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Connexin 26 (GJB2) Gene-Related Deafness and Speech Intelligibility After Cochlear Implantation

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Hypothesis: Speech intelligibility in children after cochlear implantation may depend on their deafness cause, including connexin 26 (GJB2) gene-related deafness.

Background: There is significant variability in the degree of intelligibility, or clarity, of children’s speech after cochlear implantation. GJB2 gene-related deafness may be a factor, as preliminary data suggest that pathologic changes do not affect the spiral ganglion cells, which are the neural elements stimulated by the implant, thus favoring better results.

Methods: In an observational retrospective cohort study of pediatric cochlear implantees, 38 patients with nonsyndromic deafness of unknown cause and 1 with keratitis-ichthyosis-deafness syndrome underwent GJB2 mutation analysis using polymerase chain reaction amplification and direct sequencing. The primary outcome measure assessed was Speech Intelligibility Rating score from postoperative Year 1 (n = 39) to Year 5 (n = 17). Educational setting was considered as a secondary outcome measure. Statistical analysis was double-blinded, with patients and assessors of outcome unaware of GJB2 status.

Results: Fourteen patients had GJB2-related deafness and 25 had GJB2-unrelated deafness. Comparisons at Year 3 (n = 31) revealed intelligible speech achieved by 9 of 11 with GJB2-related deafness, compared with only 6 of 20 with GJB2-unrelated deafness (p = 0.017). Ordinal logistic regression analysis on Speech Intelligibility Rating scores found statistically significantly better scores in children with GJB2-related deafness (p < 0.05) both before and after adjustment for confounding variables. A larger proportion with GJB2-related deafness also attended mainstream school (p = 0.01).


Approximately 1 in every 1,000 babies is born deaf worldwide, and the cause is genetic in 60% of cases (1). Mutations in the gap junction beta 2 gene (GJB2), also known as connexin 26, are the most common known genetic cause of prelingual deafness (2). They account for 15 to 20% of overall prelingual deafness, usually showing nonsyndromic disease (deafness with no other disability) with autosomal recessive inheritance (3). Recent evidence indicates that dominant mutations in GJB2 are associated with keratitis-ichthyosis-deafness (KID) syndrome (4), and that a 342-kb deletion in the adjoining GJB6 gene (also at locus DFNB1) can act in trans with a GJB2 mutation to also cause deafness (5). GJB2 and GJB6 are both expressed in the cochlea, producing connexin 26 and connexin 30 proteins, respectively. Connexin 26 molecules can bind to themselves, forming homomeric gap junctions, or to connexin 30, forming heteromeric gap junctions, thought to regulate recycling of endolymphatic potassium ions transiting the hair cells during transduction (6).

The relative cost of cochlear implantation in children with profound deafness is low, gaining quality-adjusted life years at acceptable costs to society of between US $12,000 and US $18,000 (7), but outcome is never guaranteed, with there being significant variability (8,9). Although speech discrimination is perhaps the most important baseline outcome in pediatric cochlear implantation, good speech intelligibility is a great desire of many parents. Speech intelligibility is dependant on auditory perception, auditory memory, and articulation and thus is at the next hierarchy of outcomes in pediatric cochlear implantation. Unfortunately, this is not achieved by all children who undergo implantation (9–11).

Three small single-center studies have been reported comparing only prelingually deaf patients with cochlear implants having GJB2-related deafness and GJB2-unrelated deafness (12–14). A study from the United States (12) found no statistically significant differences...
with any of their outcome measures, although a nonsignificant trend was noted toward better reading abilities in implant recipients with GJB2-related deafness (n = 16). Two studies from Japan found significantly better results in implant recipients with GJB2-related deafness with regard to speech perception (6-mo follow-up, n = 15, p = 0.0001) (13) and language ability (2-yr follow-up, n = 6, p = 0.03) (14). We present a larger cohort study with longer follow-up, comparing speech intelligibility in implant recipients with and without GJB2-related deafness.

PATIENTS AND METHODS

Recruitment of subjects and assignment into groups on the basis of deafness causes

The inclusion criteria for this study were nonsyndromic prelingually deaf patients with no previously known cause, or those with prelingual deafness resulting from KID syndrome who had undergone unilateral cochlear implantation by the same surgeon (J.G.T.) and who had their devices activated for at least a year, in the Northern Ireland regional cochlear implant program. Parental consent was obtained in 95% of all available patients, allowing 38 nonsyndromic children and 1 child with KID syndrome to be sampled. Data were obtained at interviews with these families and supplemented with data from case notes. Ethical approval was obtained from Queen’s University Belfast Research Ethics Committee.

