LETTER TO JMG

A novel deletion involving the connexin-30 gene, del(*GJB6*-d13s1854), found in *trans* with mutations in the *GJB2* gene (connexin-26) in subjects with DFNB1 non-syndromic hearing impairment

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Hearing impairment is a common and highly heterogeneous sensory disorder. Genetic causes are thought to be responsible for more than 60% of the cases in developed countries.[1] In the majority of cases, non-syndromic hearing impairment is inherited in an autosomal recessive pattern.[2] Thirty eight different loci and 20 genes for autosomal recessive non-syndromic hearing impairment (ARNSHI) have been identified to date.[3]

In many populations, up to 50% of all cases of ARNSHI are caused by mutations in the *DFNB1* locus (MIM 220290) on 13q12.[4] This locus contains the *GJB2* gene (MIM 121011), encoding connexin-26 (Cx26),[5] which belongs to a family of transmembrane proteins with about 20 members in humans. Hexamers of connexins (connexons) are displayed in the plasma membrane. Docking of connexons on the surfaces of two adjacent cells results in the formation of intercellular gap junction channels.[6] Several different connexins, including Cx26, have been shown to participate in the complex gap junction networks of the cochlea.[7 8] It has been postulated that these networks play a key role in potassium homeostasis, which is essential for the sound transduction mechanism.[9]

Given the high prevalence of DFNB1 deafness, molecular testing for *GJB2* mutations has become the standard of care for the diagnosis of patients with non-syndromic hearing impairment of unknown cause.[10] However, the finding of a large number of affected subjects with only one *GJB2* mutant allele complicates the molecular diagnosis of DFNB1 deafness. In different studies, these have accounted for 10–50% of deaf subjects with *GJB2* mutations.[4] It was hypothesised that there could be other mutations in the *DFNB1* locus but outside the *GJB2* gene. This hypothesis gained support by the finding of a deletion in the *DFNB1* locus outside *GJB2* but truncating the neighbouring *GJB6* gene (MIM 604418), which encodes connexin-30 (Cx30), another component of the gap junction networks of the cochlea. This deletion, named del(*GJB6*-D13S1830), was found in affected subjects either in homozygosity or in double heterozygosity with a *GJB2* mutation.[11–13] Isolation and sequencing of the deletion breakpoint junction revealed the loss of a DNA segment initially thought to be 342 kb in size but currently estimated to be 309 kb.[12 14]

In a multicentre study, it was shown that the del(*GJB6*-D13S1830) mutation is most frequent in Spain, France, the United Kingdom, Israel, and Brazil (5.9–9.7% of all *DFNB1* alleles); it is less frequent in the USA, Belgium, and Australia (1.3–4.5% of all *DFNB1* alleles), and is very rare in southern Italy.[14] Recent studies have found, however, that the deletion is present in northern Italy at frequencies similar to those of other European countries ([15] and Murgia A, Leonardi E,

Key points

- DFNB1 deafness, caused by mutations in the gene encoding connexin-26 (*GJB2*), is the most frequent subtype of autosomal recessive non-syndromic hearing impairment. Molecular testing for *GJB2* mutations has become a standard diagnostic approach for subjects with this disorder. However, 10–50% of affected subjects with *GJB2* mutations carry only one mutant allele.
- A 309 kb deletion truncating the *GJB6* gene (encoding connexin-30) was shown to be the accompanying mutation in up to 50% of deaf *GJB2* heterozygotes in different populations. We report the molecular characterisation of the breakpoint junction of a novel 232 kb deletion in the DFNB1 locus, del(*GJB6*-D13S1854), which was also found in *trans* with pathogenic *GJB2* mutations in affected subjects. The deletion arose by unequal homologous recombination, involving an AluY sequence inside *GJB6* intron 2, a mechanism which might generate other deletions at *DFNB1*.
- We developed a novel diagnostic test for the combined detection of del(*GJB6*-D13S1830) and this new del(*GJB6*-D13S1854) in a single PCR assay. The del(*GJB6*-D13S1854) mutation accounts for 25.5% of the affected *GJB2* heterozygotes which remained unresolved after screening for del(*GJB6*-D13S1830) in Spain, 22.2% in the UK, 6.3% in Brazil, and 1.9% in northern Italy. It was not found in affected *GJB2* heterozygotes from France, Belgium, Israel, the Palestinian Authority, USA, or Australia.
- Haplotype analysis revealed a common founder for the mutation in Spain, Italy, and the UK. Our data further support the complexity of the genetic epidemiology of non-syndromic hearing impairment.

