ORIGINAL ARTICLE

A genotype-phenotype correlation for *GJB2* (connexin 26) deafness

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Introduction: Mutations in *GJB2* are the most common cause of non-syndromic autosomal recessive hearing impairment, ranging from mild to profound. Mutation analysis of this gene is widely available as a genetic diagnostic test.

Objective: To assess a possible genotype-phenotype correlation for *GJB2*.

Design: Retrospective analysis of audiometric data from people with hearing impairment, segregating two *GJB2* mutations.

Subjects: Two hundred and seventy seven unrelated patients with hearing impairment who were seen at the ENT departments of local and university hospitals from Italy, Belgium, Spain, and the United States, and who harboured bi-allelic *GJB2* mutations.

Results: We found that 35delG homozygotes have significantly more hearing impairment, compared with 35delG/non-35delG compound heterozygotes. People with two non-35delG mutations have even less hearing impairment. We observed a similar gradient of hearing impairment when we categorised mutations as inactivating (that is, stop mutations or frame shifts) or non-inactivating (that is, missense mutations). We demonstrated that certain mutation combinations (including the combination of 35delG with the missense mutations L90P, V37I, or the splice-site mutation IVS1+1G>A, and the V37I/V37I genotype) are associated with significantly less hearing impairment compared with 35delG homozygous genotypes.

Conclusions: This study is the first large systematic analysis indicating that the *GJB2* genotype has a major impact on the degree of hearing impairment, and identifying mild genotypes. Furthermore, this study shows that it will be possible to refine this correlation and extend it to additional genotypes. These data will be useful in evaluating habilitation options for people with *GJB2* related deafness.

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Approximately one child in 500–650 is born deaf, making hearing impairment the most frequent sensory defect.[1][2] In more than half of these children, the cause is monogenic[3] with the inheritance pattern being autosomal dominant (20%), autosomal recessive (80%), X-linked (1%) or mitochondrial (<1%). In about 70–80% of cases other stigmata are absent and the hearing impairment is classified as non-syndromic. To date, over 70 non-syndromic loci have been mapped and 35 deafness genes have been identified.[4] Despite this heterogeneity, up to 50% of prelingual recessive non-syndromic deafness can be attributed to mutations in *GJB2* (MIM 121011).[5–9] A few specific mutations in this gene also underlie autosomal dominant non-syndromic deafness *DFNA3* (MIM 601544), but its prevalence is very low.[10][11] Although a broad spectrum of recessive deafness mutations in the *GJB2* gene is known,[12][13] one particular mutation, the 35delG mutation, is very frequent in populations of European origin.[14] This single base pair deletion results in a frameshift and a premature protein truncation. The frequency of 35delG is highest in countries close to the Mediterranean, where the carrier frequency is between 1/30 and 1/35.[14][15] In these countries, 35delG accounts for 70% of all *GJB2* alleles. Although once thought to represent a mutational hotspot within *GJB2*, background genotyping has shown that 35delG derives from a founder ancestor.[16]

The protein encoded by *GJB2* is gap junction connexin 26 (CX26). Six connexin monomers assemble to form a hexameric connexon, which localises to the cell membrane. Oligomerisation with a connexon from an adjacent cell results in channel formation, facilitating intercellular transport of small metabolites and ions. In the cochlea, CX26 is expressed in the supporting cells and fibrocytes of the cochlear duct, where it may be involved in the recycling of potassium ions from hair cells back to the endolymph. Disruption of CX26 gap junctions presumably impairs recycling of potassium ions thereby causing hearing impairment.

Although *GJB2* plays an important role in non-syndromic childhood deafness, only a few studies describe the clinical characteristics of *GJB2* associated hearing loss in detail. According to these reports, hearing impairment in prelingually deaf children with bi-allelic *GJB2* mutations affects all frequencies, varies from mild to profound even within the same family, and is most commonly non-progressive.[17–20] In the present study, we analysed audiometric characteristics associated with various types of *GJB2* mutations to delineate possible genotype-phenotype correlations.

METHODS
Clinical evaluation
Clinical data were derived from patients from Belgium, Italy, Spain, and the United States, who tested positively for bi-allelic *GJB2* mutations in routine diagnostic testing. People with hearing impairment underwent otoscopic examinations and pure tone audiometry with a diagnostic audiometer in a

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soundproof room following International Standards Organization (ISO) standards.[21] People with features of syndromic hearing impairment were excluded from the study. Individual binaural mean pure tone thresholds (dB hearing loss (HL), air conduction level) were available for 0.5, 1, 2, and 4 kHz. Out of scale measurements were coded as 120 dB hearing loss. Median threshold values were calculated for each relevant subgroup of patients. The severity of hearing impairment was defined by the degree of hearing loss in the better ear: average thresholds between 21–40 dB were considered as mild hearing impairment, 41–70 dB as moderate hearing impairment, 71–95 dB as severe hearing impairment, and >95 dB as profound deafness.[22]

In Italian children aged between 6 months and 2 years, individual binaural tone threshold was obtained by visual reinforcement audiometry, and confirmed with click evoked auditory brainstem response (ABR) also called brainstem evoked response audiometry.