 Patients were grouped according to their deafness cause after GJB2 screening. Children with two pathogenic recessive GJB2 mutations, or a combination of a pathogenic recessive GJB2 mutation together with a 342-kb deletion in GJB6 inherited from different parents, or a single pathogenic dominant negative GJB2 mutation, were classified as having GJB2-related deafness. All other children who were genetically screened were classified as having GJB2-unrelated deafness. All patients in our cohort were excluded as having had rubella virus embryopathy, cytomegalovirus embryopathy, measles, or mumps as the cause of their deafness by history taking and by cross-referencing their names against data from the Regional Virology Laboratory.

GJB2 plus GJB6 342-kb deletion mutation screening

Children underwent either venous blood or buccal smear sampling. DNA was extracted using the Puregene DNA extraction kit (Flowgen, Leicestershire, U.K.). Parental samples were also screened to assess whether mutations were inherited or de novo. Primers and polymerase chain reaction (PCR) amplification conditions are described in the Appendix. Mutation detection for the 342-kb deletion in GJB6 was performed by verification of the size of the PCR product using 1% agarose gel electrophoresis. PCR products of GJB2 Exons 1 and 2 were sequenced using dye-terminator chemistry (ABI PRISM Big-Dye Terminators v3.0 Cycle Sequencing Kit) on an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequence data were analyzed using Sequencher software (Version 4.1.2 for Macintosh, Gene Codes Corporation, Ann Arbor, MI, U.S.A.).

Group characteristics

Assessments were made of possible predictor variables in patient groups. Cochlear implant-unrelated criteria included socioeconomic status (categorized as manual or nonmanual, based on occupation of head of household), sex, congenital onset or otherwise of profound deafness, length of profound deafness, age at implantation, preoperative residual hearing before implantation (assessed by unaided close coupled audiograms, or auditory brainstem response readings), preoperative aided auditory perception, preoperative Speech Intelligibility Rating (SIR) score, contralateral (conventional) hearing aid usage, and timing of postoperative speech and language habilitation.

A congenital onset of profound deafness (bilateral > 90 dB hearing loss [HL]) was accredited if it had been recognized before the age of 1 year. This time point was taken as a cutoff to account for the practical difficulty, uncertainty, and delay in a definite earlier diagnosis of profound deafness without otoacoustic emission screening. Conversely, if less-than-profound deafness (ranging from normal hearing to 90 dB HL) was recognized in at least one ear, the patient was accredited with having noncongenital onset of profound deafness (i.e., a period of auditory experience). Preoperative aided auditory perception was measured by preoperative aided audiograms averaged over 500 Hz, 1 kHz, and 2 kHz in the better hearing ear, and an average was taken of the last two audiograms before surgery. Frequencies of 3 kHz and 4 kHz had also been tested for in all cases, but few responded, even at the maximum audiometer output of 90 dBA; thus, these two frequencies were excluded from calculations. Length of profound deafness was equivalent to age at activation of device minus age at onset of profound deafness, because this study included cases with a noncongenital onset of profound deafness. Age at implantation was taken as the age at activation of device, usually a month after surgery. Timing of postoperative speech and language habilitation was measured by the length of time between the start of our program and device activation, assessing whether the time frame in the learning curve for habilitation, when patients commenced treatment, affected their outcome. Cochlear implant-related criteria included Nucleus multichannel cochlear implant device type (Cochlear Corporation, Sydney, Australia), electrode insertion extent, processor type, coding strategy type, postoperative complications, and implant daily usage amount during waking hours.

Outcome measures

All assessments for the 39 children in this study were recorded before genetic testing. The primary outcome measure we intended to assess was speech intelligibility, using the SIR scale (9,15,16). The criteria used are described in Table 1. Periods of conversational speech elicited during assessment sessions were used to prospectively categorize many children at

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<th>Category</th>
<th>Speech intelligibility criteria</th>
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<tr>
<td>1</td>
<td>Connected speech is unintelligible. Prereognizable words in spoken language, primary mode of communication may be manual.</td>
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<tr>
<td>2</td>
<td>Connected speech is unintelligible. Intelligible speech is developing in single words when context and lip-reading cues are available.</td>
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<td>3</td>
<td>Connected speech is intelligible to a listener who concentrates and lip-reads.</td>
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<td>4</td>
<td>Connected speech is intelligible to a listener who has a little experience of a deaf person’s speech.</td>
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<tr>
<td>5</td>
<td>Connected speech is intelligible to all listeners. Child is understood easily in everyday contexts.</td>
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postoperative yearly intervals up to and including postoperative Year 5, with children also being videotaped. In other cases, the two members of the habilitation team, in agreement, retrospectively assessed SIR scores, using written case notes in one sitting at the implant center. Scores of the fixed postoperative time interval of Year 3 were chosen for comparisons between groups. The reproducibility of SIR scores, which are ordinal response variables, was assessed by an analysis of inter- and intraobserver agreements using videotape recordings of 22 children who received implants. Educational setting was also considered as a secondary outcome measure.