Abbreviations: ARNSHI, autosomal recessive non-syndromic hearing impairment

unpublished data). The deletion was also found in other studies in the USA[16–19] and Germany,[20] but not in Austria,[21] Turkey,[22 23] or China.[24] Although the finding of the del(*GJB6*-D13S1830) mutation provided an explanation for the hearing impairment in as many as 30–70% affected *GJB2* heterozygotes in some populations, it has become evident that other *DFNB1* mutations remain to be identified in most countries.[14] Here we report the molecular characterisation of a novel deletion, also truncating the *GJB6* gene, but resulting in the loss of a DNA segment shorter than in del(*GJB6*-D13S1830).

METHODS

This study was done on probands with ARNSHI and their relatives from Spain, Italy, France, Belgium, the United Kingdom, Israel, the Palestinian Authority, the USA, Brazil, and Australia. After getting written informed consent, blood samples were obtained and DNA was extracted by standard procedures.

Novel microsatellite markers were developed in the *DFNB1* region by searching for tandem repeats of the CA dinucleotide in sequence contig NT_024524.13 (National Center for Biotechnology Information database, *Homo sapiens* genome view, build 34) and by designing flanking primers:

- marker D13S1853: forward primer 5′-CAGACTGGCACAAACTTAACTG-3′; reverse primer, 5′-TGTACATCTCTTCTTACATTCATGT-3′ (annealing temperature, 56℃);
- marker D13S1854: forward primer, 5′-CTCCATCCTGGGTGACAGAGTGAG-3′; reverse primer, 5′-AGGAAGAGCTGGGGTTGCTAAGAA-3′ (annealing temperature, 58℃).

