

A DELETION INVOLVING THE CONNEXIN 30 GENE IN NONSYNDROMIC HEARING IMPAIRMENT

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ABSTRACT

Background Inherited hearing impairment affects about 1 in 2000 newborns. Up to 50 percent of all patients with autosomal recessive nonsyndromic prelingual deafness in different populations have mutations in the gene encoding the gap-junction protein connexin 26 (*GJB2*) at locus DFNB1 on chromosome 13q12. However, a large fraction (10 to 42 percent) of patients with *GJB2* mutations have only one mutant allele; the accompanying mutation has not been identified. DFNB1-linked familial cases with no mutation in *GJB2* have also been reported.

Methods We evaluated 33 unrelated probands with nonsyndromic prelingual deafness who had only one *GJB2* mutant allele. Nine subjects had evidence of linkage to DFNB1. We used haplotype analysis for markers on 13q12 to search for mutations other than the one involving *GJB2*.

Results We identified a 342-kb deletion in the gene encoding connexin 30 (*GJB6*), a protein that is reported to be expressed with connexin 26 in the inner ear. The deletion extended distally to *GJB2*, which remained intact. The break-point junction of the deletion was isolated and sequenced, and a specific diagnostic test was developed for this common mutation. Twenty-two of the 33 subjects were heterozygous for both the *GJB6* and *GJB2* mutations, including all 9 with evidence of linkage to DFNB1. Two subjects were homozygous for the *GJB6* mutation.

Conclusions A 342-kb deletion in *GJB6* is the second most frequent mutation causing prelingual deafness in the Spanish population. Our data suggest that mutations in the complex locus DFNB1, which contains two genes (*GJB2* and *GJB6*), can result in a monogenic or a digenic pattern of inheritance of prelingual deafness. (N Engl J Med 2002;346:243-9.)

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HEARING impairment affects about 1 in 1000 newborns.¹ Cases that are present before the development of speech (prelingual onset) hamper speech acquisition and, therefore, normal communication and social integration. Early detection is essential for the application of palliative treatments and special education. Since about 50 percent of the cases of hearing impairment have genetic causes,¹ molecular diagnosis and

genetic counseling are needed. However, the main obstacle to molecular diagnosis is the extreme genetic heterogeneity of nonsyndromic hearing impairment. Most cases of genetic deafness are autosomal recessive. So far, 28 loci for autosomal recessive nonsyndromic hearing impairment have been identified, which are referred to as DFNB loci, and 10 genes have been sequenced (their descriptions are available at <http://www.uia.ac.be/dnalab/hhh>). Mutations in the gene encoding the gap-junction protein connexin 26 (*GJB2*) at the DFNB1 locus on chromosome 13q12 are responsible for up to 50 percent of all cases of autosomal recessive nonsyndromic prelingual deafness in every population tested.²⁻¹³ Six monomers of connexin bind together to form a hexamer (connexon) in the plasma membrane, and each connexon binds another connexon in an adjacent cell to form an intercellular channel.^{14,15} Connexin 26 gap-junction channels are thought to have a role in recycling the potassium that enters the hair cells as part of the mechanism of auditory signal transduction.^{16,17}

Anywhere from 10 to 42 percent of patients with *GJB2* mutations have only one mutant *GJB2* allele,^{2-13,18} and some familial cases have evidence of linkage to the DFNB1 locus but have no mutation in *GJB2*.^{2,5} It was therefore postulated that another gene close to *GJB2* might be responsible for these cases.^{2,4,6,12,19} The gene encoding connexin 30 (*GJB6*) was an obvious candidate, since connexin 30 is expressed in the same inner-ear structures as connexin 26 and both connexins are functionally related.^{20,21} However, previous molecular studies did not reveal any mutation in *GJB6* that was associated with autosomal recessive hearing impairment.^{12,19,22} We sought to identify a mutation in this gene.

METHODS

Subjects

We enrolled 422 unrelated families (364 from Spain and 58 from Cuba) that had members with prelingual, sensorineural, nonsyndromic hearing impairment. A total of 167 Spanish and 26

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Cuban families had at least two affected members and an autosomal recessive pattern of inheritance (familial cases), and 197 Spanish and 32 Cuban families had only one affected member (sporadic cases). Familial cases included 52 Spanish and 5 Cuban sibships and 115 Spanish and 21 Cuban families with affected members in more than one generation.

