This is an Open Access article licensed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs 3.0 License (www.karger.com/OA-license), applicable to the online version of the article only. Distribution for non-commercial purposes only.

Original Paper

GJB2 Variants and Auditory Outcomes among Filipino Cochlear Implantees

Charlotte M. Chiong^{a, b} Eva Maria Cutiongco-de la Paz^c
Maria Rina T. Reyes-Quintos^{a, b} Celina Ann M. Tobias^e
Karen Hernandez^c Regie Lyn P. Santos-Cortez^d

^aPhilippine National Ear Institute and Newborn Hearing Screening Reference Center, National Institutes of Health (NIH), and ^bDepartment of Otorhinolaryngology, Philippine General Hospital, University of the Philippines Manila (U.P. Manila) and U.P. College of Medicine, and ^cInstitute of Human Genetics, NIH, U.P. Manila, Manila, Philippines;

^dCenter for Statistical Genetics, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Tex., USA; ^eMED-EL, Innsbruck, Austria

Key Words

Gap junction beta-2 gene • Connexin 26 protein • Cochlear implant • Genetic deafness

Abstract

Objectives: To determine the prevalence of gap junction beta-2 (*GJB2*) or connexin 26 variants in Filipino cochlear implantees, and to describe radiologic findings, audiologic results and auditory performance after cochlear implantation (CI). **Methods:** Thirty cochlear implantees with unknown etiology of congenital severe-to-profound hearing loss and 30 controls provided venous blood samples for study. Radiologic evidence of temporal bone abnormalities, residual hearing status and post-CI auditory performance are described. Statistical analysis was performed for hearing thresholds before and after CI and for Parent Evaluation of Auditory/Oral Performance in Children (PEACH) scores based on the presence of cochleovestibular anomalies, age at CI and time since CI. Genomic DNA was extracted from venous blood, PCR-amplified and sequenced for *GJB2* variants. **Results:** One patient was compound heterozygous (c.[35delG];[235delC]) for *GJB2* variants. The variants p.Gly4Asp, p.Val27Ile and p.Val37Ile were identified in both patients and controls, including 2 implantees who were homozygous for p.Val27Ile and p.Val37Ile. No significant association was found between post-CI improvement in threshold or PEACH scores and the following variables: age at CI, duration of hearing aid use prior to CI, presence of cochleovestibular anomalies and completeness of electrode insertion.

Although no significant change in audiometric thresholds due to time since CI was detected, PEACH scores were significantly improved with longer implant use at all conditions (quiet, noise and overall; $p < 0.05$). **Conclusions:** The prevalence of causal *GJB2* variants in Filipino cochlear implantees is low (3.3%). Within this population, the allele frequency of the p.Val37Ile variant in patients and controls is $>10\%$, which supports a nonpathogenic role for this variant. The low prevalence of *GJB2* variants precluded any association testing with CI outcome, although our results suggest better auditory outcome with longer CI use. Future genetic studies within the Filipino population should be able to control for population admixture.

Copyright © 2013 S. Karger AG, Basel

Introduction

Mutations in the gap junction beta-2 (*GJB2*) gene, which encodes the connexin 26 protein that is important for potassium recycling and for hair cell depolarization/repolarization, are the most common cause of congenital hearing loss of genetic origin in many populations [Estivill et al., 1998; Hutchin et al., 2005]. Among different ethnic groups, there is great variability in genotype-phenotype correlations; for example, specific variants such as c.35delG are more prevalent in Caucasians [Estivill et al., 1998], c.167delT in Ashkenazi Jews [Morell et al., 1998] and c.235delC in Asian populations [Yan et al., 2003]. In many developed countries, *GJB2* sequencing has become routine for genetic screening in hearing-impaired neonates. In contrast, in developing countries like the Philippines, infections (rubella, labyrinthitis, meningitis) and ototoxicity comprise about 50% of the known causes of congenital hearing loss, while in 24% the etiology remains idiopathic [Chiong, 2001]. It is believed that some cases of hearing loss in the latter group may be due to genetic mutations, although the proportion is not as high as in developed countries. In this report, the *GJB2* gene was sequenced in Filipino cochlear implantees with bilateral severe-to-profound hearing loss of unknown etiology and in control subjects. Radiologic abnormalities, pre-implant residual hearing and auditory performance are also described. Given the challenges to access cochlear implant services in the country, our cohort of cochlear implantees provides a unique opportunity to study prevalence rates for hearing impairment genes among Filipinos and auditory outcomes after cochlear implantation (CI). The study results will be useful for genetic counseling, prognostication and formulation of a national policy for hearing care.