American and Belgian children below the age of 2 were tested by conditioned oriented reflex audiometry, auditory brainstem response, or pure tone audiometry. There were no Spanish children below the age of 2 in our study sample.

GJB2 mutation analysis

Various methods were used for mutation analysis of the *GJB2* gene. For the Italian samples, *GJB2* mutation screening was performed by single strand conformational polymorphism (SSCP) under various conditions followed by DNA sequencing.[17] The Belgian samples were prescreened for 35delG. No further testing was done on people identified as 35delG homozygotes. If only one mutant allele was detected, the entire coding region of *GJB2* was sequenced. Samples from Spanish patients were screened first for the 35delG mutation. In heterozygotes, heteroduplex analysis of the open reading frame, splice sites and promoter of *GJB2* was completed, followed by sequencing of any DNA fragments showing abnormal gel-migration patterns. If heteroduplex analysis was normal, the entire open reading frame, splice sites and promoter of *GJB2* were sequenced.

For the samples from American patients, the initial screen was an allele-specific polymerase chain reaction (ASPCR) assay to detect the presence of the 35delG allele variant using previously described primers.[23] In people heterozygous for the 35delG allele, the coding sequence of *GJB2* (exon 2) was screened for other allele variants by SSCP using MDE gels (FMC BioProducts, Rockland, ME). All abnormal band shifts were sequenced; if no abnormal band shifts were identified, the non-coding exon of *GJB2* (exon 1) was sequenced. In cases in which the initial ASPCR screen failed to demonstrate a 35delG allele, exon 2 was screened by SSCP, again using MDE gel electrophoresis; samples were sequenced if shifts were observed. If a single coding mutation was detected, the non-coding exon of *GJB2* (exon 1) was sequenced.[24]

Mutations that were listed as non-syndromic deafness mutations on the Connexin deafness homepage[13] were all considered to be pathogenic. For sequence variants that were not described previously or for variants with an uncertain pathogenic state, the evolutionary conservation between Homo sapiens, Mus musculus, Cavia porcellus, and Ovis aries was calculated using the ConSeq web server.[25]

In families with more than one affected sibling, we considered genotypic and audiometric data from only one randomly chosen deaf individual per family. All people segregating the M34T mutation were excluded because the pathogenic nature of this mutation is not clear.[26]

Statistical analysis

To analyse whether the degree of hearing impairment was randomly distributed across the various 35delG/non-35delG and inactivating/non-inactivating genotypes (35delG/35delG, 35delG/non-35delG, non-35delG/non-35delG; inactivating/inactivating, inactivating/non-inactivating, non-inactivating/non-inactivating), χ^2 testing was performed using the hearing impairment classification of mild, moderate, severe, and profound. Missense mutations and 3 bp deletions resulting in a deletion of an amino acid were considered as non-inactivating; splice site mutations, insertions, nonsense mutations, duplications and deletions of more than 3 bp were considered as inactivating mutations. Subsequently, the 3×4 contingency tables were reduced to the appropriate 2×2 contingency table and Fisher's exact probability testing was performed to analyse whether certain classes of hearing impairment occurred more often in a specific genotype category than would be expected by chance alone.

Comparisons by hearing impairment classification between several specific mutation combinations (with n>2 observations) were made using the χ^2 test. Pairwise comparisons between subgroups were performed using Fisher's exact probability test. Therefore, the pure tone average of thresholds at 0.5, 1, 2, and 4 kHz ($PTA_{0.5–4\ kHz}$) was dichotomised around the highest value observed in the 35delG/non-35delG or non-35delG/non-35delG genotype class. The level of significance used was $p = 0.05$.

RESULTS

GJB2 mutation spectrum

This study was conducted on 277 unrelated patients with prelingual hearing impairment with bi-allelic *GJB2* mutations, who were seen at the ear, nose, and throat departments of local and university hospitals (30 Belgian, 131 Italian, 42 Spanish, and 74 American patients). The mutations in these patients comprise seven different homozygous genotypes and 50 different compound heterozygous genotypes. The changes include missense mutations, small deletions and insertions, nonsense mutations, one duplication, and one splice site mutation. Of the homozygous genotypes, five are expected to lead to an absent or truncated protein (anticipated null allele or inactivating mutation). In 16 out of 50 compound heterozygous genotypes, both changes are null alleles. The inactivating/non-inactivating and non-inactivating/non-inactivating mutation combinations represent 29 and 5 out of 50 compound heterozygous genotypes, respectively. When considering the total study sample, 78% of the patients (216/277) inherited two inactivating mutations, 19% (53/277) inherited an inactivating and a non-inactivating mutation, and 3% (8/277) inherited two non-inactivating mutations.

Sixty percent of our total sample were homozygous for 35delG, with this mutation accounting for 75.5% (418/554) of all mutant alleles. Homozygosity for 35delG was least frequent in the American sample (34/74, 46%) and most frequent in the Belgian sample (22/30, 73%). In the Spanish and Italian samples, 55% (23/42) and 66% (87/131) of patients, respectively, were 35delG homozygotes. The high percentage in the Belgian sample may reflect a selection bias since people were first screened for 35delG and mutation analysis of the complete coding region was performed only if a single 35delG allele was detected.

