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Unraveling of Enigmatic Hearing-Impaired *GJB2* Single Heterozygotes by Massive Parallel Sequencing: DFNB1 or Not?

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Abstract: The molecular etiology of nonsyndromic sensorineural hearing loss (SNHL) in subjects with only one detectable autosomal recessive *GJB2* mutation is unclear. Here, we report *GJB2* single heterozygotes with various final genetic diagnoses and suggest appropriate diagnostic strategies. A total of 160 subjects with SNHL without phenotypic markers were screened for *GJB2* mutations. Single-nucleotide variants or structural variations within the DFNB1 locus or in other deafness genes were examined by Sanger sequencing, breakpoint PCR, and targeted exome sequencing (TES) of 129 deafness genes. We identified 27 subjects with two mutations and 10 subjects with only one detectable mutation in *GJB2.* The detection rate of the single *GJB2* mutation among the 160 SNHL subjects in the present study (6.25%) was higher than 2.58% in normal hearing controls in Korean. The DFNB1 was clearly excluded as a molecular etiology in four (40%) subjects: other recessive deafness genes (N = 3) accounted for SNHL and the causative gene for the other non-DFNB1 subject (N = 1) was not identified. The etiology of additional two subjects was potentially explained by digenic etiology (N = 2) of *GJB2* with *MITF* and *GJB3*, respectively. The contribution of the single *GJB2* mutation in the four remaining subjects is unclear. Comprehensive diagnostic testing including TES is prerequisite for understanding *GJB2* single heterozygotes.

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Abbreviations: ANK1 = ankyrin 1, Cx = connexon, DFNB = nonsyndromic hearing loss and deafness, GJA1 = gap junction protein alpha 1, GJB1 = gap junction protein beta 1, GJB2 = gap junction protein beta 2, GJB3 = gap junction protein beta 3, GJB4 = gap junction protein beta 4, GJB6 = gap junction protein beta 6, GJC3 = gap junction protein gamma 3, KCNQ4 = potassium voltage-gated channel subfamily KQT member 4, MITF = microphthalmia-associated transcription factor, MYO15A = myosin XVA, OTOA = otoancorin, OTOF = otoferlin, SLC26A4 = solute carrier family 26, member 4, SNHL = sensorineural hearing loss, STRC = stereocilin, TES = targeted exome sequencing, TMC1 = transmembrane channel-like 1, USH2A = Usher syndrome type 2A, WFS1 = wolfram syndrome 1, WS2 = Waardenburg syndrome type2.

INTRODUCTION

Extreme etiologic heterogeneity exists in sensorineural hearing loss (SNHL) (http://hereditaryhearingloss.org/). Therefore, molecular genetic testing methods for efficiently screening the various deafness genes are necessary for accurately diagnosing SNHL. Various techniques, such as whole exome sequencing and targeted exome sequencing (TES), have made it possible to screen candidate genes in an extremely high-throughput manner.[1] However, making a conclusive molecular diagnosis still requires time and rigorous effort in many cases, particularly in nonsyndromic cases with rare variants. Thus, we recently suggested the importance of phenotype-driven genetic testing focusing on candidate genes according to the clinical phenotypes of the affected subjects.[2]

The predominant mutations in *gap junction protein beta 2 (GJB2)* (MIM ID: 121011) account for up to 50% of autosomal recessive nonsyndromic SNHL in some ethnicities. This significantly reduces the molecular genetic testing loads needed to reach a final genetic diagnosis through *GJB2* testing alone.[3] Although the proportion of nonsyndromic hearing loss and deafness 1 (DFNB1) among nonsyndromic sporadic or autosomal recessive severe to profound SNHL significantly varies, it still accounts for ~15% of such subjects in Koreans, making *GJB2* and *solute carrier family 26, member 4 (SLC26A4)* the most frequent causative genes of SNHL.[3–7] However, it is not always straightforward to diagnose DFNB1 in subjects carrying *GJB2* mutations. We occasionally encounter uncertain cases with a monoallelic *GJB2* mutation. It is estimated that ~6% to 20% of identified *GJB2* mutations in hearing-impaired subjects were monoallelic mutations,[8–12] although the value was reported to be up to 50% in some European populations.[13] It