Materials and Methods

Patient Recruitment and Clinical Evaluation

The study was approved by the University of the Philippines Manila – National Institutes of Health Ethics Review Board prior to study initiation. Informed consent was obtained from the adult subjects and the parents of the pediatric cochlear implantees. From an initial pool of 100 cochlear implantees, 30 cochlear implant recipients with bilateral severe-to-profound hearing loss of no definitive etiology from perinatal and maternal history as well as 30 adult control subjects (median age 32 years, range 21–59), all of Filipino descent, were enrolled in the study. Otoscopic and audiometric screening of the control subjects was performed and normal hearing results were obtained. For the cochlear implantees, all medical records including radiologic and audiometric evaluation were reviewed. CI was performed by a single surgeon, and audiometric and speech evaluation was performed in a single center. Within a soundproof setting, pure-tone thresholds for the pediatric patients were obtained by auditory steady-state response using a Biologic NavPro (Mundelein, Ill., USA) machine. In the adult

patients, audiometric evaluation based on the standard Hugh Westlake method was performed using a MADSEN Aurical Plus audiometer (Denmark). For computing the averages of the hearing thresholds, a default value of 120 dB was used to indicate profound hearing loss that is beyond the audiometer limits. In the patients with bilateral CI, thresholds for the better hearing ear were used.

Auditory outcomes were rated by Categories of Auditory Performance (CAP) [Archbold et al., 1995] and Parent Evaluation of Auditory/Oral Performance in Children (PEACH) scales [Ching and Hill, 2007]. CAP is an index consisting of eight performance categories arranged in the order of increasing difficulty, with category 8 as the highest performance. The parents of the pediatric cochlear implantees were asked to fill in a PEACH questionnaire [Ching and Hill, 2007].

Statistical analysis was performed for improvement in hearing thresholds and PEACH scores based on the presence of temporal bone anomalies, successful electrode insertion, duration of hearing aid use prior to CI, age at CI and time since CI. Improvement in hearing thresholds was derived by subtracting averaged pre-CI thresholds from averaged post-CI thresholds. The Mann-Whitney-Wilcoxon rank sum test for dichotomous independent variables and Spearman's correlation test for linear independent variables were performed using R [R Development Core Team, 2011].

DNA Extraction, PCR Amplification and Direct Sequencing

Genomic DNA was extracted from whole blood using the Qiagen QIAamp DNA Blood Midi Kit (Qiagen, Valencia, Calif., USA). The coding exon (exon 2) of the *GJB2* gene was sequenced for all participating subjects. PCR primers 5'-gAAGTCTCCCTgTTCTgTCC-3' (forward) and 5'-AATCTAACAACTgggCAATg-3' (reverse) were designed with the NetPrimer software (Premier Biosoft International, Palo Alto, Calif., USA).

PCR was performed in 45- μ l reaction mixtures, each containing 1 $\times$ PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), 2.5 mM MgCl₂, 0.2 mM of each deoxynucleotide, 0.2 μ M of the forward and reverse primers, 3 U Taq DNA polymerase (Invitrogen, Carlsbad, Calif., USA) and 1.5 μ l gDNA. PCR conditions were as follows: initial denaturation for 4 min at 94°C, 38 cycles of denaturation at 94°C for 30 s, annealing at 67.6°C for 30 s and extension at 72°C for 50 s, and a final extension for 10 min at 72°C. Amplicons were then sequenced bidirectionally. For the patient samples in which only one variant in exon 2 of *GJB2* was found, sequencing of exon 1 and adjacent intronic regions of the gene was performed.