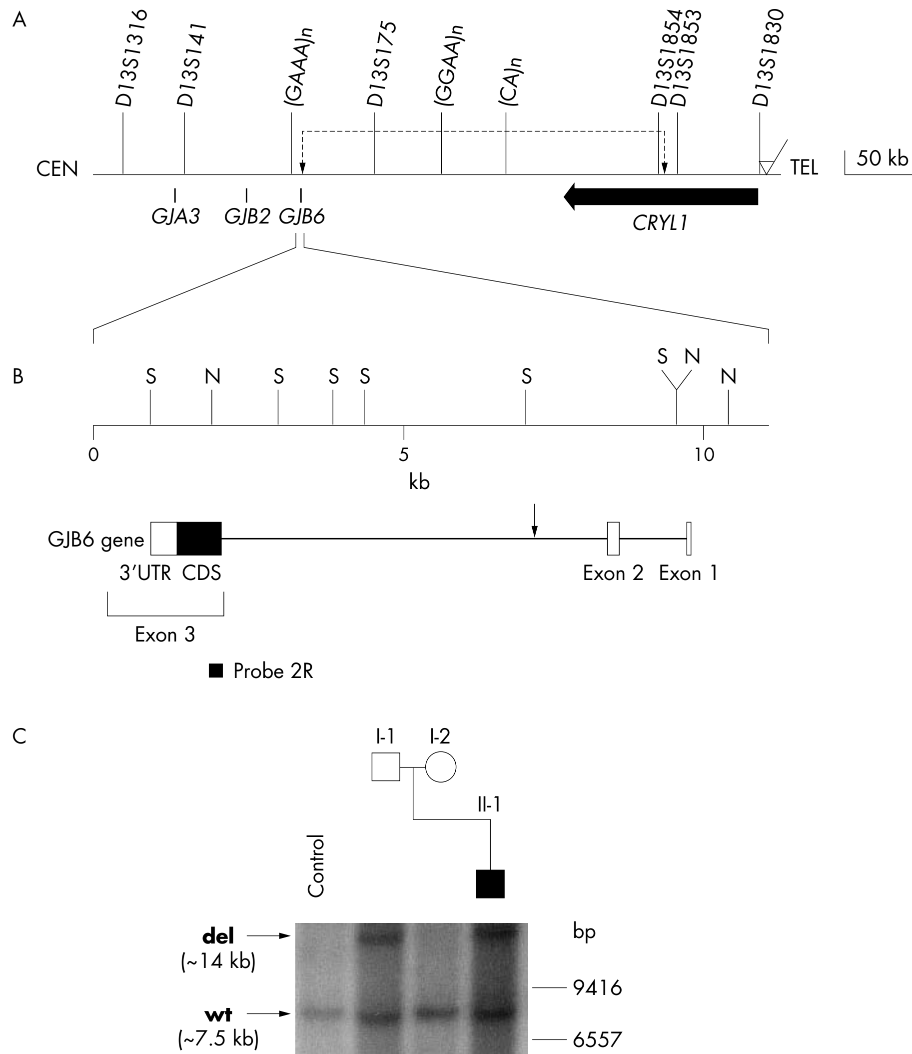

Figure 1 Map of the *DFNB1* region on 13q12, and Southern blot analysis of family E079. (A) Map of a 600 kb DNA segment including the *DFNB1* locus. The positions of polymorphic genetic markers are indicated by vertical bars. Genes in the region are depicted as horizontal bars or arrows. *GJA3* encodes connexin-46 (MIM 121015) and *CRYL1* codes for λ-crystallin. The two breakpoints of the del(*GJB6*-D13S1854) mutation are marked by vertical arrows, and the extent of the deletion is indicated by the dashed line. An empty arrowhead indicates the distal end of the previously reported del(*GJB6*-D13S1830) mutation. (B) Physical map of a 10 kb DNA segment containing the *GJB6* gene. Restriction sites are indicated by vertical bars. N, *Nsi*I; S, *Ssp*I. The structure of the *GJB6* gene is shown below the map. Exons are depicted as boxes, introns as thin lines. 3′-UTR, 3′ untranslated region; CDS, *GJB6* coding region. A vertical arrowhead marks the deletion breakpoint internal to *GJB6*. The position of probe 2R, used in the Southern blot analysis, is indicated below the gene. (C) Southern blot analysis of family E079 with probe 2R on *Nsi*I digests of genomic DNA. Polymerase chain reaction amplification of probe 2R and Southern blotting experiments were carried out as reported.[12] An approximately 7.5 kb band (wt) is revealed in all subjects of family E079 and in the control. In addition, a novel 14 kb band (del), created by the deletion, is revealed in affected subject II:1 (double heterozygote, 35delG in *GJB2*/del(*GJB6*-D13S1854)), and in his father, I:1 (del(*GJB6*-D13S1854) carrier). This band is absent in the control subject and in the proband's mother, I:2 (35delG carrier).

