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A large deletion including most of *GJB6* in recessive non syndromic deafness: a digenic effect?

Nathalie Pallares-Ruiz[1], Patricia Blanchet[2], Michel Mondain[3], Mireille Claustres[1] and Anne-Francoise Roux*,[1]

[1]*Laboratoire de Génétique Moléculaire, 34093 Montpellier cedex, France;* [2]*Département de Génétique médicale, CHU Montpellier, France;* [3]*Département ORL, CHU Montpellier, France*

Congenital profound deafness has a known genetic origin in more than 50% of all cases. The majority of the non syndromic hearing loss (NSHL) show an autosomal recessive inheritance. Mutations in the *GJB2* gene (connexin 26) account for more than 50% of the recessive non syndromic deafness (*DFNB1*) among 30 loci. Other connexin genes have been more rarely involved and attention was given here to the *GJB6* gene (connexin 30). We show that homozygous deletion of a minimal 150 kb region encompassing this gene causes NSHL. More strikingly, association of this deletion *in trans* of the *GJB2* gene 35delG or E47X mutations is also associated with NSHL.
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Introduction

The majority of the non syndromic hearing loss (NSHL) show an autosomal recessive inheritance,[1] and alterations in the *GJB2* gene, encoding connexin 26, is recognised in half of the cases. Among 50 mutations identified so far, some are major common mutations found in specific populations.[2–5] A subset of patients is reported to carry a single heterozygous mutation of this gene, possibly reflecting the carrier frequency, though digenic inheritance has also been suggested.[6] More rarely, *GJB2* has been involved in non syndromic deafness with an autosomal dominant pattern of inheritance (*DFNA3*).[7] Connexin 26 is a member of a large family of gap junction channel proteins that allow intercellular communication and regulate the passage of ions and small metabolites. The connexins form hexameric hemichannels (called connex-

on) which have to dock with a connexon from an adjacent cell to form a functional channel.[8] Three other gap junction genes *GJB1*, *GJB3* and *GJB6* respectively encoding connexins 32, 31 and 30, are known to cause deafness. One mutation in the *GJB1* gene is suspected to be responsible for deafness associated with peripheral neuropathy.[9] Mutations in *GJB3* are responsible for dominant or recessive deafness.[10,11] Finally, one mutation in *GJB6* causes hearing loss by a dominant negative effect.[12] As the two genes *GJB6* and *GJB2* are lying ~35 kb apart, they both define the *DFNA3* locus. Remarkably, *GJB2*, *GJB3* and *GJB6* are also involved in inherited skin disorders[13–15] underlining the importance of these connexins in very distinct tissues.

Connexins 26 and 30 are structurally highly related.[12,16] Moreover, expression analyses performed in mouse and rat show that Cx26 and Cx30, as well as Cx31 and Cx32, form two co-expression groups.[17,18] Co-expression of Cx26 and Cx30 in the inner ear is thought to be a prerequisite for the maturation of the cochlea.[18,19] Their co-expression is also detectable in human embryo cochlea after the 22nd week.[19,20] The co-expression of these two connexins also favoured the hypothesis that their connexons may form both homo- and/or hetero-channels.[21]

*Correspondence: Dr A-F Roux, Laboratoire de Génétique Moléculaire, Institut Universitaire de Recherche Clinique, 641, avenue du Doyen Gaston Giraud, 34093 Montpellier cedex 05, France,
Tel: +33 4 67 41 53 60; Fax: +33 4 67 41 5365;
E-mail: Anne-Francoise.Roux@igh.cnrs.fr*

Patients and methods

Patients

The five patients presented in this study were referred to us by the Genetic counselling and the Ear Nose and Throat Clinics. Clinical history of each individual was collected and audiograms were performed. For the five patients the hearing loss was sensorineural, bilateral, prelingual and profound. Informed consent was obtained from each individual.

PCR analysis

Genomic DNA was extracted from blood samples using the Nucleon BACC3 DNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions.

Mutation analysis in the *GJB2* gene was performed by PCR of the non coding exon and the coding exon, followed by DHPLC analysis (WAVE DNA Fragment Analysis System, Transgenomic) and sequencing (ABI 310 sequencer, PE biosystem).

