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Ethnicity and mutations in GJB2 (connexin 26) and GJB6 (connexin 30) in a multi-cultural Canadian paediatric Cochlear Implant Program☆

Evan Jon Propst [a,*], Tracy L Stockley [b], Karen A Gordon [a], Robert V Harrison [a], Blake Croll Papsin [a]

[a] *Cochlear Implant Program, Department of Otolaryngology, 6th Floor, Elm Wing, The Hospital for Sick Children, 555 University Avenue, Toronto, Ont., Canada M5G 1X8*
[b] *Molecular Genetics Laboratory, Department of Paediatric Laboratory Medicine, The Hospital for Sick Children, Toronto, Canada*

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Summary

Objective: To determine the relationship between ethnicity and mutations in the GJB2 and GJB6 genes in multi-cultural patients enrolled in a Canadian paediatric Cochlear Implant Program.
Methods: Blood was analyzed from 65 paediatric cochlear implant users by direct sequencing of the coding region and intron/exon boundaries of the GBJ2 gene. Individuals heterozygous for one mutation in GJB2 or in whom mutations in GJB2 were not detected were analyzed for the common 342 kb deletion mutation D13S1830 in the GJB6 gene. Information regarding ethnicity of patients' families was obtained from patient records and/or interview.
Results: GJB2 mutations were found in 36.9% of paediatric cochlear implant users tested. Nine different GJB2 mutations were identified among individuals from 14 different countries of origin. Seventy-eight percent of all identified pathogenic GJB2 mutations were 35delG. Biallelic GJB2 mutations were found in 16 cochlear implant users (66.7% of GJB2 mutations). Three novel GJB2 sequence changes were identified: (1) a missense mutation T107C (L36P) in an individual of African decent; (2) a missense mutation G475T (D159Y) in an individual of Caribbean decent; (3) a regulatory region change 1-34C to T in an individual of African decent. GJB6-D13S1830 mutations were not found in any of the patients tested. Individuals of African, Caribbean and East Indian decent had different GJB2 mutations than the remainder of individuals tested.

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* Corresponding author. Tel.: +1 416 813 7259; fax: +1 416 813 5036.
E-mail address: Evan.propst@utoronto.ca (E.J. Propst).

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Patients of Asian, Italian, Spanish, Polish and Armenian decent were not found to carry mutations in GJB2 or the common GJB6-D13S1830 mutation.
Conclusions: This study represents the largest number of biallelic GJB2 mutations isolated in a group of paediatric cochlear implant users to date. Numerous and diverse GJB2 mutations were found in this multi-cultural group of children. Even though GJB2 mutations have been widely reported in the literature, this discussion represents the first report of GJB2 mutations in a multi-ethnic population (Canadian), as compared with previous studies that investigated fairly homogeneous populations. The diversity of GJB2 mutations identified reinforces the importance of testing for changes in GJB2 by direct sequencing of the entire coding region rather than testing only for common mutations.

1. Introduction

Approximately one in 1000 children are born with moderate to profound hearing impairment [1], and it is estimated that 50% of these cases have a genetic cause. Autosomal recessive non-syndromic hearing loss (ARNSHL) is the most prevalent genetic cause of congenital hearing loss and 40—60% of cases are believed to be due to mutations in the GJB2 gene. The GJB2 gene encodes the connexin 26 (Cx26) protein, which functions as a component of gap junctions. Gap junctions form when two hemi-connexins on the surfaces of two adjacent cells join. The resultant channel permits ions and small molecules to flow between the cells. Cx26 is present in the cochlea in the stria vascularis, basement membrane, limbus and spiral prominence [2,3], and is presumed to allow potassium ions to be removed from the cochlear endolymph to the stria vascularis to maintain endolymphatic homeostasis [4]. Mutations in Cx26 are presumed to result in altered potassium recirculation, leading to the accumulation of potassium in the cochlear endolymph and causing hair cell dysfunction and deafness [5]. Approximately 100 Cx26 mutations known to cause hearing loss have been reported [6]. A 342 kb deletion in the connexin 30 (Cx30) locus of the GJB6 gene (D13S1830) has also been found to interact with a single recessive Cx26 mutation to cause deafness [7].