has been speculated that a substantial portion of DFNB1 subjects had only one detectable mutation in *GJB2* and that an occult large deletion within the DFNB1 locus in *trans* with the detected *GJB2* mutation may cause deafness in these subjects.[13–15] However, the large genomic deletion within the DFNB1 locus has not been detected in Koreans to date, suggesting that interpretation of a monoallelic *GJB2* mutation should differ depending on ethnicity. Additionally, high carrier frequencies of *GJB2* make it difficult to regard these subjects simply as DFNB1 in Korea.[4] Moreover, some researchers have reported the digenic pathogenesis of deafness involving the detected single *GJB2* mutation in combination with mutations in other deafness genes, further complicating interpretation.[16–18]

Although numerous studies have examined the clinical implications of single heterozygous mutations in *GJB2* on SNHL, there is no consensus regarding the interpretation of the results when one mutation is identified in the *GJB2* gene of the subject. Thus, we propose the use of stepwise molecular genetic approaches to clarify the contribution of the detected monoallelic *GJB2* mutation to SNHL in Koreans based on our experiences with *GJB2* single heterozygotes.

MATERIALS AND METHODS

Ethical considerations

This study was approved by the institutional review boards (IRBs) at Seoul National University Hospital (IRBY-H-0905-041-281) and Seoul National University Bundang Hospital (IRB-B-1007-105-402). Written informed consent was obtained from all of the participating subjects. For children, written informed consent was obtained from their parents or guardians on their behalf.

Clinical Evaluation

A total of 470 subjects with SNHL were subjected to genetic testing for SNHL at the otolaryngology clinics of Seoul National University Hospital and Seoul National University Bundang Hospital from September 2010 to March 2015. All of the recruited subjects were of Korean ethnicity.

Clinical characteristics of the cohort included gender, date of birth, medical history, physical examination, pure tone audiometry, and imaging studies. All of the enrolled subjects underwent audiologic evaluation using pure-tone audiometry, speech audiometry, auditory brainstem response, and auditory state response. The pure-tone thresholds were recorded at 0.25, 0.5, 1, 2, 4, and 8 kHz. However, some infants could be recorded at only limited frequencies because of poor cooperation. The hearing threshold was calculated by averaging the thresholds of 0.5, 1, 2, and 4 kHz, and was classified as subtle (16–25 dB), mild (26–40 dB), moderate (41–70 dB), severe (71–95 dB), or profound (>95 dB).

The presence of any phenotypic markers indicating syndromic SNHL was thoroughly investigated in subjects and their family members. The phenotypic markers included ophthalmologic abnormality, such as dystopia canthorum (lateral displacement of inner canthi) in Waardernburg syndrome,[19] depigmented skin lesion, freckled face, or hypopigmentation with respect to hair color. Imaging evaluations were conducted using temporal bone computed tomography or magnetic resonance imaging to identify inner ear anomalies such as an enlarged vestibular aqueduct in subjects. All family members were also investigated for the presence of any type of hearing loss.

Based on the results of the retrieved clinical and audiologic evaluations, 160 bilateral SNHL subjects without audiologic or radiological phenotypic markers and segregating either into a sporadic or possibly autosomal recessive fashion were screened for the presence of *GJB2* mutations.

DNA Samples and Sanger Sequencing of *GJB2* Coding Region

Genomic DNA was extracted from peripheral blood using standard protocols (Gentra Puregene Blood Kit, Qiagen, Venlo, Limburg, the Netherlands). Sanger sequencing of *GJB2* was performed for subjects without noticeable phenotypic markers as described previously.[4,6] Further genetic tests were conducted to determine the definitive genetic etiology of SNHL in *GJB2* single heterozygotes and their family members as described below (Figure 1).