Written informed consent was obtained from all the subjects included in the study or their parents. Family members with features of syndromic hearing impairment, as well as those with putative environmental causes, were excluded on the basis of their history and findings on clinical examination. Otoloscopic examination, tympanometry with acoustic reflex testing, and tuning-fork tests were carried out systematically to rule out a conductive hearing loss. Pure-tone audiometry was performed to evaluate air conduction (frequencies, 250 to 8000 Hz) and bone conduction (frequencies, 250 to 4000 Hz).

Genetic Techniques

DNA was extracted from peripheral blood according to standard procedures. The primers and conditions for polymerase-chain-reaction (PCR) amplification of the microsatellite markers have been described previously.²³⁻²⁵ Other primers and PCR conditions are described in Supplementary Appendix 1, available with the full text of this article at <http://www.nejm.org>. Fluorescently labeled alleles were analyzed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Mutation detection was performed by heteroduplex analysis on Mutation Detection Enhancement gels (MDE, FMC Bioproducts) as described previously.^{26,27} DNA sequencing was performed in an ABI Prism 310 Genetic Analyzer.

Southern Blotting

Total digests of genomic DNA (15 μ g) were blotted onto Zeta-Probe GT membranes (Bio-Rad). Probes were labeled by random priming with [α^{32} P]deoxycytidine triphosphate with the use of the High Prime kit (Roche). Hybridization was performed with a moderate level of stringency in Church buffer (0.5 M sodium phosphate buffer at a pH of 7.2, 7 percent sodium dodecyl sulfate, and 1 mM EDTA at a pH of 8.0) at 65°C overnight. Membranes were washed three times in 2 \times standard sodium citrate buffer (300 mM sodium chloride and 30 mM sodium citrate) with 0.1 percent sodium dodecyl sulfate at 65°C and exposed to Kodak X-OMAT AR film for 10 days at -30°C.

RESULTS

A total of 422 unrelated subjects from Spain and Cuba who had prelingual nonsyndromic hearing impairment with a mode of inheritance that was compatible with an autosomal recessive pattern were assessed for mutations in *GJB2*. Of these 422 subjects, 129 had mutations in both alleles of *GJB2*, 249 had no identifiable mutation in *GJB2*, and 44 had a mutation in one allele of the gene but no mutations in either the coding region or the splice sites of the other allele. The 44 heterozygous subjects and their relatives underwent genotyping for four microsatellite markers (D13S141, D13S175, D13S1275, and D13S292) that are close to *GJB2* on 13q12.^{23,24} Haplotype analysis for these markers ruled out linkage to DFNB1 in 11 subjects, suggesting that they were coincidental carriers of the mutation; this result was expected, given the high carrier frequency of *GJB2* mutations in the Spanish population (2.5 percent for the most frequent mutation, the deletion of guanine at position 35 [35delG])²⁸ (and unpublished data).

In 24 subjects, haplotype analysis was not informative. Finally, nine subjects (eight Spanish and one Cuban) had findings indicative of linkage to DFNB1, suggesting that another mutation on 13q12 accompanied the *GJB2* mutation.

Haplotype analysis also yielded two unexpected results. First, the lack of consistency in the segregation of the alleles of marker D13S175 in nine families suggested the presence of an unamplifiable allele. Second, the two affected daughters (Subjects II-1 and II-2 in Fig. 1) of parents who had severe deafness and who were heterozygous for the 35delG mutation in *GJB2* had inherited the wild-type *GJB2* allele from each parent, as shown by direct testing and haplotype analysis. It was not possible to amplify marker D13S175 from Subjects II-1 and II-2, with the use of either a pair of primers described in the literature²³ or an alternative primer pair designed on the basis of the sequences flanking the (CA)_n repeat. These results were consistent with the occurrence of a deletion involving at least the D13S175 marker.

To confirm and determine the extent of the deletion in this Spanish family, we tested Subjects II-1 and II-2 for sequence variance in *GJB6*, a gene very close to D13S175 (Fig. 1A). A DNA fragment corresponding to the entire coding region of *GJB6* could not be amplified by PCR in these two subjects. To determine whether the deletion of *GJB6* was total or partial, we evaluated four overlapping DNA fragments (labeled A, B, C, and D in Fig. 1B) spanning the entire coding region using PCR amplification. Fragment D was amplified, but fragments A, B, and C were not. We concluded that the deletion truncated the *GJB6* open reading frame between nucleotides 367 and 574 (the proximal break point). The partial deletion of *GJB6* was confirmed by Southern blotting (Fig. 1C and 1D).