The coding region of the *GJB6* gene (CX30) was amplified in three overlapping fragments R1, R2 and R3 of 431, 410, 333 bp, respectively. Primer sequences are as follow: (CX30-R1: ctttgcccactttgtctgt/gacccctctatccgaacctt; CX30-R2: cctccagctgatcttcgtct/gcagcaggtagcacaactct); CX30-R3: gccccaaccttgttgactgc/gttggtattgccttctggag. Primer sequences for D13S1189 analysis were retrieved from NCBI.

Quantification

We used Light Cycler Master SYBR Green I on a LightCycler system (Roche Diagnostics). Quantification analysis could not be performed for D13S175 which is a microsatellite marker. Primers used for CX26 gene analysis have been described.[2] Primers used for CX30-R1/CX30-R3 analysis are indicated above. Primers used for DMD exon 60 are already published.[22] A negative control without DNA sample and a standard curve (31.25 ng – 15.6 ng – 7.8 ng – 3.9 ng – 1.95 ng – 0.975 ng – 0.4875 ng) were included in every test. For each DNA sample, four replicate points were performed and expressed in mean$\pm$SD (standard deviation). In addition, quantification was performed twice for each sample to confirm the results.

Electronic-database information

The accession numbers and URLs for this article are as follows: http://www.iro.es/deafness/ (connexin-deafness homepage); http://www.uia.ac.be/dnalab/hhh (Hereditary Hearing Loss Homepage); *DFNB1* {MIM 220290} *DFNA3* {MIM 601544}; *GJB1* {MIM 304040} *GJB2* {MIM 121011} *GJB3* {MIM 603324} *GJB6* {MIM 604418}; Genomic sequence of the *GJB2* and *GJB6* region: http://www.ncbi.nlm.nih.gov/; Chromosome 13 map/contig view: http://www.ensembl.org/ Homo_sapiens/contigview?chr=chr13; GenBank: http://www.ncbi.nlm.nih.gov/

Results

Because no mutation in the *GJB2* gene could be found in patient SU1, a sporadic case presenting congenital profound deafness, we decided to study other deafness genes, in particular *GJB6*, because of its structural and expression similarities with *GJB2*. We then identified for the first time the deletion reported here. When PCR analysis of the *GJB6* coding region was performed, only the 3′ end of the gene (CX30-R3) could be detected, whereas the 5′ end (CX30-R1 and CX30-R2) was not amplifying, thus suggesting a homozygous deletion in *GJB6* (Table 1). We excluded uniparental disomy as a possible origin for homozygosity by microsatellite marker analyses (listed in Figure 1). Absence of amplification was then noticed for marker D13S175, lying 5′ to *GJB6*. Furthermore, this deletion appeared to extend beyond the non polymorphic marker D13S1189 (Table 1 and Figure 1) suggesting that the deleted region covers at least 150 kb, based on publicly available genomic sequence NT_009917. Discrepancies and ambiguities within the region adjacent to D13S1189 does not permit yet precise mapping of the other deletion border (Figure 1).

Independently, we studied patient SU5 who was referred for congenital profound deafness. *GJB2* sequence analysis revealed the presence of the 35delG mutation in heterozygous state with no other mutation within the gene. Family study showed that, like her unaffected brother, she had inherited the *GJB2* 35delG mutation from her unaffected mother (Figure 2A). Haplotypes analysis was then performed in the *DFNB1* region (encompassing the *GJB2* gene) to exclude or confirm involvement of this region in deafness

Table 1 PCR results

	D13S1189	*D13S175*	*CX30-R1*	*CX30-R2*	*CX30-R3*	*CX26*
SU1	−	−	−	−	+	+/+
SU5	+	hemizygote	+	+	+	+/35delG
SU47	+	hemi or homozygote	+	+	+	+/35delG
SU145	+	hemi or homozygote	+	+	+	+/35delG
SU172	+	hemizygote	+	+	+	+/E47X

