

Cx26 deafness: mutation analysis and clinical variability

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Abstract

Mutations in the gap junction protein connexin 26 (Cx26) gene (GJB2) seem to account for many cases of congenital sensorineural hearing impairment, the reported prevalence being 34-50% in autosomal recessive cases and 10-37% in sporadic cases. The hearing impairment in these patients has been described as severe or profound. We have studied 53 unrelated subjects with congenital non-syndromic sensorineural hearing impairment in order to evaluate the prevalence and type of Cx26 mutations and establish better genotype-phenotype correlation. Mutations in the Cx26 gene were found in 53% of the subjects tested, 35.3% of the autosomal recessive and 60% of the sporadic cases in our series. Three new mutations were identified. The hearing deficit varied from mild to profound even in 35delG homozygotes within the same family. No evidence of progression of the impairment was found.

Alterations of the Cx26 gene account for a large proportion of cases of congenital non-syndromic sensorineural deafness, so it seems appropriate to extend the molecular analysis even to subjects with mild or moderate prelingual hearing impairment of unknown cause.

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Congenital sensorineural hearing impairment affects approximately 1/1000 children in western countries.¹ This condition, when severe or not recognised early in infancy, is responsible for delayed development of verbal skills which prevents correct language acquisition and will eventually become a handicap for the child.

About 60% of all cases of sensorineural deafness are estimated to be of genetic origin, mainly with autosomal recessive inheritance.² The majority of these deficits are non-syndromal and an aetiological diagnosis based exclusively on clinical criteria often does not allow distinction between a genetic and an acquired cause. This has a definite influence on the quality of medical care offered to the patients and to their families, both in terms of early diagnosis and genetic counselling.³

From a genetic standpoint, non-syndromal sensorineural deafness is a highly heterogeneous condition for which, to date, 20 autosomal recessive and at least 19 autosomal dominant

loci have been mapped.⁴⁻⁶ Of particular relevance among deafness genes is *GJB2*, a gene coding for the gap junction protein connexin 26 (Cx26). This gene has recently been found mutated in cases of autosomal recessive non-syndromic sensorineural deafness.⁷⁻¹² The possibility of the Cx26 variant M34T being a dominant mutation, as reported by some authors,¹³ is controversial.¹⁰⁻¹¹ A newly detected mutation, recently described in two families with autosomal dominant deafness,¹⁴ nevertheless reopens the question of a dual mode of inheritance. The prevalence of Cx26 mutations has been reported as being 34-50% in autosomal recessive cases and 10-37% in sporadic cases. The hearing impairment in these patients has been described as severe or profound; in particular the absence of connexin 26 in cochlear cells in cases of 35delG homozygosity has been associated with profound deafness,⁹ but some mild cases have also been observed.¹⁰

These data confirm the important role played by connexin 26 in the pathogenesis of deafness but raise some important questions. Better knowledge about the actual prevalence of Cx26 mutations in sporadic deafness and a better definition of genotype-phenotype correlation in affected subjects would have an immediate impact both on clinical practice and genetic counselling for deafness.

Subjects, materials, and methods

The study was conducted on 53 unrelated subjects affected by congenital non-syndromic sensorineural hearing impairment greater than 40 dB HL. Written informed consent was obtained from the patients or from the parents in case of a minor. A detailed history was taken for each subject. A thorough clinical evaluation and audiometric assessment, together with a molecular analysis of the complete sequence of *GJB2*, were performed.

CLINICAL EVALUATION

Affected subjects aged 3 to 35 years (mean 12), 21 females and 32 males, monitored at least twice a year since diagnosis by the same audiological institution, were included in the study.

None of these subjects showed signs or other findings associated with syndromes that involve permanent hearing impairment; in particular, motor and cognitive development, thyroid hormone levels, EKG, funduscopy, and parameters of renal function were normal. Subjects with any of the following risk factors for acquired hearing deficit were excluded: (1) history or signs of infections during pregnancy (TORCH + syph + HIV); (2) birth weight

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Table 1 Parameters for clinical description of the hearing impairment

(1) Hearing impairment in dB HL (averaged on 0.5-1-2 kHz)	
Moderate	40-69 dB HL
Severe	70-94 dB HL
Profound	>95 dB HL
(2) Age of onset	
Congenital	Present at birth
Uncertain	When it was difficult to ascertain the real congenital onset
(3) Symmetrical/asymmetrical involvement	
Asymmetrical	>15 dB difference between ears in at least two frequencies
(4) Steady/progressive deficit	
Progressive	>15 dB of progressiveness in at least two frequencies or >10 dB for average of 4 frequencies (0.5-4 kHz)
(5) Configuration of audiogram	
Low frequencies	>15 dB from the better to the poorer hearing threshold at the low frequencies to the mid frequencies
Sloping	>15 dB difference between 1000 and 4000 Hz
Flat	<15 dB difference from 500 to 4000 Hz
U shaped	With >15 dB difference between the better hearing thresholds and the poorer hearing threshold(s)
None of the above configurations	