Results

Clinical Description

Of 30 cochlear implantees, 14 were male and 16 female. The median age at CI was 4 years 7 months (range 15.5 months to 27 years; average age at CI 7 years). Nineteen patients were implanted in the right ear, 7 in the left ear and 4 bilaterally. All patients were implanted with MED-EL devices, except for 1 patient who received a Cochlear Nucleus Freedom Contour device.

Half of the patients had normal cochleovestibular findings on CT and/or MRI scans, including 3 with unilateral high-riding jugular bulb. Nine patients (30%) had enlarged vestibular aqueducts (EVA), 1 patient had bilateral Mondini dysplasia and another implantee had bilateral malformed cochleae with incomplete partition. A child with bilaterally malformed cochleae, vestibules and semicircular canals also had congenitally absent cochlear and inferior vestibular nerves in the right ear. Three implantees showed evidence of superior semicircular canal dehiscence.

Fig. 1. Correlation plots for improvement in auditory thresholds and PEACH scores based on time since CI. **a** Difference between pre- and post-CI thresholds versus time since CI ($R = 0.25$, NS). **b** PEACH scores in the quiet condition versus time since CI ($R = 0.54$, $p = 0.005$). **c** PEACH scores in the noise condition versus time since CI ($R = 0.42$, $p = 0.04$). **d** Overall PEACH scores versus time since CI ($R = 0.49$, $p = 0.015$).

For 3 children, only auditory brainstem response or auditory steady-state response results were available, which showed bilateral severe-to-profound hearing loss. Among 27 patients with preoperative hearing thresholds, the average across frequencies 0.5–4 kHz was 106.6 ± 12.0 dB (median 111, range 80–120). Postoperatively, the average across frequencies for aided thresholds was 38.9 ± 10.4 dB (median 38.8, range 25–80). This means an improvement in hearing of 66.7 ± 16.6 dB on average (median 71.2, range 31.8–95). Of 3 adult implantees (age >20 years), 2 had a CAP score of 7, while 1 had a CAP score of 6. The child with bilateral inner ear malformations and right eighth nerve aplasia was implanted in the left ear and had PEACH scores of 4% for the quiet, 10% for the noise and 7% for the overall conditions. For 24 other children with PEACH scores, the average scores were as follows: quiet condition, $74.1 \pm 21.3\%$ (median 81, range 21–96); noise condition, $66.0 \pm 25.5\%$ (median 67.5, range 0–100); overall condition, $70.5 \pm 22.6\%$ (median 75, range 16–98).

The presence of temporal bone abnormalities, successful electrode insertion and length of pre-CI hearing aid use did not significantly influence post-CI threshold improvement and PEACH scores. When plotted against age at CI and time since CI (fig. 1), no significant rela-

Table 1. *GJB2* variants in implantees and controls

<i>GJB2</i> genotype	Implantees	Controls
c.[35delG];[c.235delC]	1	0
p.[(Val37Ile)];[(Val37Ile)]	1	0
p.[(Val27Ile)];[(Val27Ile)]	1	0
p.[(Val27Ile;Glu114Gly)]	0	2
p.[Gly4Asp];[–]	2	3
p.[Val27Ile];[–]	1	0
p.[Val37Ile];[–]	6	7
None	18	18
Total	30	30

tionship with post-CI improvement was found in the threshold. Likewise, no significant change in PEACH scores was detected based on age at CI. On the other hand, PEACH scores were significantly associated with time since CI at all conditions (quiet, $R = 0.54$, $p < 0.01$; noise, $p < 0.05$; overall, $R = 0.49$, $p < 0.05$; fig. 1).