**Statistical analysis**

Quantitative group characteristics and outcomes were summarized with mean and standard deviation (comparisons obtained by the independent samples *t* test) or with median and range/interquartile range (comparisons obtained with the non-parametric Wilcoxon signed-rank or Mann-Whitney *U* tests). Categorical group characteristics and outcomes were compared by χ² test with Yates correction when appropriate, or Fisher’s exact test. The κ coefficient was used in the analysis of inter- and intraobserver agreements of SIR scores. Ordinal logistic regression analyses (17) were performed to compare groups and to adjust for confounding variables, with outcome scores 3 years postoperatively used as the response variable. Tests of significance were conducted at the 5% level (*p* < 0.05). Statistical analysis was performed using SPSS 11.0 (SPSS, Inc., Chicago, IL, U.S.A.).

**RESULTS**

*GJB2* plus *GJB6* 342-kb deletion mutation screening (n = 39)

Screening for *GJB2* mutations and the *GJB6* deletion was performed in 39 children. Mutations accounting for deafness were identified in 14 cases, which were therefore classified as having *GJB2*-related deafness. The remaining 25 children were classified as having *GJB2*-unrelated deafness.

*GJB2*-related deafness group (n = 14)

Twelve nonsyndromic children had two pathogenic recessive mutations in *GJB2*: 10 homozygous for 35delG, and 2 compound heterozygotes, 35delG/169C>T. Both the 35delG mutation and the 169C>T mutation are in the coding region of *GJB2*, introducing premature termination codons. The 35delG mutation is a deletion of a guanine from a series of six guanines, resulting in a frameshift. The 169C>T mutation results in a substitution of glutamine with a stop codon (1). In all these cases, mutations were confirmed to have been transmitted from the parents. One nonsyndromic child had the 35delG mutation in *GJB2* and the 342-kb deletion in *GJB6*, a compound heterozygote for *GJB2* and *GJB6* (5). The child with KID syndrome had a de novo heterozygous mutation, 148G>A, that was absent in both parents. This results in substitution of aspartic acid with asparagine in codon 50 (D50N), a missense mutation exhibiting a dominant negative effect on the *GJB2* gap junction system in several ectodermal epithelia producing the KID phenotype (4). No other dominant mutations were found. These 14 children were classified together as having *GJB2*-related deafness.

*GJB2*-unrelated deafness group (n = 25)

Twenty-three children had neither *GJB2* mutations nor *GJB6* 342-kb deletions. A single recessive *GJB2* mutation was identified in each of two other children, confirmed as inherited from a parent. Both are novel mutations: 478G>A, changing amino acid glycine at codon 160 into serine, and 249C>G, changing amino acid phenylalanine at codon 83 into leucine. The 23 children without any mutations and the 2 with single recessive *GJB2* mutations were classified together as having *GJB2*-unrelated deafness, the latter 2 cases reflecting the frequency of single *GJB2* mutations in the general population (18).

**Outcomes measures**

The κ coefficient for interobserver agreement in the SIR scores of the two members of the habilitation team was 0.81, as assessed from videotape recordings of 22 children who received implants. The intraobserver agreement of each assessor’s videotape score, against their previously retrospectively assigned SIR score (assessed from using written case notes), for the same child was 0.81 and 0.85 for the two team members.

All 39 patients in our study have preoperative profound deafness (preoperative unaided residual hearing thresholds > 90 dB HL) when booked for surgery. Predictor variables that differed between members of the cohort, divided according to *GJB2* status, are shown and compared in Table 2. Length of profound deafness was shorter in those with *GJB2*-related deafness, and age at implantation was also lower in this group. All patients also received a full insertion of electrodes (all 22 active electrodes inside the cochlea) and used the SPEAK coding strategy. Postoperative complications were relatively more common in those with *GJB2*-related deafness. One child had a head injury and wound breakdown after a fall 2 years after primary surgery and required revision surgery involving muscle flap transfer onto the implant site. Another child required revision surgery 3 days after primary surgery, for electrode slippage. A third child developed a keloid at the skin incision, requiring excision under general anesthesia, 11 months after implantation.