Audiometric analysis of patients with bi-allelic GJB2 mutations

Genotype and hearing impairment distribution in bi-allelic GJB2 patients

Fig 1 shows the distribution of the classes of hearing impairment in the various 35delG/non-35delG and inactivating/non-inactivating genotype categories. In the 35delG/35delG genotype category, 63% (105/166) showed profound hearing impairment and 29% (47/166) were severely affected. Mild and moderate hearing impairment
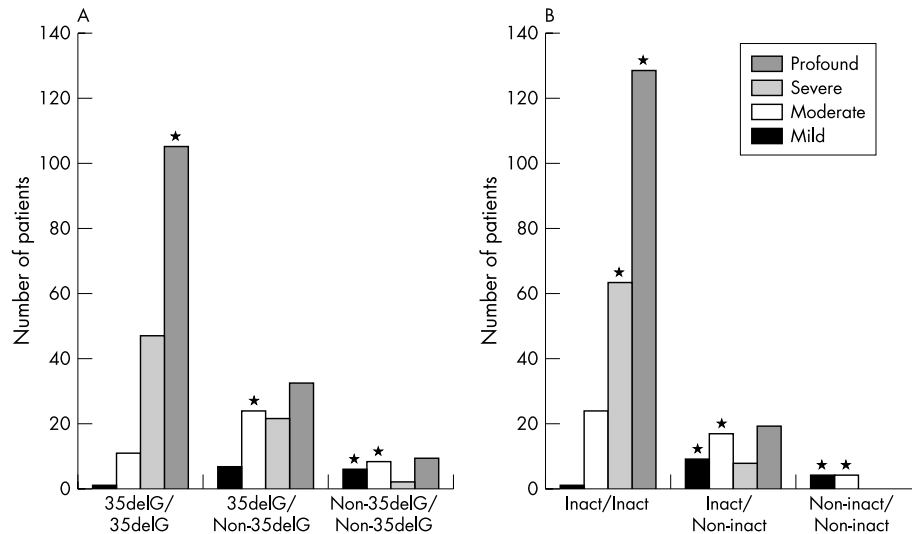
Figure 1 Distribution of the four classes of hearing impairment over the possible 35delG/non-35delG (A) and inactivating/non-inactivating (B) genotypes. The number of patients is indicated on the y axis. χ^2 testing indicated that a non-random association exists between the degree of hearing impairment and the mutation combination. For the inactivating/non-inactivating genotypes, a reduced contingency table was constructed, combining the mild and moderate category. Asterisks indicate significantly high relative frequency according to the Fisher's exact probability test.

was observed in <1% (1/166) and 8% (13/166) of 35delG homozygous people, respectively. χ^2 testing indicated that the different classes of hearing impairment were not randomly distributed across the different genotype categories (χ^2, 55.75 (df 6) and 66.19 (df 4) for the 35delG/non-35delG and inactivating/non-inactivating categories, respectively). To evaluate whether certain classes of hearing impairment occurred more frequently in combination with a certain genotype than would be expected on the basis of chance alone, Fisher's exact probability testing was performed. The 35delG/35delG genotype was significantly associated with profound hearing impairment and the inactivating/inactivating genotype category was significantly associated with profound and severe hearing impairment. In addition, the 35delG/non-35delG and non-35delG/non-35delG genotypes showed significant associations with mild or moderate hearing impairment and so did the inactivating/non-inactivating and non-inactivating/non-inactivating mutation combinations.

Comparison of threshold values between specific *GJB2* genotypes

To determine whether sample differences existed between people with a similar mutation combination, $PTA_{0.5-4\ kHz}$ values for 35delG homozygotes and for people with an inactivating/inactivating genotype were compared between countries. We found no significant difference between people from different countries in either the 35delG/35delG group (χ^2 test) or the inactivating/inactivating mutation combination group (χ^2 test, data not shown). The threshold measurements of all 35delG homozygotes were therefore combined into one group to compare thresholds of people with different specific mutation combinations. The P5 (lower 5th centile of the 35delG/35delG patients) and P10 (lower 10th centile of the 35delG/35delG patients) PTA levels for the combined group were at ~60 and 70 dB HL respectively (data not shown). As illustrated by fig 2, patients with 35delG/delE120, 35delG/IVS1+1G>A, 35delG/L90P, 35delG/V37I, or V37I/V37I genotypes clearly showed significantly less severe hearing impairment with respect to 35delG homozygotes (Fisher's exact test). Remarkably, it was possible to show that some genotypes that occurred only once were also associated with significantly less severe hearing impairment compared to 35delG homozygotes (313del14/313del14,

W24X/313del14, L90P/V95M, R184P/IVS1+1G>A, L90P/V153I, V63M/D159N, Y65X/L90V, V37I/R143W; fig 2). No significant differences were found between 35delG homozygotes and people with a 35delG/R143W, 35delG/E147K, 35delG/313del14, 35delG/W24X, 35delG/V95M, 35delG/W77R, 35delG/E47X, 35delG/167delT, or 35delG/R184P mutation combination (fig 3). Although the genotypes 35delG/167delT and 35delG/R184P showed no significant difference with 35delG homozygotes when dichotomised around the highest value (fig 3), they did show a significant excess of cases with relatively mild hearing impairment when dichotomised around another value (respectively p = 0.006 and p = 0.014 when dichotomising around 51.25 dB for 35delG/167delT and 70 dB for 35delG/R184P). This is probably the consequence of the relatively wide variation in the degree of hearing impairment for these two genotypes. There was no substantial difference in thresholds between people with the same mutation combination coming from different countries (data not shown).