GJB2 Genotypes

Of 30 cochlear implantees who were sequenced for *GJB2*, only 3 patients were homozygous or compound heterozygous for *GJB2* variants (table 1). One implantee was compound heterozygous for the c.35delG and c.235delC mutations. A second implantee was homozygous for the p.Val37Ile variant. However, the p.Val37Ile variant was found in the heterozygous state at high allele frequencies in both patients (10%) and controls (11.7%; table 1). One implantee was homozygous for p.Val27Ile, while 2 controls carried both the p.Val27Ile and p.Glu114Gly variants in the heterozygous state. Previous reports have concluded that p.Val27Ile was a polymorphism, either as a single variant or within a haplotype with p.Glu114Gly [Kudo et al., 2000; Park et al., 2000]. Another known variant, p.Gly4Asp [Hwa et al., 2003], was identified in the heterozygous state in both implantees and controls at allele frequencies of 3.3 and 5%, respectively. Sequencing of exon 1 of *GJB2* in 9 implantees who were heterozygous for a single variant in exon 2 did not detect additional variants.

The patient who was compound heterozygous for c.[35delG];[235delC] was partly of Spanish descent. Based on oral history, multiple individuals from both sides of the patient's family also have hearing impairment. His temporal bone CT revealed bilateral EVA (fig. 2a). Although he was implanted in the right ear at the age of 6 years, he had been using hearing aids since he was 2 years old. He had excellent post-CI thresholds (average 41.2 dB; fig. 2b) and PEACH scores of 90–92% at all conditions.

The implantee who was homozygous for p.Val37Ile had EVA in the left ear (fig. 2c) and was implanted bilaterally at the age of 4 years. His average for aided thresholds was 25 dB (fig. 2d) and his PEACH scores ranged from 66–70%.

Discussion

Whether the p.Val37Ile variant damages hearing function or not remains controversial. This variant has been associated with mild-to-moderate hearing impairment in multiple individuals [Snoeckx et al., 2005b]. Bioinformatics analysis using PolyPhen-2 [Adzhubei et al., 2010] predicts the possibly damaging effect of the variant, while the SIFT [Ng and Henikoff, 2001] program labels p.Val37Ile as tolerated. On the other hand, functional studies indicate that the p.Val37Ile variant disables induction of the formation of homotypic gap junction

Fig. 2. **a, b** Temporal bone CT showing bilateral EVA (black arrowheads) in the patient with the *GJB2* c.35delG/c.235delC genotype. **c** Visual reinforcement audiometry (blue hashed lines) in the same patient at the age of 5 years showed severe hearing impairment, while aided thresholds (letter S) indicated hearing within the speech spectrum. Red, right ear; blue, left ear. **d** Thresholds further improved 3.5 years after CI in the right ear. **e** Temporal bone CT of the patient who is homozygous for the *GJB2* p.Val37Ile variant (black arrowhead). **f** Unaided thresholds indicate profound hearing loss in the right ear (red circles) and severe hearing impairment in the left ear (blue crosses). Thresholds obtained with hearing aids (blue S) were within the hearing range at 500–4,000 Hz but not at 500 and 8,000 Hz. Implant-aided thresholds (red S) were within the speech range across all frequencies.

channels in *Xenopus* oocytes and may even have a dominant negative effect [Bruzzone et al., 2003; Palmada et al., 2006]. However, the allele frequency in both patients and controls in this study is >10%, and thus p.Val37Ile may be considered a common variant among Filipinos. It also appears to be equally prevalent among hearing-impaired individuals and controls in East Asian populations [Hwa et al., 2003; Han et al., 2008]. Likewise, both the p.Val27Ile and p.Val37Ile variants have high allele frequencies in Indonesians [Snoeckx et al., 2005a] and Malaysians [Zainal et al., 2012]. Given this evidence, it is possible that the patient in this study was homozygous for the p.Val37Ile variant because of high variant allele frequencies within the population, and that the profound hearing loss in this patient has a different etiology.

Because only 1 patient carried recessive *GJB2* mutations in two alleles, the prevalence of hearing impairment due to *GJB2* variants within this cohort of Filipino cochlear implantees is low (3.3%). The prevalence of causal variants in *GJB2* is similarly low in other Southeast Asian countries such as Indonesia [Snoeckx et al., 2005a] and Malaysia [Zainal et al., 2012], where the general Filipino population originated historically. Since *GJB2* variants are not a common cause of hearing impairment within the Filipino population, *GJB2* sequencing will not be cost-efficient as a genetic screening tool.