To determine the distal break point of the deletion, we used PCR to amplify a set of sequence-tagged sites, which have been described or were developed by us during this work, from Subjects II-1 and II-2 (Fig. 1A). Combining the results obtained for each sequence-tagged site with the data provided by the Southern blotting, we identified an interval that should contain the distal break point. We developed a specific PCR assay to amplify the break-point junction of the deletion. As expected, no PCR product was obtained from control subjects with normal hearing, but a 460-bp DNA fragment was obtained from both of the children and their parents. Sequencing of the PCR product from each parent yielded the same break-point junction (Fig. 2A), indicating that both children were homozygous for the deletion. The deletion spanned 342 kb, and an examination of the break-point junction showed that the deletion involved moderately similar sequences along a very short

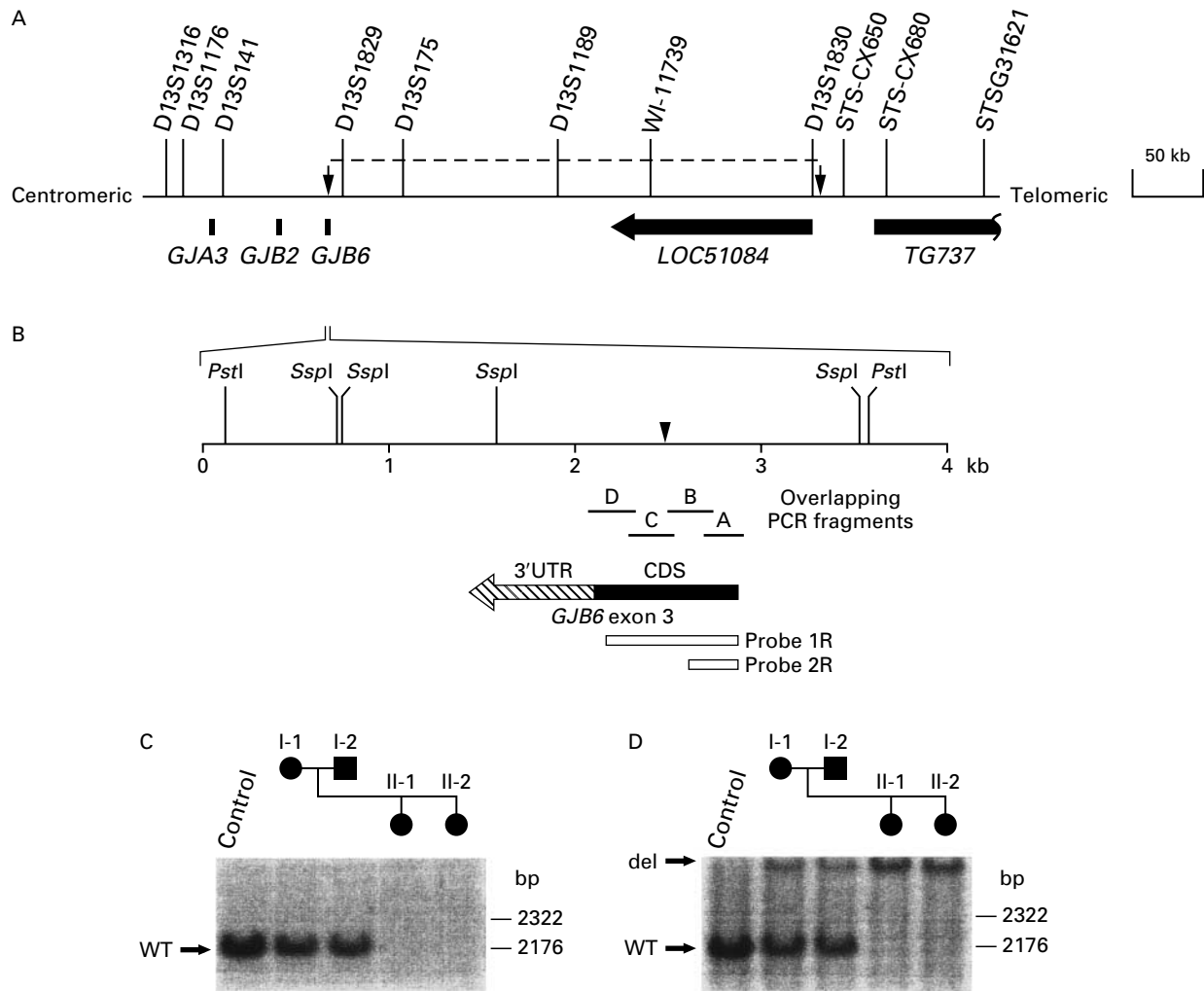


Figure 1. Map of the Region of *GJB6* on Chromosome 13q12 Affected by the 342-kb Deletion, Referred to as the $\Delta(GJB6-D13S1830)$ Deletion (Panel A); Map of Exon 3 of *GJB6* (Panel B); and Pedigree and Results of Southern Blot Analysis of One Spanish Family (Panels C and D).

Panel A shows a 600-kb DNA segment that includes the segment affected by the $\Delta(GJB6-D13S1830)$ deletion and flanking sequences (National Center for Biotechnology Information accession number, NT_009917.3). The positions of the seven markers and four sequence-tagged sites (STS) are indicated. The location of two other genes in the region is shown: the gene encoding λ -crystallin (*LOC51084*) and the gene that encodes probe hTg737 (*TG737*), which has been implicated in polycystic kidney disease. The two break points of the deletion are marked by vertical arrows, and the extent of the deletion is indicated by the dashed line. Panel B shows the 4-kb segment of DNA that contains the third exon of the *GJB6* gene and flanking sequences. UTR denotes untranslated region, and CDS coding sequence. Restriction sites, either *PstI* or *SspI*, are indicated. The positions of the probes used in the Southern blot analysis are indicated below exon 3. The arrowhead indicates the deletion break point within *GJB6*. Panel C shows the results of Southern blot analysis in a Spanish