(−) Absence of amplification; (+) amplification observed. Hemizygosity for D13S175 could be deducted from SU5 and SU172 family studies. CX26 genotype is indicated.
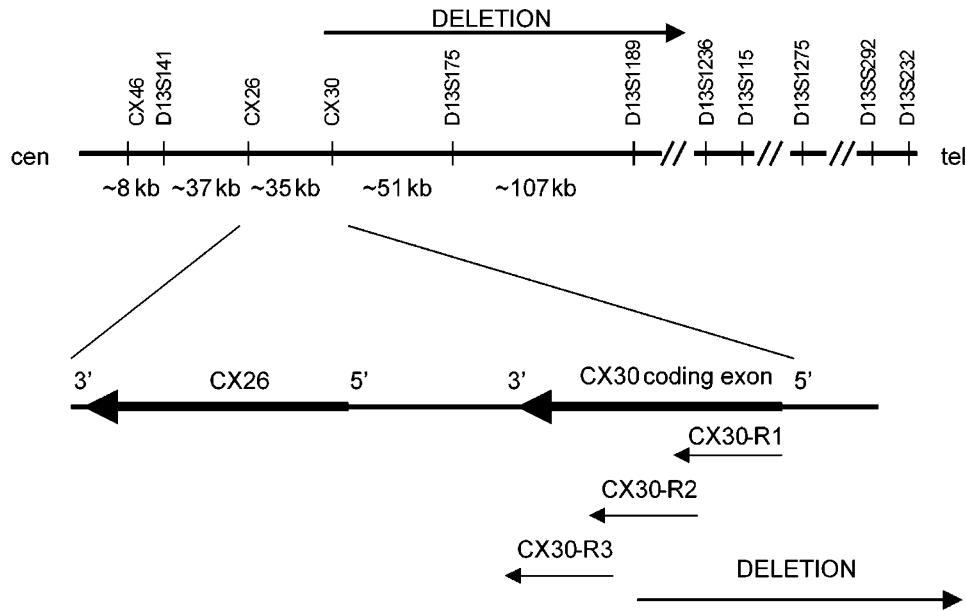
Figure 1 Schematic representation of the DFNB1 region and the deletion. The order of the markers is given according to the contig view (Ensembl project from the Sanger Institute). The distances between genes and markers from NT_009917 contig are indicated. CX26 (*GJB2*) and CX30 (*GJB6*) genes are indicated by arrows. The deletion is indicated: the centromeric breakpoint is within fragment CX30-R2 in the coding exon, the telomeric breakpoint is unknown but lies beyond marker D13S1189.
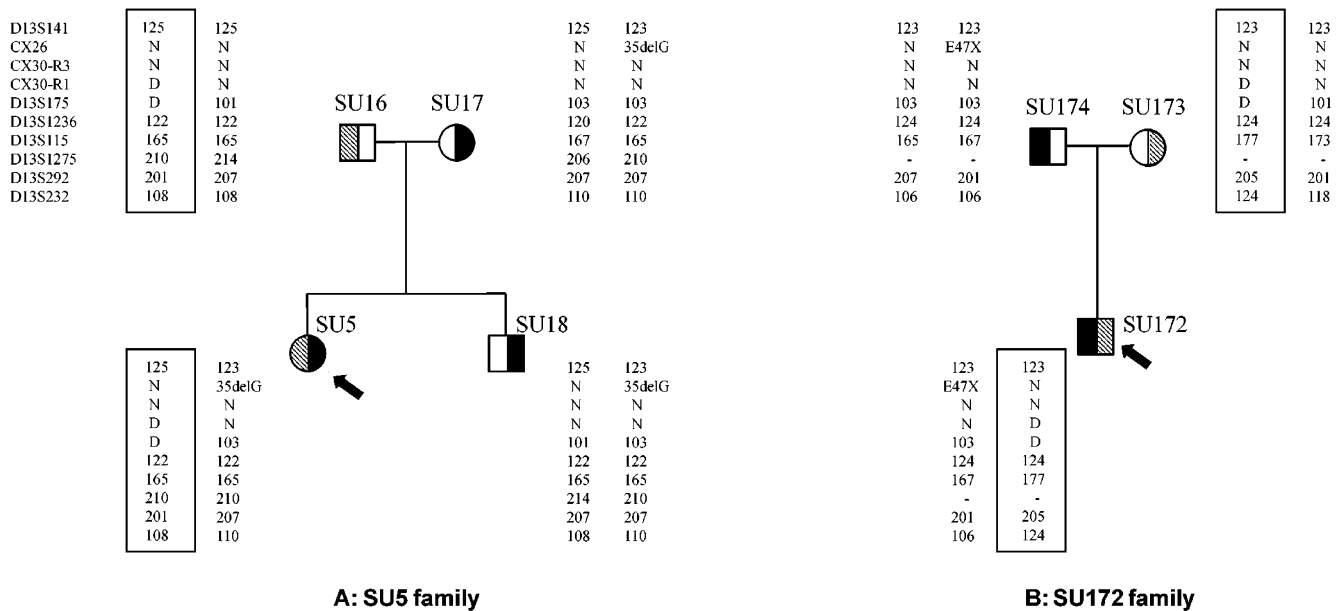
Figure 2 Haplotypes analysis in the DFNB1 region. (**A**) SU5 family and (**B**) SU172 family. Carriers of the *GJB2* 35delG or E47X mutation are indicated by black fillings; carriers of the *GJB6* deletion are indicated by diagonal hatching. Haplotypes carrying the deletion are boxed. The size of each polymorphic allele is indicated in base pair (bp). The proposita (SU5) and propositus (SU172) are indicated by a thick arrow. N: normal; D: deleted.