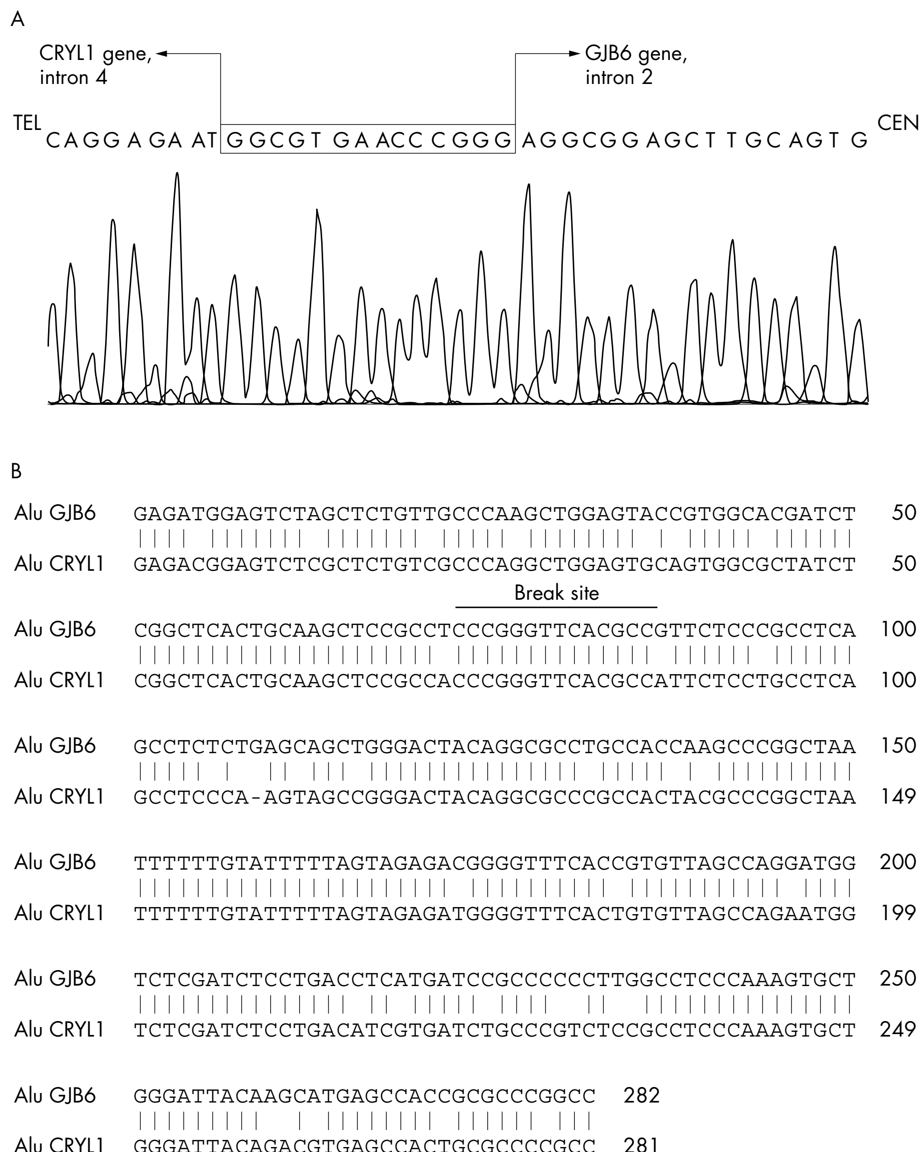

Figure 2 Breakpoint junction of the del(*GJB6*-D13S1854) deletion. Sequencing of the breakpoint junction was done directly on the polymerase chain reaction product obtained with primers DelBK1 and DelBK2. We used sequencing primer DelBK3, 5′-TCTTCTGTATGTATGTCACTTTTCA-3′. (A) Electropherogram of a DNA segment containing the breakpoint junction (boxed) and flanking sequences. The relative positions of the centromere (cen) and telomere (tel) of the long arm of chromosome 13 are indicated to show the orientation of the sequenced DNA segment. (B) Alignment of the sequences flanking the proximal and distal deletion breakpoints. The alignment was performed using BLASTN software (National Center for Biotechnology Information), which reported an identity of 88% along 282 nucleotides.

RESULTS

We reported previously on 39 unrelated Spanish subjects with ARNSHI, who were heterozygous for one *GJB2* mutant allele and did not carry the del (*GJB6*-D13S1830) mutation.[14] All these cases carried alleles which are considered unambiguously pathogenic.[10] After excluding 11 cases not linked to *DFNB1* on the basis of haplotype analysis of siblings, there remained 28 unelucidated heterozygotes.[14] We genotyped the proband, parents, and siblings from these 28 cases for microsatellite markers D13S175[25] and D13S1830,[26] both of which are deleted in the del(*GJB6*-D13S1830) mutation (fig 1A). In two multiplex cases (S591, S630) and two simplex cases (E079, E262), haplotype analysis revealed inconsistencies in the segregation of alleles of marker D13S175, suggesting the presence of an unamplifiable allele. The same results were obtained when using an alternative primer pair[12] flanking the microsatellite at D13S175. Conversely, haplotype analysis revealed no segregation inconsistencies, but there was heterozygosity for marker D13S1830 in either the proband or the parents of all four cases. Together, these data suggested the existence of (at least) a novel deletion at the *DFNB1* locus, involving marker D13S175 but not D13S1830.