Previous studies investigating the frequency of Cx26 mutations have examined fairly homogeneous populations and have found an association between the type of Cx26 mutation and country of origin (Table 1). Even though Cx26 mutations have been investigated in over 35 countries, the frequency of Cx26 mutations in a multi-cultural population, such as in Canada, has never been reported.

The population of the city of Toronto, Canada, is 4.6 million [49]. Approximately one half of these individuals are immigrants, with over 400,000 arriving in the past 15 years [49]. A recent census listed 222 separate places of birth for Canada's immigrants, with each of 106 nations listed as the place of birth for more than 1000 Toronto residents [49]. The most common places of birth of Toronto's immigrants in decreasing order were as follows: United Kingdom (9.8%), Italy (8.1%), Hong Kong (6.1%), India (5.5%), Jamaica (4.8%), China (4.8%), Portugal (4.5%), Philippines (4.5%), Poland (4.3%), Guyana (3.4%), Sri Lanka (3.0%), Viet Nam (2.8%), other (38.4%).

The Cochlear Implant Program at The Hospital for Sick Children in Toronto, Canada, services Toronto's ethnically diverse population, and consequently offers a unique opportunity to further explore the relationship between mutations in Cx26 and country of origin. The purpose of this study was to determine the relationship between ethnicity and mutations in GJB2 and GJB6 genes in multi-cultural Canadian patients enrolled in a paediatric Cochlear Implant Program.

2. Methods

2.1. Subjects

Subjects were 65 consecutive non-syndromic paediatric cochlear implant users implanted for severe to profound hearing loss at The Hospital for Sick Children, Toronto, Canada, from September 2003 to September 2004. This project was approved by The Hospital for Sick Children Ethics Review Board which adheres to the ''Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans''. Blood was obtained intraoperatively at the time of implantation after obtaining written consent from each child's parent/guardian. Ethnicity was obtained from parents during completion of the cochlear implant intake questionnaire or via interview by the lead author during discussion of genetic results.

Table 1 Overview of GJB2 (connexin 26) mutations by country/territory as reported by previous studies