family with severe deafness. Probe 2R was used on *SspI* digests of genomic DNA. A 2.2-kb wild-type (WT) band is present in the control subject and the parents. This band is absent in both children. Panel D shows the results of Southern blot analysis in the same family with probe 1R on *SspI* digests of genomic DNA. In addition to the 2.2-kb band, a novel 2.9-kb band, created by the deletion (del), appears faintly in the parents (both of whom are heterozygous) and more clearly in the children (both of whom are homozygous) and is absent in the control subject. Circles indicate female family members, and squares male family members.

TABLE 1. RESULTS OF SCREENING FOR THE 342-kb DELETION IN *GJB6*.*

SUBJECTS	SPANISH	CUBAN	TOTAL
	SUBJECTS	SUBJECTS	
	no. with deletion/total no. screened (%)		
Subjects with one mutant <i>GJB2</i> allele	20/30 (67)	2/3 (67)	22/33 (67)
Evidence of linkage to DFNB1	8/8 (100)	1/1 (100)	9/9 (100)
Linkage to DFNB1 uncertain	12/22 (55)	1/2 (50)	13/24 (54)
Subjects with no mutant <i>GJB2</i> alleles	1†/210 (0.5)	0/39	1†/249 (0.4)
Control subjects with normal hearing	0/200	0/0	0/200

*The deletion is referred to as $\Delta(GJB6-D13S1830)$.

†This patient was homozygous for the $\Delta(GJB6-D13S1830)$ deletion.

DISCUSSION

Nonsyndromic prelingual hearing impairment is difficult to diagnose by molecular means, because many genes are involved and there is insufficient knowledge of the individual contribution of each gene and its mutations. Therefore, most genetic analyses include routine molecular diagnosis for mutations in the *GJB2* gene, since such mutations are the cause of up to 50 percent of the cases. However, the diagnostic techniques reveal only one mutant *GJB2* allele in a substantial proportion of patients. Although unexplained cases may be attributable in part to intrinsic drawbacks in the techniques for the detection of mutations or to the high frequency of carriers in the population, it has long been suspected that other mutations are present in a gene or genes in the same chromosomal region. We identified a novel mutation — a deletion that truncates the *GJB6* gene but does not affect the *GJB2* gene — that frequently accompanied a mutation in a single *GJB2* allele (i.e., a double heterozygous state) in our group of subjects with unexplained cases of nonsyndromic prelingual hearing impairment.