To simplify the search for the deletion breakpoints, we assumed that the deletion in these four cases would be the same. Haplotype analysis for other microsatellite markers from the *DFNB1* locus[11] (fig 1A) revealed inconsistencies in the segregation of alleles, which allowed us to map the telomeric breakpoint distal to $(CA)_n$, and the centromeric breakpoint between markers $(GAAA)_n$ and D13S175. These results suggested that the centromeric breakpoint could be inside the *GJB6* gene. We tested this hypothesis by Southern blotting (fig 1, panels B and C). Probe 2R was assayed on *Ssp*I digests of genomic DNA from the proband and parents of

case E079 in order to investigate whether the *GJB6* coding region, fully contained in exon 3 (fig 1B), was intact. This probe did not show any change in dosage of *GJB6* exon 3 or in the *Ssp*I restriction pattern when comparing deletion carriers with control subjects (data not shown). To investigate whether the deletion could involve other parts of the *GJB6* gene, we assayed probe 2R on *Nsi*I digests of genomic DNA from deletion carriers and control subjects (fig 1, panels B and C). In addition to the expected 7.5 kb band, a novel 14 kb band, created by the deletion, was observed in the deletion carriers (fig 1C). As this 14 kb band has the expected size based upon the predicted restriction map, these findings led us to conclude that the deletion truncated *GJB6*.

To locate the deletion distal breakpoint, we searched for novel microsatellite markers in the interval between $(CA)_n$ and D13S1830 (see Methods). In all four cases with the deletion, genotyping and haplotype analysis revealed heterozygosity and consistent segregation for marker D13S1853, but inconsistencies in the segregation of alleles of marker D13S1854 (fig 1A). These data placed the distal deletion breakpoint between D13S1854 and D13S1853, an interval of about 9.5 kb. Thus we undertook a BLASTN comparison of the sequence stretch containing *GJB6* intron 1, exon 2, and intron 2 with the sequence spanning the interval between D13S1854 and D13S1853. This analysis revealed the existence of a 282 bp Alu sequence inside *GJB6* intron 2 sharing 88% identity with another Alu repeat located in direct orientation inside the D13S1854–D13S1853 interval. We designed primers flanking this candidate breakpoint junction, and a polymerase chain reaction (PCR) product of about 560 bp was obtained only from DNA samples of deletion carriers. Sequencing of this PCR product revealed the deletion breakpoint junction (fig 2A), which was the same in all four studied cases.

This novel deletion was named del(*GJB6*-D13S1854). Examination of the breakpoint junction supported the hypothesis that it originated from homologous recombination between two Alu sequences which belong to the Y subfamily, as shown by RepeatMasker software[27] (fig 2B). The proximal repeat is located in *GJB6* intron 2, and the distal repeat is in intron 4 of the gene encoding λ-crystallin (*CRYL1*, GenBank AF077049). The exact breakpoints could not be determined as the breaks could have taken place at any point of two identical 14 bp stretches (fig 2B). The deletion spans