Country/territory	Connexin mutation	References
Australia	35delG, V37I, M34T*, L90P, C53R^, W24X, T123N•, 167delT, W77R, R143W, R127C•, R184W, I35S^, 333-334delAA	Dahl et al. [8]
	delE118, R148P	Denoyelle et al. [9]
Austria	35delG, W24X, A88S, L90P, delE120, V153I*	Frei et al. [10]
	35delG, L90P, IVS1 + 1G to A, 314del14, V84L, I20T, R143Q, R75W^,167delT, G160S*, M163V•, M34T*	Janecke et al. [11]
Belgium (with Italy, Spain)	35delG, 167delT, L90P, IVS1 + 1G to A, R184P, E147K, 313del14^, V37I, W77R, R143W, W24X, V95M, delE120	Cryns et al. [12]
Brazil	35delG, V95M, V27I*, V37I, delE120	Oliveira et al. [13]
China	235delC, 299-300delAT, V37I, 35delG	Liu et al. [14]
Finland	35delG, M34T*, V37I	Lopponen et al. [15]
France	35delG, L90P, 167delT, 313del14^, E47X, Q57X, W24X, R32H, V37I, A40E, W77R, V95M, delE120, S139N, R184P, C211X^, 235delC, 269insT, 333-334delAA	Feldmann et al. [16]
	35delG, E47X, 312del14, N206S, R184P, W24X, C64X, delE120, Q57X, R143W, W44X, IVS1 + 1G to A	Roux et al. [17]
France (Corsica)	35delG	Lucotte et al. [18]
Ghana	R143W, V178A, I203K, L79P, R184Q(dominant), A197S, L214P, 35insG	Hamelmann et al. [19]
Greece	35delG, R127H, R184P, K224Q•	Antoniadi et al. [20]
Hungary	35delG, W24X, 31del14, E47X, 167delT, 235delC, 313del14, L90P, V37I, M34T*, S19T, R32C, G59V^, S17Y^, R127H*, A149T^	Toth et al. [21]
India	W24X, 35delG, R143W	Maheshwari et al. [22]
Italy	35delG, E47X, L90P, D159V^ 284ins/dup^ V95M, 167delT, R184P, delE120, I20T	Gualandi et al. [23]
	35delG, 167delT, L90P, R184P, IVS1 + 1G to A	Mesolella et al. [24]
Italy (Sicily)	35delG (half the rate of northern Italy), 167delT	Salvinelli et al. [25]
Iran	35delG, W24X	Najmabadi et al. [26]
Iran (Kurdish)	35delG, R32H, delE120, IVS1 + 1G to A, R184P, R127H*	Mahdieh et al. [27]
Israel (Jewish)	35delG, 167delT, 51del12insA	Sobe et al. [28]
Japan	235delC, V37I, G45E, Y136X, 176-191del16, 299-300delAT, R143W, T86R^, 605ins46	Ohtsuka et al. [29] Yan et al. [30]
Jordan	35delG	Medlej-Hashim et al. [31]
Kenya	G to A at 3426^	Gasmelseed et al. [32]
Korea	235delC, 35delG, 176-191del16, 299-300del2^, S85P, R143W, I203T*	Park et al. [33]
Lebanon	35delG	Denoyelle et al. [9]
Morocco	35delG	Lench et al. [34]
Oman	Absence of 35delG and 167delT	Simsek et al. [35]
New Zealand	35delG, 31del38	Denoyelle et al. [9]
Palestinian Authority	35delG, 167delT, T229C^, IVS1 + 1G to A, 235delC	Shahin et al. [36]
Poland	35delG, M34T*, Q47X^, R184P, 313del14^	Wiszniewski et al. [37]
Russia	35delG	Zinchenko et al. [38]
Slovakia	W24X, R127H, 35delG, V153I*, L90P, V37I	Minarik et al. [39]
Spain (with Italy)	35delG, 167delT, R184P, R143W, 333-334delAA, L90P, E47X, V37I, 312del14, M1V, 35insG, G12V, S19T, W24X, M34T*, Y65X, W77R, F83L*, E114X^, delE120, R127H, A149T^, P173R^	Rabionet et al. [40]
Sudan	35delG, 3318-34C to T^, 3697G to A^	Gasmelseed et al. [32]
Switzerland	35delG, 167delT, 313del14^, V37I, delE120, M34T*	Gurtler et al. [41]
Taiwan	235delC, 299-300delAT, R184Q(dominant)	Wang et al [42]
Thailand	235delC, W24X, M34L*	Kudo et al. [43]

Table 1 (*Continued*)

Country/territory	Connexin mutation	References
Tunisia	35delG, E47X	Ben Arab et al. [44] Denoyelle et al. [9]
Turkey	35delG, W24X, 235delC, delE120, R127H, Q80R	Uyguner et al. [45]
United Kingdom	35delG, 167delT	Parker et al. [46]
United States of America	Caucasians: 35delG, 167delT, M34T*, W44C(dominant), K15T, E47X, L90P, 269insT, H100Y, 312del14, 182M, V84M^, IVS1 + 1G to A, L10P^, 31del14, R32C, P70A^ P70S^, V95M, 299-300delAT, delE120, R143W, E147K, R184Q^, V84A•, V27I + E114G, K224Q Hispanic: 35delG, V37I, R143W, N206S, Y152X^, K122I, R32C, E47X African American: 35delG, R143W Asian: V27I + E114G, 35delG, V37I, 235delC, 408insA^ Ashkenazi Jewish: 167delT, 35delG	Pandya et al. [47] Morell et al. [48]

Mutations for each country/territory are listed in order of frequency. GJB2 mutation names are listed as per Ballana et al. [6]. Mutations marked by a (*) are polymorphisms not leading to disease; mutations marked by a (•) are changes with unknown relation to disease; and mutations marked by a (∧) were not described as of December 11, 2004.