with the possible presence of a cryptic mutation (Figure 2A). Involvement of *DFNB1* region could not be excluded as the proposita had inherited from her unaffected father a different chromosome than her brother. However, and surprisingly, the proposita SU5 showed a single peak for D13S175 defining allele 103 suggesting either a neomutation of the paternal

Table 2 Quantification results: dosage of CX30 gene and D13S1189

	CX26 Mean ± SD	DMD Mean ± SD	DMD/CX26	D13S1189 Mean ± SD	D13S1189/CX26	CX30-R1 Mean ± SD	CX30-R1/CX26	CX30-R3 Mean ± SD	CX30-R3/CX26
SU5	11.26 ± 0.42	22.26 ± 1.32	**1.97**	5.45 ± 0.43	**0.48**	5.83 ± 0.43	**0.51**	10.52 ± 1.32	**0.93**
SU16	3.06 ± 0.18	3.49 ± 0.07	**1.14**	1.78 ± 0.22	**0.58**	1.38 ± 0.16	**0.44**	3.98 ± 0.27	**1.29**
SU17	2.14 ± 0.17	5.10 ± 0.22	**2.38**	2.09 ± 0.18	**0.97**	2.16 ± 0.16	**1.0**	2.35 ± 0.16	**1.09**
SU18	2.09 ± 0.26	2.77 ± 0.23	**1.32**	2.48 ± 0.28	**1.18**	2.09 ± 0.08	**1.0**	2.44 ± 0.17	**1.16**
SU47	2.14 ± 0.26	4.80 ± 0.28	**2.24**	0.97 ± 0.09	**0.45**	1.06 ± 0.10	**0.49**	2.50 ± 0.37	**1.17**
SU145	1.49 ± 0.25	3.24 ± 0.34	**2.17**	0.61 ± 0.06	**0.4**	0.75 ± 0.06	**0.50**	1.92 ± 0.21	**1.28**
SU172	4.19 ± 0.63	4.12 ± 0.07	**0.98**	ND		2.33 ± 0.23	**0.55**	ND	
SU173	7.09 ± 0.82	13.23 ± 0.99	**1.86**	ND		3.51 ± 0.29	**0.49**	ND	
SU174	4.84 ± 0.29	3.90 ± 0.28	**0.8**	ND		5.47 ± 0.28	**1.13**	ND	

CX26 gene is the reference gene; each patient is heterozygous for the 35delG or the E47X mutation (except for SU16: father of SU5 patient; and SU173: mother of SU172 patient), assessing the presence of the two CX26 alleles. An X-linked gene, DMD exon 60, is used as control of correct gene dosage, DMD/CX26 visualizes the 2 DMD genes present in females (SU5, SU17, SU47, SU145, SU173) *vs* one copy of DMD gene present in males (SU16, SU18, SU172, SU174). For each sample, ratio/CX26 is indicated in **bold** and corresponds to the copy number of the particular PCR tested. In *italics*: patients with congenital profound deafness (SU5, SU47, SU145 and SU172), females are underlined. SD: standard deviation, ND: not determined.

inherited allele or absence of its amplification. The father had been first defined as homozygous 101/101.

Putting these data together, we then wondered if D13S175 could be deleted along with the major part of *GJB6* in SU5, as in SU1, but in heterozygous state. To address this hypothesis, we performed real time quantitative PCR using Light-Cycler (Roche diagnostics) on all the deleted PCR fragments observed in SU1 except D13S175 marker. The results, presented on Table 2, show that SU5 presents two copies of the 3′ end of the *GJB6* gene (CX30-R3) but only one copy of the 5′ coding region of the *GJB6* gene (CX30-R1) and D13S1189 and therefore concluded that only one allele was present for D13S175 (Tables 1 and 2). Moreover, quantitative PCR performed on the parents and the brother of the proposita showed that this deletion was inherited from the unaffected father (SU16) who was, therefore, hemizygote for D13S175 (Figure 2A and Table 2).