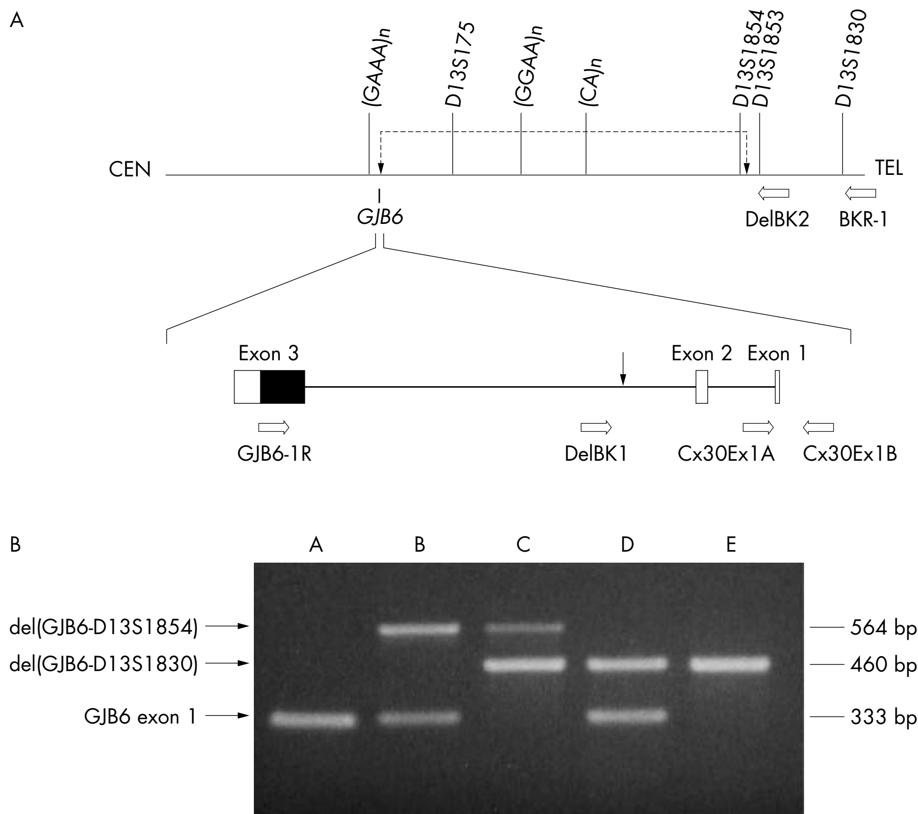

Figure 3 Single test for the detection of del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854). The rationale of the method is to amplify DNA segments containing the breakpoint junction of each deletion, as well as a segment containing *GJB6* exon 1, which is used as a control to check the efficiency of the polymerase chain reaction (PCR) and to distinguish heterozygosity *v* homozygosity for any of the two deletions (*GJB6* exon 1 is removed by both deletions). (A) Schematic drawing showing the location of the primers used in the multiplex PCR assay. GJB6-1R, 5′-TTTAGGGCATGATTGGGGTGATTT-3′, and BKR-1, 5′-CACCATGCGTAGCCTTAACCATTTT-3′ (for amplification of the del(*GJB6*-D13S1830) breakpoint junction); DelBK1, 5′-TCATAGTGAAGAACTCGATGCTGTTT-3′, and DelBK2, 5′-CAGCGGCTACCCTAGTTGTGGT-3′ (for amplification of the del(*GJB6*-D13S1854) breakpoint junction); Cx30Ex1A, 5′-CGTCTTTGGGGGTGTTGCTT-3′, and Cx30Ex1B, 5′-CATGAAGAGGGCGTACAAGTTAGAA-3′ (to amplify *GJB6* exon 1). PCR was carried out using the following programme: one cycle of denaturation at 95°C for five minutes; five touchdown cycles of denaturation at 94°C for 40 seconds, and annealing for 40 seconds at 65°C for the first cycle and a 1°C reduction per cycle; 25 cycles of denaturation at 94°C for 40 seconds, and annealing at 60°C for 40 seconds; and a final extension step of 72°C for seven minutes. The reaction took place in a final volume of 15 μl, at a final concentration of 1.5 mM $MgCl_2$, using Fast Start Taq DNA polymerase (Roche). (B) Separation of the PCR products by electrophoresis in a 1.5% agarose gel. The position of the PCR products corresponding to the deletion breakpoint junctions and to *GJB6* exon 1 are indicated by arrows on the left, and their sizes in base pairs (bp) are shown on the right. All the PCR products obtained in this multiplex reaction were sequenced to confirm their identities and validate the test. A, Wild type (wt); B, del(*GJB6*-D13S1854)/wt heterozygote; C, del(*GJB6*-D13S1830)/del(*GJB6*-D13S1854) compound heterozygote; D, del(*GJB6*-D13S1830)/wt heterozygote; E, del(*GJB6*-D13S1830) homozygote.

Table 1 Results from the screenings for the del(*GJB6*-D13S1854) mutation

Country/laboratory	No of *DFNB1* heterozygotes carrying del(*GJB6*-D13S1854)/No of *DFNB1* heterozygotes	Accompanying *DFNB1 mutant allele (No of cases)*
Spain	12/47 (25.5%)	35delG (10), V37I (1), del(GJB6-D13S1830) (1)
Italy	1/53 (1.9%)	35delG (1)
France	0/40	
Belgium	0/20	
United Kingdom	4/18 (22.2%)	35delG (4)
Israel	0/11	
USA/Virginia	0/92	
USA/Iowa	0/88	
Brazil	1/16 (6.3%)	V37I (1)
Australia	0/27	

232 kb. It could create a chimeric gene by joining *CRYL1* intron 4 with *GJB6* intron 2. The hypothetical chimeric mRNA would contain the first three exons of *CRYL1* and *GJB6* exon 3. However, no product is likely as there is an in-frame stop codon in between the two open reading frames.

