Our work also highlights the importance of performing haplotype analysis, when possible, in subjects with only one mutant *GJB2* allele, to identify carriers whose hearing impairment might be due to mutations in a different gene or to hypothetical epistatic interactions between *GJB2* mutations and other unlinked gene(s). This step would also help to concentrate research on the elucidation of cases consistent with linkage to *DFNB1* by investigating other parts of *GJB2* (promoter, 3′ UTR) and by searching for hypothetical point mutations in *GJB6* or other DNA rearrangements in the *DFNB1* locus.

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Electronic-Database Information

The accession number and URLs for data presented herein are as follows:

Connexin-Deafness Homepage: http://www.crg.es/deafness/
Hereditary Hearing Loss Homepage, http://www.uia.ac.be/dnalab/hhh/
National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/ (for contig NT_009799)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for DFNB1, *GJB2*, *GJB6*, and *GJB3*)

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