<1500 g; (3) neonatal Apgar scores <0-4 at one minute or <0-6 at five minutes; (4) need of neonatal mechanical ventilation; (5) hyperbilirubinaemia requiring transfusion; (6) use of ototoxic medication (aminoglycosides, antibiotics, etc) in multiple courses; (7) bacterial meningitis or other infections associated with sensorineural hearing impairment; (8) history of head trauma associated with skull fractures or loss of consciousness; (9) signs of neurodegenerative disorders.

Families included in the study were classified as having autosomal recessive inheritance when there were at least two hearing impaired sibs with normal hearing parents. Cases in which a detailed history did not indicate any other childhood sensorineural hearing loss were referred to as sporadic.

Twenty five cases in the study were sporadic, 17 could be classified as autosomal recessive, while in the remaining 11 cases the family history was positive for the presence of at least one relative with prelingual permanent sensorineural hearing impairment. These latter cases that did not fit our definition of autosomal recessive were generically defined as familial cases.

Family members of unrelated hypoacusis subjects who tested positive for *Cx26* mutations were subsequently offered a molecular and audiological evaluation.

The audiological assessment was aimed at the definition of several parameters, such as age of onset and severity of the deficit, symmetrical/asymmetrical involvement, stability of the damage, and frequency involved (table 1).

MOLECULAR ANALYSIS

This was performed by PCR amplification of genomic DNA, analysis of the amplified products by PAGE (polyacrylamide gel electrophoresis), and by two alternative methods designed for the detection of unknown point mutations, single strand conformation polymorphism (SSCP) and conformation sensitive gel electrophoresis (CSGE).¹⁵ PCR products that showed shifted SSCP bands or heteroduplexes on CSGE analysis were characterised by direct sequencing.

High molecular weight DNA was extracted by standard protocols from peripheral blood leucocytes. The entire coding sequence of the

Cx26 gene was amplified in overlapping fragments using primers as previously described.¹²

Amplification reactions were performed in a final volume of 50 µl containing 200 ng of genomic DNA, 200 µmol/l dNTPs, 20 pmol each primer, 1 mmol/l MgCl₂, 2% DMSO, and 2 U *Taq* polymerase. After five minutes of denaturation at 95°C, 30 PCR cycles were carried out as follows: 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with final extension at 72°C for seven minutes.

Amplification products were then subjected to SSCP analysis on polyacrylamide gels, 6-8%, 2% cross linker, with and without 5% glycerol. Samples were loaded with formamide 60% final concentration and electrophoresed for two to three hours in a water jacketed apparatus with 0.5 × Tris-borate buffer at 20 W constant power. The fragments were visualised on the gels with silver staining

CSGE analysis of PCR products was performed on 6% polyacrylamide gels polymerised in 10% ethylene-glycol, 15% formamide, in Tris-aurine buffer. Before electrophoresis EDTA was added to a final concentration of 10 mmol/l; samples were denatured at 98°C for five minutes and incubated at 68°C for one hour, as described by Ganguly *et al.*¹⁵ Bands were visualised by silver staining.

Direct sequencing of the PCR products on both strands was performed, after purification, on an automated DNA sequencer (ABI Prism 310, Perkin-Elmer).

Statistical analysis was performed by Fisher's exact test.

Results

Mutations of the *Cx26* gene were found in 28 (53%) of the unrelated subjects in our series (table 2). Fifteen cases were sporadic and six were autosomal recessive. Seven positive cases had a family history of deafness but did not fit our definition of autosomal recessive. Sixteen subjects, 30% of our hearing impaired population, were homozygous for the most frequent mutation, the deletion of one of a series of six guanine nucleotides between position 30-35 of the coding sequence (35delG). This mutation accounts for 71% of the *Cx26* mutated alleles. Eight heterozygotes for the 35delG mutation were identified, but the second *Cx26* mutation was detected in only four of them.

Table 2 Summary of Cx26 mutations detected in affected subjects

Mutations	No of subjects	Sporadic cases	Autosomal recessive cases	Familial cases
35delG/35delG	16	8	3	5
35delG/139 GT	3	2	1	
35delG/269 TC	1			1
358-360delGAG/167delT	1	1		
35delG/ negative	4	3	1	
269 TC/ negative	1		1	
551GC/ negative	1	1		
31del14/ negative	1			1
Total	28	15	6	7

In four patients no 35delG alleles were found; mutations on both alleles were identified in only one case (358-360delGAG/167delT), while in three cases only one mutation was detected.