2.2. Mutation detection

DNA was extracted from whole blood using high salt (Gentra Systems Inc., Minneapolis, MN, USA) or spin column (Qiagen Inc., Valencia, CA, USA) methods. Mutation detection for GJB2 was performed by direct sequencing of the coding regions of exons 1 and 2 and the intron/exon boundaries of the gene. PCR reactions contained in a 50 μl volume 200 ng genomic DNA, 10 pmol each primer, 2.0 mM buffer (2.0 mM $MgCl_2$, 500 mM KCl, 100 mM Tris—HCl, pH 8.8), 200 μM each dNTP (dATP, dCTP, dTTP, dGTP, Amersham Pharmacia Biotech), and 1.5 U Taq polymerase (Amplitaq Gold, Roche Pharmaceuticals, Nutley, NJ, USA). PCR conditions used were initial denaturation of 95 °C 10 min; touchdown step cycle (15 cycles) 95 °C 30 s, 64.5 °C to 57 °C decreasing 0.5 °C per cycle, 72 °C 30 s; cycle (20 cycles) 95 °C 30 s, 57 °C 30 s, 72 °C 30 s; and final extension of 72 °C 2 min. PCR products were purified with a PCR purification kit (PCR Purification Kit, Qiagen) and 5 ng used in fluorescent dye terminator cycle sequencing (BigDye Terminator kit, Applied Biosystems (ABI), Foster City, CA, USA). Cycle sequencing was performed with PCR conditions of 95 °C, 2 min, cycle (25 cycles) 96 °C 10 s, 50 °C 5 s, 60 °C 4 min. Sequencing products were purified (CetriSep columns, Princeton Separations Inc., Adelphia, NJ, USA) separated on an ABI 3100 and results analyzed using GeneScan and SeqScape software (ABI). Samples were sequenced in both directions.

Individuals heterozygous for one mutation in GJB2 or in whom no mutations in GJB2 were detected were analyzed for the common 342 kb deletion D13S1830 in the GJB6 gene. A PCR assay specific for the GJB6 deletion was used. PCR reactions contained 50 pmol normal primer (AGGAATGATTTCAAAGACATAGA), which anneals within the deleted region, 20 pmol mutant primer (GAATTTGACGAGCCTTTATGTAT) which anneals outside the deleted region, and 50 pmol common reverse primer (GCCTAGAAATAAAGCAGTCAACA). The normal and mutant primer were labeled with the fluorescent dye 6-FAM for detection of PCR products on an automated sequencer. PCR reactions were performed in a 25 μl volume with 2.0 mM buffer (2.0 mM $MgCl_2$, 500 mM KCl, 100 mM Tris—HCl, pH 8.8), 200 μM each dNTP (dATP, dCTP, dTTP, dGTP, Amersham Pharmacia Biotech), and 1.5 U Taq polymerase (Amplitaq Gold, Roche). PCR conditions used were initial denaturation of 95 °C 10 min; touchdown step cycle (15 cycles) 95 °C 30 s, 64.5 °C to 57 °C decreasing 0.5 °C per cycle, 72 °C 30 s; cycle (20 cycles) 95 °C 30 s, 57 °C 30 s, 72 °C 30 s; and final extension of 72 °C 2 min. PCR products were separated on an automated sequencer (ABI 377 or ABI 3100, ABI), and analyzed using GeneScan software (ABI). Normal and mutant alleles were distinguished by size (normal, 220 bp; mutant 117 bp).