Comparison of threshold values between the various 35delG/non-35delG and inactivating/non-inactivating genotypes with exclusion of genotypes that cause significantly less severe hearing impairment

Because some specific genotypes were associated with significantly less severe hearing impairment with respect to 35delG homozygotes, the statistically significant differences we noted in fig 1 could reflect the presence of less severe mutation combinations within specific subgroups. To assess this possibility, we repeated the analyses pertaining to fig 1, after excluding all the genotypes that had been found to be significantly less severe in comparison to 35delG/35delG (fig 2). After exclusion, it appeared that hearing impairment overall was still not randomly distributed across the various categories of 35delG/non35delG (χ^2 6.98 (df 2)) and inactivating/non-inactivating (χ^2 19.61 (df 2)) genotypes. Despite this finding, Fisher's exact probability testing indicated that each of the different 35delG/non-35delG genotypes separately did not occur more frequently in combination with certain classes of hearing impairment. Inactivating/non-inactivating and non-inactivating/non-inactivating genotypes, however, were still significantly associated with
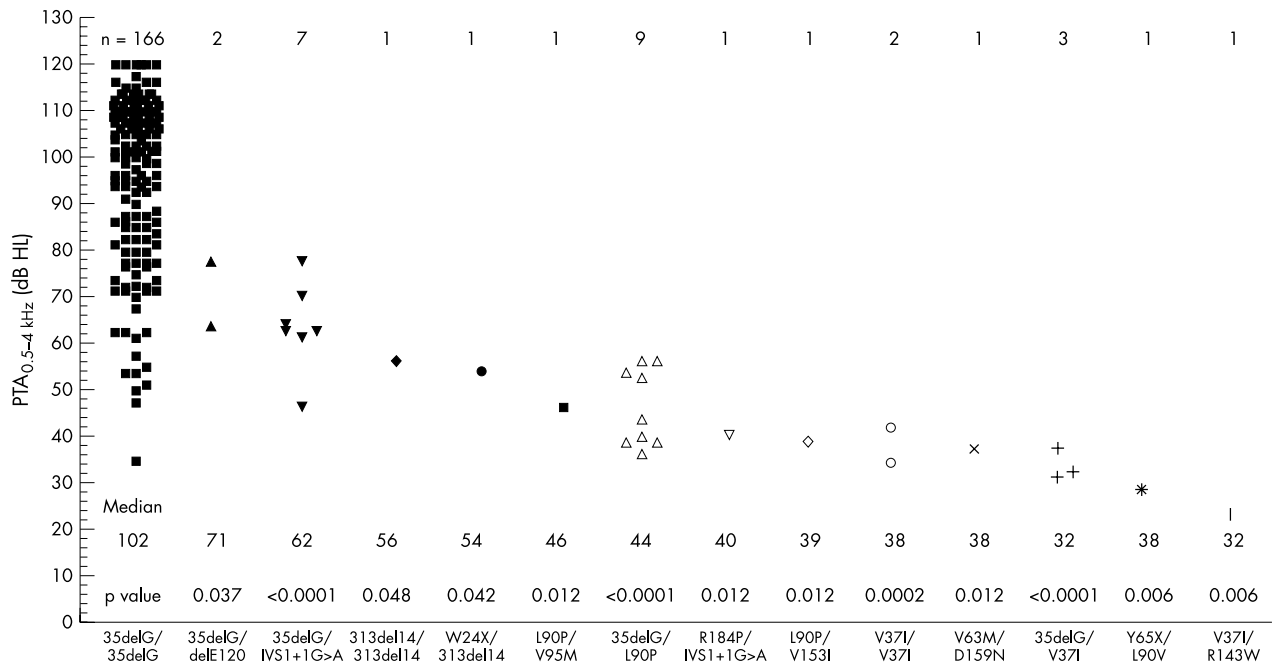
Figure 2 Binaural PTA$_{0.5–4 kHz}$ (dB HL) for 35delG homozygous patients compared with subgroups of patients with specific testable *GJB2* genotypes that showed significantly less severe hearing impairment in comparison to 35delG homozygotes in Fisher's exact probability testing. The number of observations (n), the median value (dB HL) and the p value are indicated for each subgroup.

moderate hearing impairment, while all other significant associations indicated in fig 1B disappeared (fig 4).