**Group characteristics**

All children in both groups had prelingual profound deafness (preoperative unaided residual hearing thresholds > 90 dB HL) when booked for surgery. Predictor variables that differed between members of the cohort, divided according to *GJB2* status, are shown and compared in Table 2. Length of profound deafness was shorter in those with *GJB2*-related deafness, and age at implantation was also lower in this group. All patients also received a full insertion of electrodes (all 22 active electrodes inside the cochlea) and used the SPEAK coding strategy. Postoperative complications were relatively more common in those with *GJB2*-related deafness. One child had a head injury and wound breakdown after a fall 2 years after primary surgery and required revision surgery involving muscle flap transfer onto the implant site. Another child required revision surgery 3 days after primary surgery, for electrode slippage. A third child developed a keloid at the skin incision, requiring excision under general anesthesia, 11 months after implantation.

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All 39 patients in our study have preoperative SIR scores and postoperative Year-1 SIR scores available, 36 have postoperative Year-2 SIR scores, 32 have postoperative Year-3 SIR scores, 24 have postoperative Year-4 SIR scores, and 17 have reached at least Year 5 (Tables 3 and 4). Comparisons of preoperative values with Year-3 scores showed significant differences within each group (*GJB2*-related deafness group, *p* < 0.001 [Fig. 1]; *GJB2*-unrelated deafness group, *p* < 0.001 [Fig. 2]). One child with *GJB2*-related deafness has developmental verbal dyspraxia (19) (not known to be associated with *GJB2* mutations) and was excluded from the intergroup comparison of Year-3 SIR scores. There was a signifi-
cant difference between groups in the proportion achieving intelligible speech (Category 3 or greater); 82% (9 of 11) with GJB2-related deafness, compared with 30% (6 of 20) with GJB2-unrelated deafness (p = 0.017). There was also a significant difference between groups in median SIR scores; patients with GJB2-related deafness (median, 3; range, 2–4) had significantly higher scores than those with GJB2-unrelated deafness (median, 2; range, 1–4) (p = 0.004) (Fig. 3).

Ordinal logistic regression analysis on postoperative SIR scores was used to control for factors likely to confound the above results. As seen in Table 5, the unadjusted odds ratio for higher postoperative SIR scores in the GJB2-related deafness group compared with the GJB2-unrelated deafness group was 9.4 (95% confidence interval, 1.8–47.8; p = 0.007). After adjustment for confounders (age at implantation, socioeconomic status, and baseline preoperative SIR score), the odds ratio remained statistically significant: 8.8 (95% confidence interval, 1.5–53.9; p = 0.018).

Educational setting was known for the 32 patients at the 3-year postoperative interval. There were significant differences between groups (p = 0.01): half of those with GJB2-related deafness were attending mainstream schools, with the rest in partial hearing units; whereas only 2 of those with GJB2-unrelated deafness were attending mainstream schools, 12 were attending partial hearing units, and 6 of the remainder were in schools for the deaf.

**DISCUSSION**

The single patient with KID syndrome, despite being syndromic, was classified together with the nonsyndromic patients as having GJB2-related deafness because of the underlying GJB2 mutation as the cause of deafness.
Postoperative Year-3 SIR scores and educational settings were selected as outcome variables, as this allowed time for genuine differences to manifest between groups, and reasonable numbers to allow analysis had reached Year 3. This SIR scale is applicable to ages ranging from 1-year-olds to 15-year-olds and is easily translated into any language. This facilitates comparisons between all children in one cochlear implant center, or between different centers, provided they are compared at fixed postoperative time intervals. Although the SIR scale has been found to be a reliable means of assessing speech intelligibility in other pediatric cochlear implant programs in the United Kingdom (9,15,16), a potential weakness of the study is the accuracy of the retrospectively assessed SIR scores, by the two members of the habilitation team, before genetic analysis commenced. This was feasible because the SIR scale represents “real-life” situations and thus can be retrospectively assessed, com-

TABLE 4. Individual Year-3 Speech Intelligibility Rating scores for children with GJB2-unrelated deafness

<table>
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<tr>
<th>Subject</th>
<th>Preoperative SIR</th>
<th>Year-1 SIR</th>
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Median (interquartile range) 1 (1–1) 1 (1–2) 2 (1–2) 2 (1–3) 2 (2–3) 3 (2–3)

aCategory 3 or greater = intelligible speech.
SIR, Speech Intelligibility Rating.