3. Results

Patients represented 14 different ethnicities: other Caucasian Canadian (43.1%), Asian (12.3%), East Indian (10.8%), Ashkenazi Jewish (6.2%), African (6.2%), Arabic (4.6%), Italian (4.6%), Caribbean (3.1%), Armenian (1.5%), Russian (1.5%), Spanish (1.5%), Greek (1.5%), German (1.5%), Polish (1.5%).

The distribution of GJB2 mutations across cochlear implant users is depicted in Fig. 1. GJB2 mutations (disease causing or of unknown signifi-

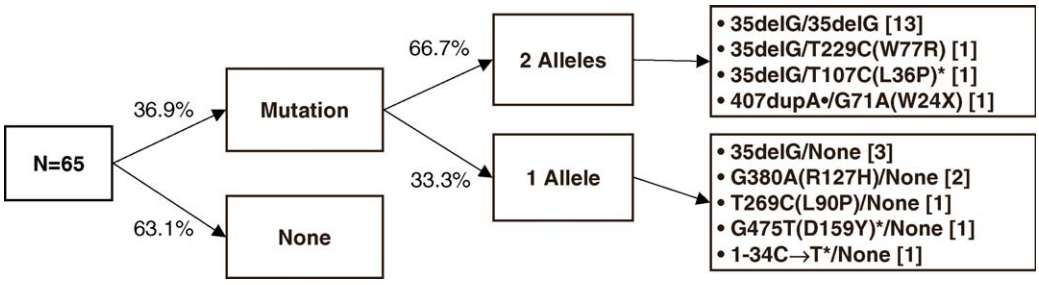

Fig. 1 Distribution of GJB2 (connexin 26) mutations across cochlear implant users. Only disease-causing mutations are listed as per Ballana et al. [6]. Mutations are listed as nucleotide change followed by protein change/mutation name in round brackets. The number of individuals with each type of mutation combination is indicated in square brackets. Changes marked by (*) are of uncertain clinical significance. Changes marked by (•) have been described previously but do not appear on the connexins and deafness homepage.

cance, excluding known polymorphisms as per Ballana et al. [6]) were found in 24 (36.9%) of paediatric cochlear implant users tested: 16 (66.7%) were biallelic and 8 (33.3%) were monoallelic. Nine different GJB2 mutations were identified: six have previously been described as disease-causing mutations and three were novel mutations which are of uncertain clinical significance.

Seventy-eight percent of all identified pathogenic GJB2 mutations were 35delG. Three novel GJB2 mutations were identified: (1) a missense mutation T107C (L36P) in an individual of African decent; (2) a missense mutation G475T (D159Y) in an individual of Caribbean decent; (3) a regulatory region change 1-34C to T in an individual of African decent. The clinical significance of these three changes is unclear. All 49 cochlear implant users with one or no GJB2 mutations tested negative for the common D13S1830 mutation in the GJB6 gene.

The percentages of each type of GJB2 mutation by country of origin are illustrated in Table 2. Individuals of African, Caribbean and East Indian decent had different GJB2 mutations than the remainder of individuals tested. Patients of Asian, Italian, Spanish, Polish and Armenian decent did not have mutations identified in GJB2 or the GJB6-D13S1830 mutation.

4. Discussion

This is the first report of the genetic profile of hearing loss due to mutations in the GJB2 gene in a multi-ethnic population. The finding that patients represented 14 different countries of origin including Italy, India, Poland, Asia and the Caribbean, demonstrates that the sample is representative of Toronto's diverse immigrant population. Unfortu-