Analysis of progression in bi-allelic *GJB2* patients

The threshold distribution in each nationality was similar (data not shown), as were age distributions across countries (median ages, 4–7 years; range, 0–67 years). We performed linear regression analysis of thresholds on age and found no significant age effect on threshold with only one exception—the Italian patients showed slight but significantly less severe hearing impairment with increasing age (data not shown). Because it can be difficult to reliably assess pure tone thresholds in very young children, we repeated regression analysis of the Italian sample after excluding patients aged <2 years and found the threshold-age effect no longer significant.
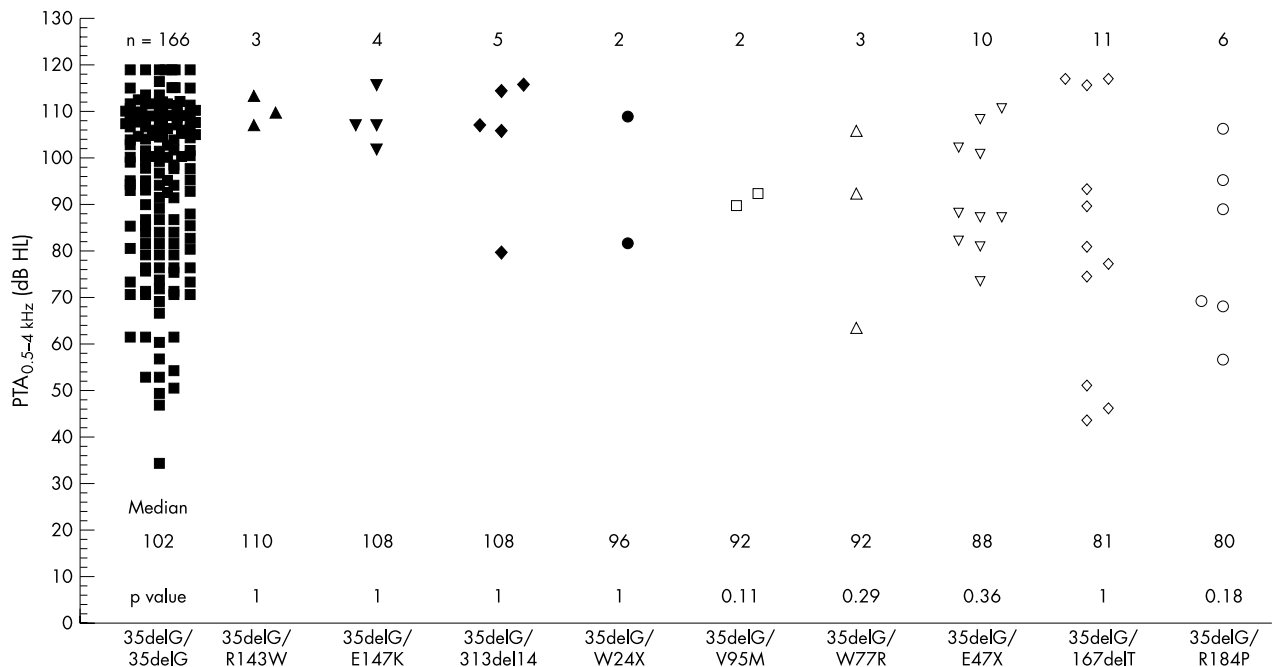

Figure 3 This shows the same as fig 2, but now including the subgroup of patients with specific testable *GJB2* genotypes who did not show significantly less severe hearing impairment in comparison to 35delG homozygotes in Fisher's exact probability testing. The number of observations (n), the median value (dB HL) and the p value are indicated for each subgroup. The PTA$_{0.5–4 kHz}$ values were dichotomised as described. Note that 35delG/167delT and 35delG/R184P show an excess of patients with remarkably mild degrees of hearing impairment.

DISCUSSION

Mutations in *GJB2* account for up to 50% of prelingual recessive non-syndromic deafness in the white population. Screening of this gene is relatively easy, thereby allowing molecular diagnosis for non-syndromic deafness. In the present study, we investigated the audiological characteristics in a large number of *GJB2* hearing-impaired people originating from Belgium, Italy, Spain, and the United States, to delineate potential genotype-phenotype correlations. Several interesting features emerged. Although hearing loss in people with two *GJB2* allele variants ranged from mild to profound, the non-random distribution pattern of different categories of hearing impairment over the various 35delG/non-35delG (fig 1A) and inactivating/non-inactivating genotypes (fig 1B) suggested a correlation between the type of mutation and the severity of hearing impairment. Statistical analysis indeed confirmed that certain classes of hearing impairment occurred significantly more often in association with certain genotypes than would be expected by chance alone. As no significant difference existed in the degree of hearing impairment between people with similar mutation combinations originating from different countries, hearing thresholds in people with similar *GJB2* genotypes were combined to analyse the effect of specific mutation combinations. Interestingly, 35delG homozygotes showed significantly more hearing impairment with respect to 35delG/delE120, 35delG/IVS1+1G>A, 35delG/L90P, 35delG/V37I, and V37I/V37I genotypes (fig 2). A significant difference was also seen with a number of mutation combinations that occurred only in a single patient (313del14/313del14, W24X/313del14, L90P/V95M, R184P/IVS1+1G>A, L90P/V153I, V63M/D159N, Y65X/L90V, V37I/R143W; fig 2). However, as the latter findings are based on a single patient, they should be interpreted cautiously and should be corroborated by additional data. The genotypes involving V37I and V153I raise questions. The V37I variant has been reported as a mutation and as a polymorphism,[5 27–29] and it seems to be relatively frequent in the Asian population. It was noted that the prevalence in the Asian hearing loss population is lower than expected based on the carrier-frequency.[30] Therefore, Bason et al[30] suggested that this discrepancy is caused by a