FIG. 1. Trends of improving SIR scores in patients with GJB2-related deafness, before and up to 5 years after implantation. At Year 3, the only child who scored in Category 1 has developmental verbal dyspraxia.
pared with performance tests conducted in more laboratory-type settings (20). The results of our videotape analysis of intra- and interobserver agreements also show good reproducibility for each member’s assessments between their videotape assessment and their retrospectively assessed SIR score of the same child from written case notes and also between different assessors.

Univariate analyses demonstrate that children with GJB2-related deafness who received implants had more intelligible speech, and a larger proportion attended mainstream school, reflecting good capabilities in communicating in an oral-only mode. Our numbers were large enough to use regression-based analyses to adjust for potential confounders and tease out contributions made by deafness etiology group to speech outcome. Postoperative complications were significantly higher in the group with GJB2-related deafness; however, this was unlikely to account for better results in this group. Length of profound deafness was significantly shorter and age at implantation significantly lower in patients with GJB2-related deafness and thus likely to positively favor outcomes in this group. These two latter variables are closely related, with a single exception. Thus, in the regression analysis, only one of these two (age at implantation) was assessed, together with socioeconomic status, a known confounder, and baseline preoperative SIR scores, as potential confounders of the comparison between deafness etiology groups. The best outcomes were observed in those with GJB2-related deafness (Table 5). Although most of our analyses were performed at Year 3 postoperatively, follow-up of those who had had implants for 4 or more years shows interesting trends. Most children in the GJB2 group achieved Category 3 or greater, indicating “intelligible speech” by Year 4 or 5 postoperatively and continued to improve (Fig. 1), whereas the performance of the non-GJB2 group tended to plateau between Category 2 and Category 3 at the same time intervals (Fig. 2).
Our findings may be explained by differing pathologic changes in the auditory pathway in the different groups of children. The cochlear implant directly electrically stimulates the auditory nerve cell bodies and the spiral ganglion cells and thus bypasses the affected organ of Corti in sensorineural deafness (21). Survival of the spiral ganglion cells has traditionally been viewed to play a significant role in outcome after cochlear implantation; however, increasingly, the importance of the central auditory pathway is also being recognized (22). In human (23) and mouse cases (24) with GJB2-related deafness, temporal bones studied suggest that these cases may have normal spiral ganglion cell counts. However, the evidence is anecdotal, as only one of the human temporal bones studied was from a person with GJB2-related deafness, whereas four were from persons with GJB2-unrelated deafness (23). Clinically, patients with GJB2-related deafness also appear to have no neurologic problems other than deafness, despite the absence of GJB2 expression in their brains (25). Thus, in our study, our fairly homogeneous group of patients with GJB2-related deafness may have the advantage of a relatively normal auditory pathway from spiral ganglion cells to primary auditory cortex. Temporal bones studied in patients with GJB2-unrelated deafness have revealed low spiral ganglion cell counts (23). These individuals may be further disadvantaged by having mutations in other nonsyndromic deafness genes such as OTOF (26), GJB3 (connexin 31) (27), or KCNQ4 (28) associated with pathologic changes in the auditory nerve and/or central auditory pathway. These changes may thus exist in our relatively heterogeneous group of patients with GJB2-unrelated deafness, perhaps explaining their poorer outcomes.

Before genetic testing, most children in our cochlear implant program had a deafness of unknown cause. The frequency of GJB2-related deafness in this study was 14 of 39 in the cohort (approximately 30%), and thus it is the most common known cause of deafness. Although it is possible for another single cause to be as common among patients with GJB2-unrelated deafness, this is unlikely because of the extreme heterogeneity that exists in genetic deafness (3). No other cause of deafness is likely to have the same impact on cochlear implant outcome as GJB2. Previous studies have found statistically better results in pediatric implant recipients with GJB2-related deafness regarding speech perception (13) and language ability (14). We have used a new outcome measure in comparing these groups, speech intelligibility, and have again found statistically better results in pediatric implant recipients with GJB2-related deafness. GJB2 appears to be a good marker of outcome after cochlear implantation.

### CONCLUSION

The immediate implication of this study is that parents of children with GJB2-related deafness can receive better preoperative counseling regarding good speech intelligibility outcomes.