We developed a single test for the detection of both deletions, which is useful for routine molecular diagnosis (fig 3). The del (*GJB6*-D13S1854) mutation was found in *trans* in 12 of 47 Spanish unrelated affected subjects which were unresolved *DFNB1* heterozygotes (25.5 %) (table 1). Their hearing impairments ranged from mild to profound. Interestingly, one subject was a compound heterozygote for the two deletions (fig 3, lane C). We also screened 604 additional Spanish unrelated subjects with ARNSHI who were not carriers of *DFNB1* mutations. One of these was found to be heterozygous for del(*GJB6*-D13S1854). The deletion was not found in 100 control subjects with normal hearing. After this screening, the unelucidated heterozygotes in our sample represent 12.2% of the total number of subjects with at least one *DFNB1* mutation (36/295). Excluding 11 cases not linked to *DFNB1*, this figure drops to 8.5% (25/295). With a frequency of 2.2% (12/548), the del(*GJB6*-D13S1854) mutation is among the five most common *DFNB1* alleles in our Spanish sample.

A multicentre study was conducted to investigate the prevalence of the novel deletion in different countries (table 1). The del(*GJB6*-D13S1854) mutation was found to account for 22.2% of affected *GJB2* heterozygotes who were unresolved after screening for del(*GJB6*-D13S1830) in the United Kingdom, for 6.3% in Brazil, and for 1.9% in northern Italy. It was not found in screening carried out on samples from France, Belgium, Israel, the USA, or Australia. The novel deletion was not found in 159 Israeli Jewish and 40 Palestinian Arab unrelated subjects with ARNSHI, who did not carry any *GJB2* mutation.

DNA sequencing confirmed that the breakpoint junction was the same in all the positive cases found in Spain, Italy, the United Kingdom, and Brazil. We investigated the evolutionary origins of the deletion by studying haplotypes associated with this mutation (table 2). All chromosomes carrying the deletion share a core haplotype composed of allele 209 from marker $(GAAA)_n$ (frequency of this allele in Spain, 0.415), and allele 204 of marker D13S1853 (frequency of this allele in Spain, 0.411) (table 2). These markers are very close to the deletion breakpoints, at distances of only 9 and 6 kb, respectively. An expanded haplotype with all the four markers revealed four variants associated with the deletion, the most frequent being haplotype A, from which the other three could have arisen through single recombination events (table 2). Our results show that all the studied chromosomes carrying the del (*GJB6*-D13S1854) mutation in Spain, the United Kingdom, and Italy share a common founder.

DISCUSSION

The hypothesis of digenic inheritance of DFNB1 hearing impairment has received theoretical support from several observations. Both Cx26 and Cx30 are expressed in the same inner ear structures.[28 29] Moreover, connexons composed of Cx26 can bind connexons composed of Cx30 to form heterotypic gap junction channels.[30] It was also reported that a *GJB6* mutation results in autosomal dominant hearing impairment in humans,[31] and that Cx30 deficient mice lack the endocochlear potential and have a severe constitutive hearing impairment.[32] However, the fact that point mutations in *GJB6* have not yet been found in cases of ARNSHI in

Table 2 Haplotypes associated with the del(*GJB6*-D13S1854) mutation

Marker†	Heterozygosity‡ (%)	Haplotype* A	B	C	D	Genotype for CEPH individual 134702
$(TG)_n$	65	208	204	206	208	206/208
$(GAAA)_n$	79	209	209	209	209	209/216
D13S1853	66	204	204	204	204	202/202
D13S1830	71	153	153	153	156	153/156
Number and geographical distribution of haplotypes (n = 14)		6 Spain 2 UK 1 Italy **Total: 9**	1 Spain 1 UK **Total: 2**	1 Spain **Total: 1**	2 Spain **Total: 2**	