Three additional unrelated patients (SU47, SU145 and SU172) also referred for profound congenital deafness showing one heterozygous mutation in the *GJB2* gene (35delG or E47X) were then tested for the D13S175 and revealed either hemizygosity or homozygosity for one allele (Table 1). Consequently, analysis of the region by quantitative real time PCR evidenced that they were also deleted in the same region including marker D13S1189 (Table 2). Finally, family study revealed that SU172 had inherited the deletion from his mother SU173, *in trans* of the E47X *GJB2* mutation (Figure 2B).

Discussion

A point mutation in the *GJB6* gene has been involved in non syndromic deafness (*DFNA3*) by dominant inhibition of the activity of wild-type GJB6 channels,[12] underlining the critical function of GJB6 in normal hearing. Expression studies suggest that *GJB2* and *GJB6* have a similar regulation in the

cochlea, but a possible redundancy of the two encoded proteins seems excluded by the data from Grifa *et al*.[12] This prompted us to suppose that the total absence (homozygous state) of GJB6 protein in itself results in a severe phenotype.

Because we deal with a deletion spanning a large genomic region upstream and including most of the *GJB6* gene, several mechanisms could lead to the deafness phenotype. First, removing two doses of *GJB6* or removing one dose of *GJB6* and one dose of *GJB2* result in a reduced number of homomeric or heteromeric connexons that is not sufficient to ensure the correct transport of the potassium ions; then digenic inheritance is the etiology of deafness in the heterozygous cases presented here. Second, the deletion removes the regulatory elements specific for cochlea and/or changes the chromatin conformation, resulting in a functionally null or deficient *GJB2* allele. Then deafness results from a *GJB2* deficiency alone. Third and last, the profound deafness phenotype is caused by a combination of both mechanisms. Therefore, in addition to the deficit of *GJB6*, the reduced level of wild type *GJB2* may aggravate the phenotype in homozygotes. Then in heterozygotes an allele carrying a *GJB2* deafness-causing mutation *in trans* of a reduced (or null) allele of *GJB2*, in addition to a deficit of *GJB6* causes profound deafness. Expression studies are necessary to evaluate whether this deletion results in a functionally reduced or null *GJB2* allele but they remain difficult because of the accessibility of the specific tissue.

There is no doubt that we deal with a similar deletion in the five unrelated patients but we do not know yet whether this deletion is ancestral or recurrent, or both. Finding this mutation in five unrelated patients referred to our clinics prompt us to suppose that this deletion is not rare. Because it requires an appropriate screening it can remain undetected in the heterozygous cases. Although SU1 and SU5 are known to originate respectively from Spain/Italy and Spain/France, the ethnic background of the other patients, who live in France,

is unknown. Population studies will be necessary to estimate the occurrence of this deletion and the confirmation of its recessive expression, though the latter is strongly suggested by the data presented here. However, until the exact deletion boundaries are known, large scale screening will remain laborious.

The characterisation of this new *GJB6* deletion enabled us to fully determine the molecular basis of deafness in four of the six families initially labelled as 'heterozygous for a *GJB2* mutation.' Actually, the two remaining families present a distinct deafness etiology. Therefore, at least in specific populations, we emphasise that the screening of this deletion in deaf patients will solve a number of cases heterozygous for one mutation in the *GJB2* gene and thereby constitute a significant improvement of the genetic testing and diagnosis.

In conclusion, we describe a new large deletion destroying the *GJB6* gene. Considering the importance of *GJB6* for normal hearing, we suggest that the homozygous absence of this gene is responsible for severe hearing impairment and therefore *DFNB1*, as *DNFA3*, should also include the *GJB6* gene. Moreover, this deletion present *in trans* of a *GJB2*-deficient allele (35delG or E47X) also leads to profound deafness. That the mechanisms underlying the deafness phenotype reside in a pure digenic effect or in a diminished expression of the normal *GJB2* allele, or a combination of both still remain to be proven.

While this manuscript was in review, another group, Lerer *et al*. (*Hum Mutat* 2001; **18**: 460) has identified a large deletion 5′ of the *GJB6* gene in Jewish Ashkenazi families. Though the authors do not show that the deletion lies within *GJB6* coding region, their results tend to suggest that the deletion they identified is similar to the deletion presented here.

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