distortion of the V37I frequency in the control population, a selection bias towards individuals with more severe hearing impairment, decreased penetrance, or the fact that V37I is a polymorphism but not a mutation. Also the pathogenic state of V153I is uncertain. It is listed as a rare polymorphism,[13] and valine at position 153 shows an average conservation between Homo sapiens, Mus musculus, Cavia porcellus, and Ovis aries. However, Kenna et al[31] reported that this variant may represent a mild recessive mutation and we detected it only once in 1348 Italian individuals who were screened for *GJB2*. If V37I and V153I are polymorphisms, it is not surprising that the hearing impairment of patients with a 35delG/V37I, V37I/V37I and L90P/V153I genotype was significantly less than that of 35delG homozygous patients. However, as we demonstrate in the present study that the phenotypic variability of *GJB2* patients can largely be attributed to their genotype, it is possible that V37I and V153I are pathogenic variants leading to a mild phenotype.

A compilation of audiological data from previous studies confirms to some extent that specific relevant *GJB2* genotypes are associated with relatively mild hearing impairment (for references see table 1). Like the present study, Janecke et al[34] found that the distribution of homozygotes for truncating *GJB2* mutations and other genotypes among the categories of hearing impairment differed significantly. In addition, they reported that the distribution of genotypes with and without L90P differed significantly. They concluded that the L90P mutation in trans with any other recessive mutation is predominately associated with mild or moderate hearing impairment, thereby corroborating our results. Although their findings were variable, they suggested that hearing impairment in people cosegregating the 35delG/L90P genotype is progressive and may even be associated with episodes of sudden sensorineural hearing loss. Regression analysis on our 9 35delG/L90P compound heterozygotes (aged 1–35) failed to find significant progression (data not shown), not supporting the findings of Janecke et al, and none of these people reported episodes of sudden sensorineural hearing loss. Also Denoyelle et al[19] reported that the distributions of compound heterozygotes and 35delG homozygotes among the hearing-loss subgroups differed significantly, which is in
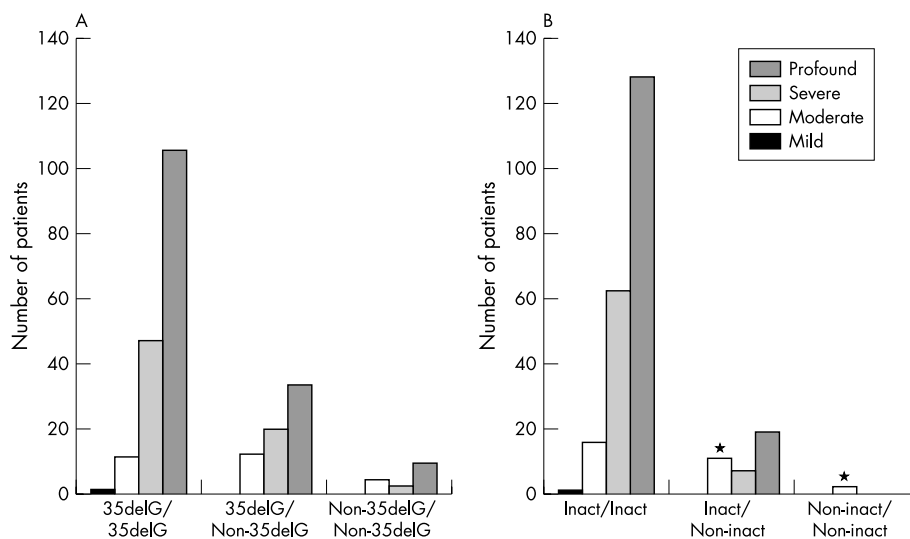

Figure 4 This shows the same as fig 1, but now with exclusion of specific genotypes that are associated with significantly less severe hearing impairment compared to the 35delG homozygous genotype as indicated in fig 2. The number of patients is indicated on the y axis. χ^2 testing indicated that hearing impairment classes were non-randomly distributed across the different 35delG/non35delG and the inactivating/non-inactivating genotypes. For both genotype categories, a reduced contingency table was constructed, combining the mild and moderate category as well as the 35delG/non35delG and non-35delG/non-35delG genotypes or the inactivating/non-inactivating and the non-inactivating/non-inactivating categories. Asterisks indicate significantly high relative frequency according to the Fisher's exact probability test.