Examination of *GJB2* Noncoding Region

To exclude the presence of pathogenic *GJB2* mutations outside of the coding regions, Sanger sequencing for the four known pathogenic mutations, c.-22-2A>C,[20] c.-23G>T,[21] c.-23+1G>A (formerly referred to as IVS1+1G>A),[22] and c.-259C>T[23] was performed using two primer sets: GJB2-AF (5′-GGCGGGAGACAGGTGTTG-3′), GJB2-AR (5′-CCAAG-GACGTGTGTTGGTC-3′) and G259F (5′-AGCGCTCA-TAAATGCCAAGT-3′), G259R (5′-GCCGCAACACCTGTCT CC-3′).

Next, we conducted a multiplex breakpoint PCR assay for two previously reported large genomic deletions (del[GJB6-D13S1830] and del[GJB6-D13S1854]).[13] To detect other structural variations involving 5-kb regions upstream of *GJB2* and the *GJB6* region within the DFNB1 locus, we also verified the raw data of TES using Integrative Genomic Viewer (http://www.broadinstitute.org/igv/home (Figure 2).

Studies of Other Deafness Genes: TES and gap Junction Gene Studies

The presence of causative mutations in other deafness genes was investigated by targeted capture of exons and flanking sequences of 129 deafness genes, followed by massively parallel sequencing of DNA libraries as previously described.[24] Variant detection was accomplished as described.[24]

To exclude the possibility of capture failure or structural variations involving the exons of any of the 129 target genes, we also analyzed the coverage status of TES during the second phase of TES data analysis. Sanger sequencing of exons showing poor coverage (<10 ×) was performed with primer sets of *Otoferlin (OTOF), Stereocilin (STRC), and Otoancorin* (OTOA) (see Table S1, Supplemental Content, http://links.lww.com/MD/A877, which illustrates primer sequences for Sanger sequencing to screen exons of *OTOF*, *STRC*, and *OTOA* showing poor coverages by TES) so that variants that may reside in these regions could be detected (see Table S2, Supplemental Content, http://links.lww.com/MD/A877, which illustrates regions showing significantly low depth of coverage in TES: *OTOF*, *STRC*, and *OTOA*).

Several gap junction genes, including gap junction protein alpha 1 (GJA1), gap junction protein beta 1 (GJB1), GJB2, gap junction protein beta 3 (GJB3), gap junction protein beta 4 (GJB4), and gap junction protein beta 6 (GJB6), were screened either by TES or Sanger sequencing. Particularly, Sanger sequencing of Gap junction protein gamma 3 (GJC3), which was not included in our TES deafness panel, was separately