Overall, in seven of our cases, of whom four were heterozygous for the common 35delG mutation, the presumed Cx26 partnering mutation was not detected (12.5% of the Cx26 chromosomes analysed).

Two new mutations were found, a missense mutation, 269T→C, and a deletion of 14 nucleotides, 31del14. The T to C transition at nucleotide 269 of the coding sequence changes a leucine at codon 90 of the second transmembrane domain to a proline (L90P); it was found in two unrelated subjects, associated with a 35delG in one case, as the only detected muta-

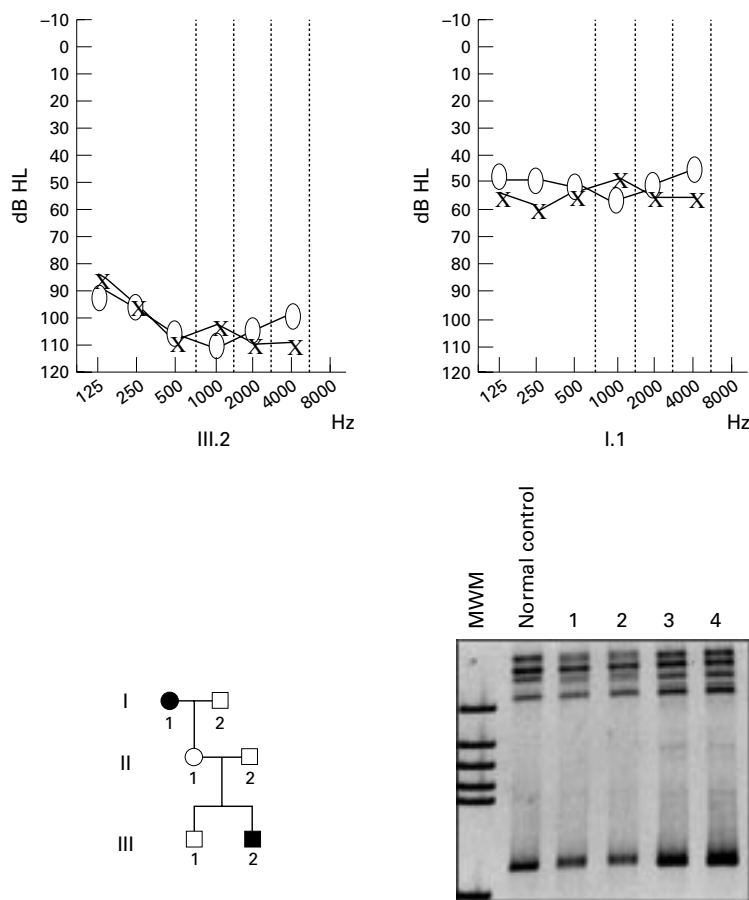


Figure 1 Tonal audiometric curves and SSCP analysis of two 35delG homozygotes in the same family. Affected subjects in the family pedigree are indicated by filled symbols. Subject III.2, 4 years old, has a profound bilateral hearing impairment; subject I.1, 70 years old, has only a moderate hearing deficit. SSCP analysis of the heterozygous 35delG normally hearing subjects II.1 and II.2 is also shown. MWM, molecular weight marker pBR322/HaeIII.

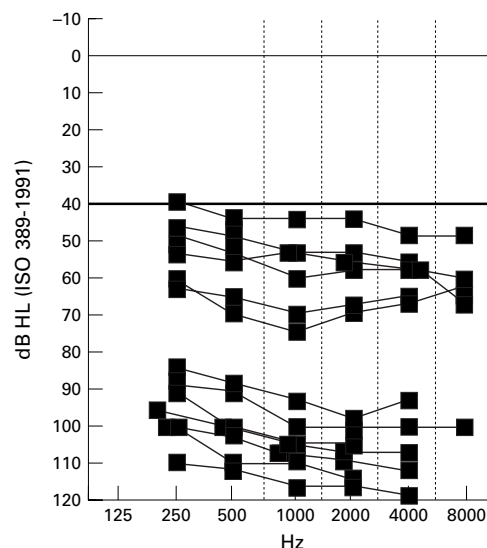


Figure 2 Tonal audiometric curves of 15 homozygotes for the 35delG mutation. Each tonal measurement corresponds to the better ear threshold of a single subject. Arrowheads indicate no measurable thresholds for immediately following frequencies.

tion in the second case. The 14 nucleotide deletion, also detected as a single mutation, involves the stretch of six Gs that represents a possible mutational hot spot in the gene.⁷ Cx26 mutations were found in 35% of the autosomal recessive and in 60% of our sporadic cases; this difference is not statistically significant (Fisher's exact test, p=0.208).