Table 1 Overview of hearing impairment associated with specific *GJB2* mutations as reported by previous studies. The upper panel represents mutations that are associated with significantly less severe hearing impairment with respect to 35delG homozygotes; the lower panel represents mutations that do not show significant differences with respect to 35delG homozygotes

Genotype	This study	Previous studies	References
35delG/V37I	Mi (3)	Mi, Mo, Se (2), High frequency	29 32 33
35delG/L90P	Mi (4), Mo (5)	Mi, Mo (15), High frequency (2), Se (2), Pr	19 29 34 32 35–37
35delG/delE120	Mo, Se	Se (2), Se to Pr	28 36 38
35delG/ IVS1+1G>A	Mo (6), Se	Mo (3), Se, Pr (3)	34 25 32 35
V37I/V37I	Mi, Mo	Mi (2), Mo (4), Se, Se to Pr	28–31 32
35delG/W24X	Se, Pr	Mo, Se (2), Pr	36 37
35delG/E47X	Se (6), Pr (4)	Se, Pr (3)	31 32 36
35delG/167delT	Mo (3), Se (5), Pr (3)	Mo (4), Se (3), Pr (8)	29 31 32 36 39 40
35delG/W77R	Mo, Se, Pr	Mo, Pr (13)	31 36 39
35delG/V95M	Se (2)	/	
35delG/ 313del14	Se, Pr (4)	Se to Pr (3), Pr (6)	6 34 32 38 39
35delG/R143W	Pr (3)	Pr	31
35delG/E147K	Pr (4)	/	
35delG/R184P	Mo (3), Se, Pr (2)	Se to Pr (7), Pr (2)	28 37

The number of patients is indicated between brackets in cases where more than one patient with the respective phenotype has been reported.
Mi, mild; Mo, moderate; Se, severe; Pr, profound.

line with our findings. However, they included several affected individuals from certain families, which can lead to a potential bias, due to a shared genetic background. The main difference with the current study is that they only made a classification based on the number of 35delG alleles and were not able to identify specific *GJB2* genotypes that are associated with a remarkably mild phenotype. Based on their results they concluded that the hearing loss due to *GJB2* could not be predicted, even within families. In the study of Cohn et al,[18] 167delT homozygotes were significantly more severely affected than 35delG homozygotes. Also, in this study multiple affected individuals from certain families were taken into account. In addition, they concluded that hearing impairment is very variable and suggested that other factors modify the phenotypic effects of *GJB2* mutations. Considering previous publications on the *GJB2* phenotype, some reports have given some evidence of a genotype-phenotype correlation for *GJB2*, but these studies are limited compared to the current study.

In contrast to the above listed genotypes leading to mild hearing impairment, no significant differences could be detected between 35delG homozygotes and 35delG/ 313del14, 35delG/R143W, 35delG/E147K, 35delG/W24X, 35delG/V95M, 35delG/W77R, 35delG/E47X, 35delG/167delT or 35delG/R184P compound heterozygous genotypes (fig 3). These findings have also been confirmed by other studies to some extent (table 1), and the combined data indicate that *GJB2* related hearing impairment is mostly determined by the specific mutation combination. Variation in hearing impairment within each genotype category suggests that environmental factors or modifier genes, or both, are also involved but their contribution appears to be less important.

The finding that some *GJB2* genotypes are associated with significantly less severe hearing impairment compared with 35delG homozygous mutations suggests that the significant differences between the different 35delG/non35delG and inactivating/non-inactivating categories (fig 1) are mainly due to the presence of milder genotypes in some subgroups. We confirmed this subgroup effect by repeating our comparisons with the exclusion of these mild mutation combinations. After exclusion, most but not all of the significant differences had disappeared (fig 4). Based on these results, we believe that it is very likely that the

inactivating/non-inactivating and non-inactivating/non-inactivating categories still included remarkably mild mutations after the present exclusion. In table 2, we have listed all genotypes that were detected in single people with moderate hearing impairment and $PTA_{0.5–4\ kHz}$>57.5 dB. These genotypes are not included in fig 2 because Fisher's exact probability testing only produced significant results between our group of 35delG homozygotes and single genotypes if the latter category was present in people with $PTA_{0.5–4\ kHz}$<57.5 dB. Nevertheless, these genotypes might eventually be labelled as significantly less severe compared to the 35delG/35delG genotype if a second person with the same

Table 2 List of mutation combinations in single patients showing moderate hearing impairment. Fisher's exact probability testing only produced significant results between our group of 35delG homozygotes and single genotypes if the latter category was present in people with $PTA_{0.5–4\ kHz}$<57.5 dB. The genotypes listed below might eventually be labelled as significantly less severe compared to the 35delG/35delG genotype if a second person with the same genotype is found and if both people have $PTA_{0.5–4\ kHz}$⩽83 dB

GJB2 genotype	$PTA_{0.5–4\ kHz}$
35delG/V84L	63
35delG/M93I	61
35delG/S138N*	70
35delG/R184W	65
35delG/N206S	65
W24X/R184P	58
R32C/E47X	59
V84L/R184P	60
T123N/T123N*	60

*The pathogenic nature of S138N and T123N is not proven. S138N was not described previously, and T123N was reported as a change with an unknown relation to disease.[13] These variants were not found in 100 Belgian (S138N) or 400 American (T123N) control chromosomes respectively. The amino acids involved in both variants are not evolutionary conserved between Homo sapiens, Mus musculus, Cavia porcellus, and Ovis aries.