The frequent occurrence of subjects who were heterozygous for both the $\Delta(GJB6-D13S1830)$ deletion and point mutations in *GJB2* could be explained on the basis of either a monogenic or a digenic pattern of inheritance. In the case of a monogenic mode of inheritance, there must be a regulatory element that is essential for the expression of the *GJB2* gene in the inner ear. This hypothetical element would be located far upstream of *GJB2* and *GJB6*, and the deletion of this element would suppress the level of expression of *GJB2* enough to produce a phenotype of hearing impairment. However, the existence of the *GJB2* regulatory element remains merely hypothetical. An alternative interpretation would be that the

deletion inactivates a second gene whose protein product is functionally related to connexin 26. Substantial experimental evidence supports the hypothesis that *GJB6* is this postulated second gene. First, connexin 26 and connexin 30 are both expressed in the spiral limbus, the spiral ligament, and the stria vascularis and among the supporting cells of the organ of Corti in rat cochlea.^{20,21} Both connexins were also detected in the lateral wall of the inner ear in a 22-week-old human fetus.²¹ Second, connexin 26 and connexin 30 monomers can bind each other to form heterotypic, or mixed, gap-junction channels.²⁹ Third, a mutation in *GJB6* was reported in a family with autosomal dominant hearing impairment.²² Finally, in the current study, we identified three deaf patients (two patients in the family whose pedigree is shown in Fig. 1 and one additional patient) who lacked a functional *GJB6* gene but who had two intact *GJB2* alleles.

Altogether, these data support the concept that DFNB1 is a complex locus containing two genes (*GJB2* and *GJB6*) and that the loss of any two of the four alleles from these genes results in hearing impairment. In other words, patients with a prelingual hearing impairment could be homozygous for point mutations that inactivate *GJB6* alleles or heterozygous for both the $\Delta(GJB6-D13S1830)$ deletion and mutant *GJB2* alleles. This type of complex pattern of inheritance has already been reported in other recessive disorders, notably retinitis pigmentosa.³⁰ However, these hypothetical mutations in *GJB6* have not yet been identified.^{12,19,22} Although the presence of the $\Delta(GJB6-D13S1830)$ deletion may have hampered the detection of point mutations in some screening studies, the high frequency of negative results suggests that if these mutations exist, they must be rare. Screening for *GJB6* mutations in other populations should help clarify this point.

Currently, only two genes in the region affected by the deletion have been sequenced: *GJB6* and the gene encoding λ -crystallin (*LOC51084*), a component of the lens of the eye (the sequence is available at <http://www.ncbi.nlm.nih.gov/LocusLink>). Different missense mutations in *GJB6* are responsible for autosomal dominant hearing loss²² or autosomal dominant hidrotic ectodermal dysplasia,³¹ probably because they encode dysfunctional proteins or cause dominant negative effects. However, to our knowledge, no pathogenic mutation in *LOC51084* has been reported. Although we found no signs of skin or eye disorders in the three subjects who were homozygous for the Δ (*GJB6*-D13S1830) deletion, all of them are still children. Thus, careful follow-up of their clinical status will be needed to settle this point.

Our findings indicate that the Δ (*GJB6*-D13S1830) deletion is the second most frequent (after the 35delG mutation in *GJB2*) genetic cause of nonsyndromic prelingual hearing impairment in the Spanish population. The frequency of this deletion in other populations remains to be determined, but the deletion of marker D13S175 has been demonstrated in at least one familial case of prelingual deafness in New Zealand.³² When the current report was in press, Lerer et al.³³ reported a deletion involving the *GJB6* gene in seven patients with nonsyndromic hearing loss from four unrelated Ashkenazi Jewish families. Since they did not isolate the break-point junction of the deletion, we do not know whether the mutation is the same as the one we report in the current article. All these reports, taken together, should provide new insight into the role of connexins in the auditory system. The relatively large percentages worldwide of patients with unexplained cases of prelingual deafness who are heterozygous for the *GJB2* mutation suggest that the Δ (*GJB6*-D13S1830) deletion or other, similar mutations are also widespread. Our results also indicate that the deletion of large portions of a chromosome can easily be missed with the use of the usual mutation-detection assays, even though they may have a high prevalence in human disease.

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