*We only report here those cases in which the haplotype associated with the deletion could be determined unambiguously. Allele sizes were determined by DNA sequencing of a control sample, which 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.[25]
†Relative order and physical distances are as follows: $(TG)_n$ – 110 kb – $(GAAA)_n$ – 9 kb – deletion proximal breakpoint – 232 kb – deletion distal breakpoint – 6 kb – D13S1853 – 60 kb – D13S1830.
‡Calculated from 100 Spanish control chromosomes.

humans argues against this hypothesis. In addition, $Cx26^{+/-}$/$Cx30^{+/-}$ double heterozygous mice have only a moderate hearing impairment,[33] in contrast with the phenotype observed in humans, where most double heterozygotes for del(*GJB6*-D13S1830) and a *GJB2* mutation have severe or profound hearing impairment.[11–13 15 16 19 20 34 35]

An alternative hypothesis postulates the existence of a *cis* acting regulatory element which would activate the expression of *GJB2* in the inner ear. This regulatory element would have been removed by the deletions, and its absence would have dramatic effects on the expression of *GJB2*, to the point that an otherwise normal allele would behave as a null allele. Both hypotheses can be combined—that is, the main pathogenic effect of the deletions might be caused by the *GJB2* expression deficit, but haploinsufficiency for Cx30 may contribute to worsening of the phenotype.

The 232 kb sequence stretch removed by del(*GJB6*-D13S1854) is still too large to search for a regulatory element. Molecular characterisation of other DNA rearrangements in the *DFNB1* locus leading to hearing impairment may help to define a smaller interval. Under the hypothesis of the regulatory element, it is predicted that another class of deletions, leading to hearing impairment but not truncating *GJB6*, might also be present in the *DFNB1* locus. After screening for the deletions so far reported, affected *GJB2* heterozygotes still represent 8–30% of all subjects with mutations in *GJB2* in different populations ([14] and this study). These figures are far from what should be expected if these *GJB2* heterozygotes were just coincidental carriers. Although hypothetical epistatic interactions between *GJB2* mutations and other unlinked gene(s) might contribute to this situation, additional mutations in *DFNB1*, not yet identified, are also likely to exist. The AluY sequence contained in *GJB6* intron 2 has the potential of generating deletions affecting this gene, by homologous recombination with other highly similar repeats along the *DFNB1* locus. Alu/Alu recombination leading to deletion is a common disease causing mechanism.[36]

Both del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) inactivate the *CRYL1* gene and remove the sequence interval between *GJB6* and *CRYL1*, where no additional genes have been reported so far. The *CRYL1* gene is widely expressed, and its product, λ-crystallin, shows similarity with 3-hydroxyacyl-CoA dehydrogenase.[37] The contribution of λ-crystallin to DFNB1 hearing impairment, if any, remains enigmatic. To date, subjects carrying either del(*GJB6*-D13S1830) or del(*GJB6*-D13S1854) do not present with any eye disorder ([12] and this study).

Our multicentre study reveals significant differences in the frequency of each of the deletions, and also different patterns of geographical distribution. The del(*GJB6*-D13S1830) mutation, found in many populations over the world, is much more frequent than del(*GJB6*-D13S1854), which is for the present restricted to a few countries. Both mutant alleles are frequent in Spain and the United Kingdom (the combined frequency of the two deletions in our Spanish sample is 10.6% (58/548) of the *DFNB1* mutant alleles; in the United Kingdom they total 9.8% of the *DFNB1* alleles and both are among the five most common mutations); in France, del(*GJB6*-D13S1830) is very frequent, whereas del(*GJB6*-D13S1854) has not been found to date; and in Belgium, del(*GJB6*-D13S1830) is not a common allele, while del(*GJB6*-D13S1854) has not been detected so far ([14] and this study). The situation in Italy is even more striking, the del(*GJB6*-D13S1830) being a frequent allele in the north and very rare in the south ([14 15] and Murgia A, Leonardi E, unpublished data). These differences between neighbouring countries, and even between regions of the same country, further illustrate the complexity of the genetic epidemiology of non-syndromic hearing impairment.

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