Table 2 GJB2 mutations by ethnicity

Ethnicity	*N*	35delG/35delG	35delG/W77R	35delG/L36P*	407dupA/W24X	35delG/none	R127H/none	L90P/none	D159Y*/none	1-34C->T*/none	None/none
African	4		1	1						1	1
Arabic	3	1									2
Armenian	1										1
Ashkenazi Jewish	4	1									3
Asian	8										8
Caribbean	2				1				1		
East Indian	7	1				1	2				3
German	1	1									
Greek	1	1									
Italian	3										3
Polish	1										1
Russian	1	1									
Spanish	1										1
Other Caucasian	28	7				2		1			18

N is the total number of individuals in each ethnic group. Mutations marked by (*) are novel.

nately, Portugal, Guyana and Sri Lanka, three countries from where substantial amounts of Toronto's immigrant population derive, were not represented in the sample population studied.

The prevalence of GJB2 mutations in 36.9% of our paediatric cochlear implant users concurs with other studies in the literature [16,21,23,41]. Biallelic GJB2 mutations were found in 16 cochlear implant users, representing 66.7% of all GJB2 mutations identified, and the largest number of biallelic GJB2 mutations reported in pediatric cochlear implant users to date. The finding of nine different GJB2 mutations among 14 different ethnic groups reflects the vast diversity of Toronto's ethnic makeup. In actuality, the variety of mutations in the present study may under-represent the diversity of mutations in Toronto's community, since this study only looked at children with severe to profound hearing loss, and not individuals with mild or moderate hearing loss in whom hearing loss is often due to different, less severe connexin mutations [12]. Nevertheless, the finding of numerous different types of connexin mutations reinforces the importance of testing by direct sequencing of the entire GJB2 gene rather than only common mutation analysis. The finding that individuals of African, Caribbean and East Indian decent harboured different mutations in GJB2 than the remainder of individuals tested may reflect a common origin for such individuals or a decreased propensity for cross-cultural mating.

4.1. Previously described disease-causing mutations

Six previously described disease-causing GJB2 mutations were identified in the present study. The finding that 78% of all identified pathogenic GJB2 mutations were 35delG is not surprising, since the 35delG mutation remains the most frequent mutation in the hearing impaired population worldwide. The 35delG mutation involves the deletion of a guanine residue in a stretch of six guanines beginning at position 30 leading to a frameshift mutation [50]. In our population, the 35delG mutation was found in individuals of African, Arabic, Ashkenazi Jewish, East Indian, Greek, Russian, and other Caucasian decent. The 35delG mutation is estimated to be about 500 generations or 10,000 years old [51]. A founder effect for the 35delG mutation has been described [51], which explains the variable frequency of this mutation across different populations rather than resulting from a mutational hotspot.

The T229C (W77R) mutation involves a transition of T to C at nucleotide 229 resulting in the exchange from Tryptophan to Arginine at position 77 [52]. This mutation was first described in a Muslim Israeli-Arab village in the lower Galilee, and has also been described in individuals of Spanish decent [40]. In our sample group, the T229C (W77R) mutation was found in trans (heterozygous with one mutation on each of the two homologous chromosomes) with 35delG in an individual of African decent. The G71A (W24X) mutation involves a substitution from G to A at nucleotide 71 resulting in the exchange from Tryptophan to a premature stop codon at position 24 [22]. This mutation was first described in two consanguineous Pakistani families, and has since been described in individuals of Hungarian [21], East Indian [22], Iranian [26], Slovak Romany [39], Thai [43], and Turkish [45] decent. In our population, the G71A (W24X) mutation was found in trans with 407dupA in an individual of Caribbean decent. The G380A (R127H) mutation involves a transition of G to A at nucleotide 380 resulting in the exchange of Arginine to Histamine at position 127 [53]. While the R127H mutation has been described as both a disease-causing mutation and a polymorphism, functional analysis has demonstrated that the R127H mutation results in the formation of defective Cx26 gap junctions [53]. This mutation has been described in families of Greek [20], Hungarian [21], Kurdish Iranian [27], Slovak Romany [39], Turkish [45], Spanish and Balkan [20,54] decent. In our sample group, the G380A (R127H) was isolated from two individuals of East Indian decent. The T269C (L90P) mutation involves a transition of T to C at nucleotide 269 resulting in the exchange of Leucine to Proline at position 90 [55]. This mutation was first described in two unrelated individuals from Italy, and later in individuals of Austrian [10,11], Hungarian [21], Slovak Romany [39] and Caucasian American [47] decent. In our population, the T269C (L90P) mutation was identified in a monoallelic individual of Caucasian Canadian origin. The 407dupA is an insertion of an A nucleotide after position 407 that would predict a nonsense change of Tryptophan to a stop at codon 136 and would be considered a disease-causing mutation. This mutation was previously described as 408InsA in monozygotic twins of Asian decent [47], but does not appear in the connexins and deafness homepage [6]. In this study, the 407dupA was isolated in trans with G71A (W24X) in an individual of Caribbean decent.