Although the *GJB6* gene is known to cause hearing impairment through digenic inheritance with *GJB2* [Lerer et al., 2001], the *GJB6* gene was not tested in this study. Similarly, only

a few individuals were sequenced for exon 1. Worldwide, the prevalence rates for mutations within *GJB2* exon 1 and *GJB6* remain low, and such mutations are only common in individuals from select populations who have heterozygous mutations in *GJB2* [Del Castillo et al., 2003; Sirmaci et al., 2006; Yuan et al., 2010].

Earlier studies suggested better post-CI outcomes in children with *GJB2* mutations [Matsushiro et al., 2002; Bauer et al., 2003], but more recent studies demonstrate that such outcomes in *GJB2*-positive children are mainly due to duration of CI use or younger age at implantation [Connell et al., 2007; Lalwani et al., 2009]. Although the number of pediatric cochlear implantees in this study was small, we were able to show an improvement in PEACH scores with increasing time since CI, but not with age at implantation.

Due to the low prevalence of *GJB2* mutations, it was not possible to test if there is an association between the presence of *GJB2* mutations and post-CI auditory outcomes. However, merely increasing the sample size may not be a cost-efficient strategy in conducting future genetic research. The identification of the c.35delG and c.235delC variants in 1 individual due to mixed ancestry may reflect population admixture within the general Filipino population, which is to be expected considering the country's migration and colonization history. This forebodes difficulties in designing case-control studies for this population and can result in false-positive associations due to population admixture. Thus, future genetic studies within the Filipino population require a study design and statistical analysis that properly control for population admixture and substructure.

Acknowledgements

This study was funded in part by MED-EL Philippines and U.P. Manila – NIH grant 2008-005.

Disclosure Statement

C.A.M.T. is presently with MED-EL Innsbruck, Austria, but she and the rest of the authors have no proprietary or other interests that could be construed to affect the results of this study.

References

- Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al: A method and server for predicting damaging missense mutations. *Nat Methods* 2010;7:248–249.
- Archbold S, Lutman ME, Marshall DH: Categories of auditory performance. *Ann Otol Rhinol Laryngol Suppl* 1995;166:312–314.
- Bauer PW, Geers AE, Brenner C, Moog JS, Smith RJ: The effect of *GJB2* allele variants on performance after cochlear implantation. *Laryngoscope* 2003;113:2135–2140.
- Bruzzone R, Veronesi V, Gomès D, Bicego M, Duval N, Marlin S, et al: Loss-of-function and residual channel activity of connexin26 mutations associated with non-syndromic deafness. *FEBS Lett* 2003;533:79–88.
- Ching TY, Hill M: The Parents' Evaluation of Aural/Oral Performance of Children (PEACH) scale: normative data. *J Am Acad Audiol* 2007;18:220–223.
- Chiong CM: Cochlear implantation: the Philippine General Hospital experience. *Philipp J Otorhinolaryngol Head Neck Surg* 2001;9–13.
- Connell SS, Angeli SI, Suarez H, Hodges AV, Balkany TJ, Liu XZ: Performance after cochlear implantation in *DFNB1* patients. *Otolaryngol Head Neck Surg* 2007;137:596–602.