### Acknowledgments

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### APPENDIX

In all children, Exon 2 of GJB2 (including all of the coding region) and flanking intronic segments were screened for mutations by polymerase chain reaction (PCR)-based DNA sequencing using the following: Exon 2 forward primer 5'-GTAAGAGGTT-GTGTGTCTAGG-3' with Exon 2 alt.reverse primer 5'-TTGGTTTTGATCTCCTCGAT-3', and Exon 2 alt.forward primer 5'-GAAGAGGAGG-AAGTTCACTAAGGG-3' with Exon 2 reverse primer 5'-AGGTCAAGAATCTTTGTTGGG-3'.

All children with only one or no mutations in Exon 2 of GJB2 were screened for mutations in Exon 1 of GJB2 and flanking intronic segments by PCR amplification using the following: Exon 1 forward primer 5'-GTAACCT-TCCAGTCTTGAGG-3' with Exon 1 reverse primer 5'-AGAAACGCCCGCTCCAGAAGG-3'. All children with one or no mutations in Exon 2 of GJB2 were also screened for the large 342-kb deletion, which includes most of the GJB6 gene. This latter screening involved PCR amplification of all these test cases for the breakpoint sequence, together with a positive control obtained from Ignacio Del Castillo, Ph.D., Unidad de Genética Molecular, Hospital Ramón y Cajal, Madrid, Spain (5), and (GJB6–IR) GJB6 forward primer 5'-TITAGGGCAT-

### TABLE 5. Results of ordinal logistic regression on Year-3 Speech Intelligibility Rating scores expressed as odds ratios for group comparisons with adjustment for confounding variables (n = 31)\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Year-3 SIR score</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adjusted for</td>
<td>Odds ratio</td>
<td>(95% CI)</td>
</tr>
<tr>
<td>Unadjusted</td>
<td>9.4</td>
<td>(1.8–47.8)</td>
<td>0.007</td>
</tr>
<tr>
<td>Age at implantation</td>
<td>8.5</td>
<td>(1.4–50.5)</td>
<td>0.018</td>
</tr>
<tr>
<td>Socioeconomic status</td>
<td>9.4</td>
<td>(1.8–47.9)</td>
<td>0.007</td>
</tr>
<tr>
<td>Preoperative SIR score</td>
<td>10.7</td>
<td>(2.0–55.9)</td>
<td>0.005</td>
</tr>
<tr>
<td>Age at implantation + socioeconomic status + preoperative SIR score</td>
<td>8.8</td>
<td>(1.5–53.9)</td>
<td>0.018</td>
</tr>
</tbody>
</table>

\(^a\)The odds ratio estimates the odds of achieving a higher postoperative Year-3 category in the SIR scale in a patient having GJB2-related deafness, relative to having GJB2-unrelated deafness. SIR, Speech Intelligibility Rating; CI, confidence interval.
GATTGGGGTGATT-3’ with (BKR-1) GJB6 reverse primer 5’-CACCATGCTAGCCCTAAACCTTTT-3’.

Amplification conditions were as follows: reaction volume was 10 μl including 25 ng of genomic DNA (12.5 ng of the Spanish control sample only for GJB6 breakpoint sequence amplifications), each primer at 0.3 μmol/L, Qiagen HotstarTaq DNA Polymerase at 0.25 U, dNTPs at 200 μmol/L, PCR buffer (x1) and solution Q (x1). For the reaction involving GJB2 Exon 2 alt.forward primer with GJB2 Exon 2 reverse primer, after the initial denaturation step at 96°C for 10 minutes, samples were amplified under the following thermal conditions: 95°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute, for 10 cycles. This was followed by 95°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute, for 35 cycles, with a final extension time at 72°C for 7 minutes. For reactions involving GJB2 Exon 2 forward primer with GJB2 Exon 2 alt.reverse primer, GJB2 Exon 1 forward primer with GJB2 Exon 1 reverse primer, and (GJB6–1R) GJB6 forward primer with (BKR-1) GJB6 reverse primer, after the initial denaturation step at 96°C for 10 minutes, samples were amplified under the following thermal conditions: 95°C for 1 minute, 63°C for 1 minute, and 72°C for 1 minute, for 10 cycles. This was followed by 95°C for 1 minute, 61°C for 1 minute, and 72°C for 1 minute, for 35 cycles, with a final extension time at 72°C for 7 minutes. The control sample from Spain was initially successfully amplified on its own using 25 ng, 10 ng, and even 5 ng of genomic DNA. This attempted to ensure that when subsequently amplified together with 25-ng test samples, if any test sample appeared negative, this was not because of primer insensitivity or incorrect reaction conditions.

REFERENCES