4.2. Novel mutations

Three novel GJB2 mutations were identified. The T107C (L36P) is a missense mutation isolated from an individual of African decent. The T to C transition at nucleotide 107 would result in the substitution of

Leucine with Proline. Even though, in this study, the T107C (L36P) mutation was identified in trans with 35delG, its clinical significance remains to be determined. The G475T (D159Y) is a missense mutation isolated from an individual of Caribbean decent. This G to T transversion at nucleotide 475 would predict a change of aspartic acid to tyrosine at position 159. In this study, the G475T (D159Y) mutation was found on one allele with no connexin mutations on the other allele and its clinical significance remains to be determined. The 1-34C > T is a regulatory region change isolated from an individual of African decent. The C to T transition occurring 34 nucleotides upstream of the translational start site of the GJB2 gene was found on one allele with no connexin mutations on the other allele. The clinical significance of 1-34C > T remains to be determined. Neither of the GJB2 mutations identified in our study in individuals of African decent were found in a previous study of 365 unrelated individuals from Africa [19]. Population studies of individuals of Caribbean decent need to be conducted to determine the clinical significance of the G475T (D159Y) mutation.

4.3. Polymorphisms

Three additional GJB2 sequence changes were found in this patient group that have been labeled as non-disease-causing polymorphisms [6]. The clinical significance of the T101C (M34T) substitution remains unclear [56,57], with reports of the mutation in deaf and hearing individuals. In our group of children, the M34T mutation was monoallelic, isolated from one individual with severe to profound hearing loss. Other polymorphic mutations identified in our sample were the V153I and I203T, each of which was isolated in monoallelic state in an individual with severe to profound hearing loss.

4.4. GJB6 testing

Previous studies have reported a digenic cause of deafness (GJB2/GJB6) among heterozygotes ranging from 2.6% in a mixed North American population [47] to 67% in a Spanish population [7]. The absence of the common GJB6-D13S1830 mutation in our sample group may be due to the diverse ethnicities of the eight patients with monoallelic GJB2 mutations or to the small sample size of each ethnic population in the present study. A novel deletion involving the GJB6 gene (D13S1854) has recently been discovered, which may account for some of the hearing loss in this group of individuals [58].

4.5. Groups with no connexin mutations detected

The inability to detect mutations in GJB2 and the common GJB6-D13S1830 mutation in individuals of Asian, Italian, Spanish, Polish and Armenian decent may reflect the small sample size within each ethnic group and thus require testing of a larger number individuals to identify mutations, or may introduce an exciting opportunity to search for new genes that may be predisposing these individuals to hearing loss.

5. Conclusions

This discussion represents the first report of GJB2 mutations in a multi-ethnic population as compared with previously-investigated homogeneous populations, and describes the largest number of biallelic GJB2 mutations in pediatric cochlear implant users to date. The finding of numerous and diverse GJB2 mutations in this unique multi-cultural group reinforces the importance of testing for hearing loss by direct sequencing of the entire coding region rather than testing only for common mutations in the GJB2 gene.

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