- Del Castillo I, Moreno-Pelayo MA, Del Castillo FJ, Brownstein Z, Marlin S, Adina Q, et al: Prevalence and evolutionary origins of the del(GJB6-D13S1830) mutation in the DFNB1 locus in hearing-impaired subjects: a multicenter study. *Am J Hum Genet* 2003;73:1452–1458.
- Estivill X, Fortina P, Surrey S, Rabionet R, Melchionda S, D'Agruma L, et al: Connexin-26 mutations in sporadic and inherited sensorineural deafness. *Lancet* 1998;351:394–398.
- Han SH, Park HJ, Kang EJ, Ryu JS, Lee A, Yang YH, et al: Carrier frequency of GJB2 (connexin-26) mutations causing inherited deafness in the Korean population. *J Hum Genet* 2008;53:1022–1028.
- Hutchin T, Coy NN, Conlon H, Telford E, Bromelow K, Blaydon D, et al: Assessment of the genetic causes of recessive childhood non-syndromic deafness in the UK – implications for genetic testing. *Clin Genet* 2005;68:506–512.
- Hwa HL, Ko TM, Hsu CJ, Huang CH, Chiang YL, Oong JL, et al: Mutation spectrum of the connexin 26 (GJB2) gene in Taiwanese patients with prelingual deafness. *Genet Med* 2003;5:161–165.
- Kudo T, Ikeda K, Kure S, Matsubara Y, Oshima T, Watanabe K, et al: Novel mutations in the connexin 26 gene (GJB2) responsible for childhood deafness in the Japanese population. *Am J Med Genet* 2000;90:141–145.
- Lalwani AK, Budenz CL, Weisstuch AS, Babb J, Roland JT Jr, Waltzman SB: Predictability of cochlear implant outcome in families. *Laryngoscope* 2009;119:131–136.
- Lerer I, Sagi M, Ben-Neriah Z, Wang T, Levi H, Abeliovich D: A deletion mutation in GJB6 cooperating with a GJB2 mutation in trans in non-syndromic deafness: a novel founder mutation in Ashkenazi Jews. *Hum Mutat* 2001;18:460.
- Matsushiro N, Doi K, Fuse Y, Nagai K, Yamamoto K, Iwaki T, et al: Successful cochlear implantation in prelingual profound deafness resulting from the common 233delC mutation of the GJB2 gene in the Japanese. *Laryngoscope* 2002;112:255–261.
- Morell RJ, Kim HJ, Hood LJ, Goforth L, Friderici K, Fisher R, et al: Mutations in the connexin 26 gene (GJB2) among Ashkenazi Jews with nonsyndromic recessive deafness. *N Engl J Med* 1998;339:1500–1505.
- Ng PC, Henikoff S: Predicting deleterious amino acid substitutions. *Genome Res* 2001;11:863–874.
- Palmada M, Schmalisch K, Böhmer C, Schug N, Pfister M, Lang F, et al: Loss of function mutations of the GJB2 gene detected in patients with DFNB1-associated hearing impairment. *Neurobiol Dis* 2006;22:112–118.
- Park HJ, Hahn SH, Chun YM, Park K, Kim HN: Connexin26 mutations associated with nonsyndromic hearing loss. *Laryngoscope* 2000;110:1535–1538.
- R Development Core Team: R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2011. <http://www.r-project.org>.
- Snoeckx RL, Djelantik B, Van Laer L, Van de Heyning P, Van Camp G: GJB2 (connexin 26) mutations are not a major cause of hearing loss in the Indonesian population. *Am J Med Genet A* 2005a;135:126–129.
- Snoeckx RL, Huygen PLM, Feldmann D, Marlin S, Denoyelle F, Waligora J, et al: GJB2 mutations and degree of hearing loss: a multicenter study. *Am J Hum Genet* 2005b;77:945–957.
- Sirmaci A, Akcayoz-Duman D, Tekin M: The c.IVS1+1G>A mutation in the GJB2 gene is prevalent and large deletions involving the GJB6 gene are not present in the Turkish population. *J Genet* 2006;85:213–216.
- Yan D, Park HJ, Ouyang XM, Pandya A, Doi K, Erdenetungalag R, et al: Evidence of a founder effect for the 235delC mutation of GJB2 (connexin 26) in east Asians. *Hum Genet* 2003;114:44–50.
- Yuan Y, Yu F, Wang G, Huang S, Yu R, Zhang X, et al: Prevalence of the GJB2 IVS1+1G>A mutation in Chinese hearing loss patients with monoallelic pathogenic mutation in the coding region of GJB2. *J Transl Med* 2010;8:127.
- Zainal SA, Md Daud MK, Abd Rahman N, Zainuddin Z, Alwi Z: Mutation detection in GJB2 gene among Malays with non-syndromic hearing loss. *Int J Pediatr Otorhinolaryngol* 2012;76:1175–1179.