Table 3 Functional properties of the more frequent *GJB2* mutations as described in the literature. The effect on the degree of hearing impairment of these mutations in combination with a 35delG allele is also included

Mutation	Effect on hearing impairment (current study)	Functional properties	References
35delG	Mild to profound, with most patients showing severe or profound hearing impairment	No expression	41
IVS1+1G>A	Significantly less severe compared with 35delG	No expression	42
V37I	Significantly less severe compared with 35delG	Complete loss of channel activity	43
W77R	No significant difference compared with 35delG	Complete loss of channel activity	43–46
V84L	Possibly associated with less severe hearing impairment	Conductance similar to wild-type CX26	43 47
L90P	Significantly less severe compared with 35delG	Complete loss of channel activity	41 43 48
V95M	No significant difference compared with 35delG	Conductance similar to wild-type CX26	47
delE120	Significantly less severe compared with 35delG	Complete loss of channel activity	43
R143W	No significant difference compared with 35delG	Conductance similar to wild-type CX26	47
R184P	No significant difference compared with 35delG	Complete loss of channel activity	43 48

genotype is found and if both people have $PTA_{0.5-4\ kHz} \leqslant 83$ dB. It is thus possible that the list of genotype-phenotype correlations described in the present report can be easily extended to additional genotypes. Our study clearly demonstrates the feasibility of identifying specific mild *GJB2* genotypes based on audiometric data from a relatively small number of patients. We would recommend establishing a large multicentre study to collect such data from as many people with *GJB2*-related deafness as is possible. Additional data may also make it feasible to predict the expected degree of hearing impairment more accurately.

For some of the more frequent *GJB2* mutations, including 35delG, V37I, W77R, V84L, L90P, V95M, delE120, R143W, R184P, and IVS1+1G>A, functional studies have been conducted. These studies analyse the expression and inter-cellular communication properties (including junctional conductance) of mutant CX26 protein in communication deficient cells (Xenopus oocytes, HeLa cells, or N2A cells). We attempted to relate results from these studies to our findings (table 3) but found this correlation difficult to make. For example, expression studies reveal that the 35delG and IVS1+1G>A mutations do not yield any detectable CX26 protein and mRNA respectively.[41 42] Based on this result, a reasonable expectation would be that both mutations would have the same effect on hearing impairment. However, we observed 35delG/IVS1+1G>A compound heterozygotes to have significantly (p<0.0001) less severe hearing impairment compared to 35delG homozygotes. As the conclusion that there is no mRNA for the IVS1+1G>A mutation is based on a DNA sequencing result,[42] the presence of a very small amount of mRNA cannot be excluded, possibly providing an explanation for this discrepancy. Functional studies of the other mutants indicate that all are expressed with at most only a minor effect on translation. A complete loss of channel activity has been demonstrated for V37I, W77R, L90P, delE120, and R184P while V84L, V95M, and R143W mutant proteins induce a junctional conductance similar to wild-type CX26. There are numerous discrepancies between our data and these results (table 3), reflecting the limitations of in vitro systems when trying to mimic cellular conditions in the cochlea. Furthermore, these systems investigate junctional conductance and not changes in the pore structure that may influence permeability and gap junction properties.

Several studies have shown that CX26 forms homomeric and homotypic channels, but it may also interact with other inner ear connexins to form heteromeric and heterotypic channels.[49 50] The formation and function of heteromeric and heterotypic channels have not been investigated. It is also possible that interactions with other unidentified inner ear proteins exist. At a minimum, these discrepancies between functional data and our genotype-phenotype data indicate that it would be useful to analyse the validity of in vitro systems as a model for in vivo conditions.

In conclusion, our results demonstrate for the first time on a systematic basis that people cosegregating specific *GJB2* genotypes have significantly less severe hearing impairment compared with 35delG homozygotes. The delineation of this genotype-phenotype correlation, together with data obtained by large multicentre follow up studies, may be valuable within the framework of universal newborn hearing screening. The purpose of this screening is the early detection and habilitation of children with congenital hearing loss. As *GJB2* accounts for up to 50% of non-syndromic recessive hearing impairment, mutation analysis of this gene is routinely carried out in many newborns with hearing impairment in many countries. Children with congenital hearing loss are often referred at the age of 1–3 months for follow up screening and therapeutic intervention.[51] Complementing physiological testing of hearing with the genotype-phenotype correlation we describe in this report may facilitate the selection of appropriate habilitation strategies for people with *GJB2* related deafness. Furthermore, our study also demonstrates that *GJB2* is clearly involved in mild and moderate hearing impairment, and we suggest that *GJB2* mutation analysis is indicated in all patients with putative autosomal recessive non-syndromic hearing impairment, regardless of the severity of hearing impairment. In addition, the entire coding region as well as the splice site should be screened instead of only the recurrent mutations.

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