The audiological assessment of subjects carrying Cx26 mutations showed that the hearing impairment can vary from moderate to profound, ranging from 45 dB HL to over 120 dB HL (averaged on central frequencies, 500-1000-200 Hz). A variable level of impairment was observed even in 35delG homozygotes within the same family (fig 1). The audiograms of our 35delG homozygotes clearly show the variable level of deficit and, surprisingly, seem to identify two prevalent areas of cochlear damage, the first one centred around 60 dB and the second one higher than 95 dB of loss (fig 2).

Even though the majority of our patients had a severe to profound deficit, the percentage of subjects with Cx26 mutations found in the three groups of hearing loss severity in which patients were classified was: 35.5% for patients with profound loss, 20% for severely impaired, and 33.3% for moderately impaired subjects.

The cochlear deficit associated with Cx26 mutations involves all frequencies and the morphology of the audiometric curve is either flat (77% of the cases) or slightly decreasing towards the high frequencies; in only two cases was the damage asymmetrical, probably because of an acquired cause. Threshold measurements were repeatedly performed over the course of a period that varied from 1 to 20 years, depending on the age of the patients. The auditory impairment has never been found to be progressive, according to parameters described in table 1. The vestibular function was not specifically tested but motor

milestones were normally acquired and the patients never reported problems with balance.

Discussion

Although non-syndromal congenital hearing impairment is estimated to be of genetic origin in about 60-70% of cases, only 20% actually show a positive family history and at least 25% are aetiologically unexplained.¹ Since different causes may have indistinguishable clinical effects, an aetiological diagnosis can be very difficult in the majority of sporadic cases. The recent finding that *Cx26* mutations are responsible for a significant proportion of congenital non-syndromic deafness opens up new diagnostic possibilities through which the clinical approach to these patients can be significantly modified.¹⁶

It is crucial to provide precise and definite information about the actual prevalence of *Cx26* mutations; there is in fact a general consensus about prevalence in cases with documented autosomal recessive inheritance but reports regarding sporadic cases are still controversial. It is also important to improve current knowledge on genotype-phenotype correlation; *Cx26* hearing loss was originally described as profound and few studies have specifically addressed this problem.

The aim of our study was to verify the relevance of alterations of this gene as the cause of deafness in our population, to define clinical features, and delineate the audiometric pattern of expression of the specific deficit. In agreement with previous studies we found a high frequency of *Cx26* mutations in patients with presumed autosomal recessive sensorineural hearing impairment. In particular, our prevalence of 35% is identical to that found by Kelley *et al*¹⁰ in 58 multiplex families with living normally hearing parents and at least two affected children diagnosed with non-syndromal hearing impairment.

We also found a particularly high frequency of mutations in apparently sporadic cases (60%). The different prevalence of *Cx26* mutations between autosomal recessive and sporadic cases in our study is not statistically significant, even though noticeable. A possible reason for these data is the small average family size in our population (67% of the probands were the only child of their parents) and indeed, by combining recessive and sporadic families, with exclusion of the probands, the number of affected sibs we observed is identical to that expected for an autosomal recessive trait.

On the other hand the high prevalence of *Cx26* mutations we detected in the subset of sporadic cases appears to be in contrast with previously published papers that reported lower prevalence figures of 10 or 37%.^{9,17} Two possible reasons are, first, a very stringent exclusion of cases with indicators of acquired hearing impairment which would eventually lead to an underestimate of the actual prevalence of *Cx26* mutations and, second, the variable phenotypic expression of *Cx26* deficit.

The phenotypic variability that was observed by Kelley *et al*¹⁰ in 35delG homozygotes has

been found to be quite significant in 35delG homozygotes and compound 35delG/G139T heterozygotes of our series, even within the same family. It is very tempting to hypothesise that undetected, very mild cases of hearing impairment in family members could lead to mistakes in the definition of some sporadic families.

Subjects with *Cx26* mutations usually showed a symmetrical auditory deficit. According to our audiometric parameters, no signs of progression of hearing loss were observed. Mutation analysis could not predict the degree of hearing impairment, which varied from moderate to profound, even in 35delG homozygotes within the same family, and the prevalence of *Cx26* mutations found in different categories of deafness severity was similar.

Our data indicate that alterations of *Cx26* do account for a large proportion of congenital non-syndromic sensorineural impairments including mild deficits. The prevalence of these mutations is high both in recessive and sporadic cases when indicators of acquired hearing defects are excluded.

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