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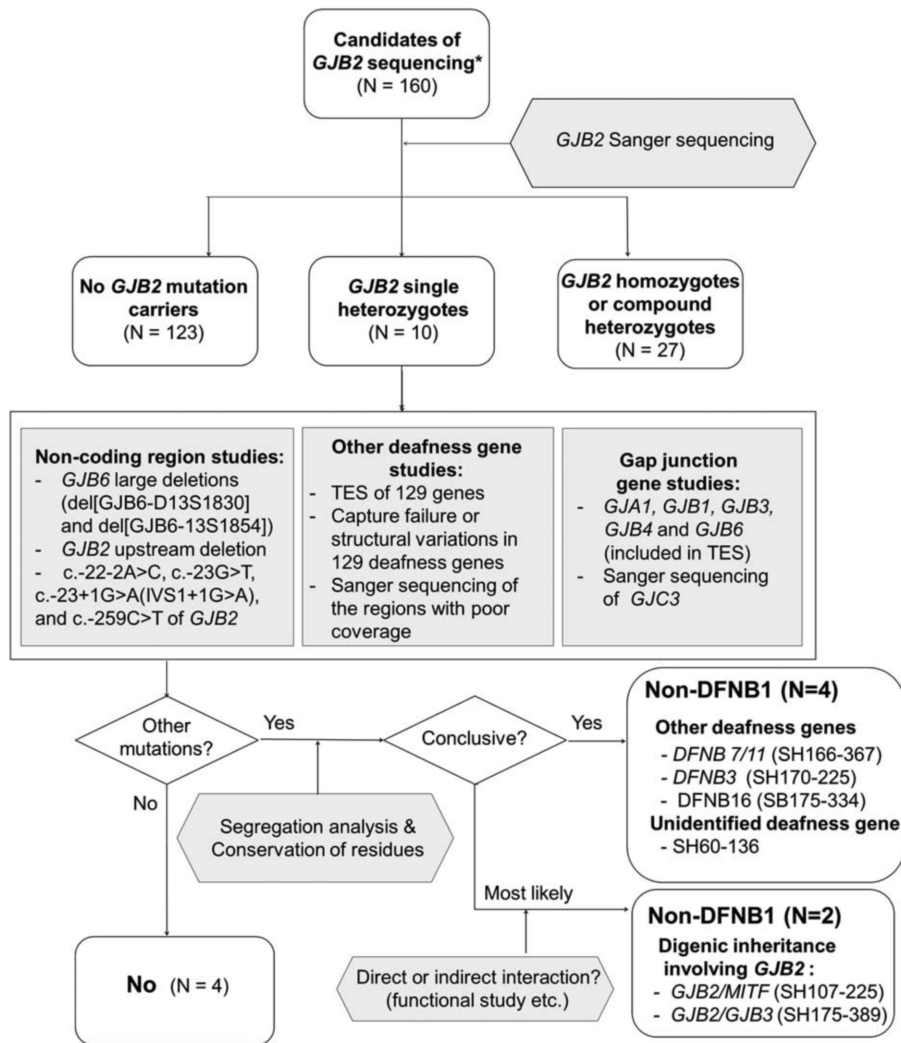

FIGURE 1. Molecular diagnostic flow of the present study: *GJB2* single heterozygotes were analyzed using a stepwise and comprehensive molecular diagnostic protocol including massive parallel sequencing of 129 known deafness genes and Sanger sequencing of additional gap junction genes as well as screening of known *GJB6* large deletions. Approximately half of *GJB2* single heterozygotes were not DFNB1. Completely different causative genes or even digenic etiology involving the *GJB2* mutation accounted for these non-DFNB1 subjects. DFNB1 = nonsyndromic hearing loss and deafness 1, GJB2 = gap junction protein beta 2, GJB6 = gap junction protein beta 6.

conducted to explore other mutations in gap junction genes.[25] The GJC3 primer sequences were as follows: GJC3-1F (5′-CCTTGGATTAGGAGTGACAAGG-3′), GJC3-1R (5′- CCC TGGGACATCTGTGTTG-3′), GJC3-2F (5′- AAGGCTG CCTGCTTCGAT-3′), GJC3-2R (5′-TCTTTAGGAAAATGGT CTTCTCA-3′), GJC3-3F (5′-CCTGGGGTTGCAGTACCAC-3′), GJC3-3R (5′- TTGTACTTCCCAGAAAGGTGA-3′), GJ C3-4F (5′-ATGGGTGGCACCTAAAGTGT-3′), and GJC3-4R (5′-GTCCCAGTTGTCGGTTATGC-3′).

Investigation of Genetic Contributions of Detected Variants and/or Mutations

Segregation analysis, evaluating variants in the Korean normal hearing control group, and evaluating the conservation of residues among species were performed for variations and/or mutations obtained in the genetic studies described earlier. The possibility of digenic inheritance of SNHL resulting from the detected monoallelic *GJB2* mutation with other deafness genes was also considered based on previous studies.[16–18]

RESULTS

Recruitment of *GJB2* Single Heterozygotes and Clinical Evaluations

Among the 160 subjects with moderate to profound SNHL without any phenotypic markers, 37 subjects (23.1%, 37/160) harbored at least one mutant allele in *GJB2*. Twenty-seven subjects were confirmed to be DFNB1 with two definitely pathogenic mutant alleles in *GJB2* either as homozygotes or compound heterozygotes. The remaining 10 subjects (27.0%, 10/37) presented monoallelic *GJB2* mutations or suspicious variants (p.T123N from SH94-208) (Tables 1 and 2). Five kinds of *GJB2* mutations including one possibly pathogenic variant (p.T123N) were detected in these subjects: p.V37I (SB 175-334), p.R143W (SH60-136 and SB201-389), p.V193E (SH118-

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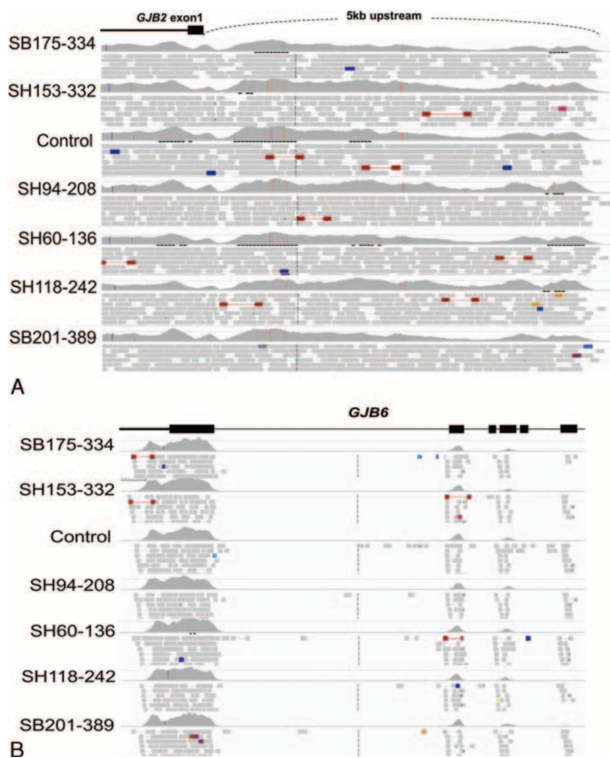

FIGURE 2. Integrative Genomic Viewer findings of noncoding upstream regions of *GJB2* (A) and *GJB6* regions (B) from six *GJB2* single heterozygotes (five group III subjects and SH60-136) with prelingual SNHL and a control with normal hearing: six *GJB2* single heterozygotes with SNHL showed no noticeable difference in terms of coverage in these regions compared with the control, indicating that no structural variations were *in trans* with the detected single heterozygous *GJB2* mutation in these regions. GJB2 = gap junction protein beta 2, GJB6 = gap junction protein beta 6, SNHL = sensorineural hearing loss.

242, SH184-416, SH170-377, and SH175-389), c.235delC (SH153-332, SH166-387, and SH107-225), and p.T123N (SH94-208).

None of the 10 single heterozygotes of *GJB2* carried pathogenic structural variations of *GJB6*/upstream regions of *GJB2* (Figure 2) or previously reported single-nucleotide variations in the noncoding regions of *GJB2* (data not shown). Next, we analyzed the TES data to identify causative point mutations of other deafness genes, if any, in these subjects.

GJB2 Single Heterozygotes where DFNB1 was Excluded as a Final Molecular Diagnosis: A Fortuitously Detected *GJB2* Mutation (Group I)

There were three subjects (SH166-367, SH170-377, and SB175-334) with two recessive mutations, presumed to be pathogenic, in completely different deafness genes. One of the children with a heterozygous c.235delC mutation (SH 166-367) was identified to carry a predominant founder mutation, p.R34X (c.100C>T) (rs121908073), and a novel variant, p.W482R of *Transmembrane channel-like 1 (TMC1)* (NM_138691), in a *trans* configuration (Table 1). The recessive mutation p.R34X was previously reported as a single founder mutation of *TMC1*.[27,28] The other novel missense variant, c.1444T>C (p.W482R), was also strongly considered pathogenic because the residue was highly conserved among various species including zebrafish and *Caenorhabditis elegans* as indicated by the high GERP score (6.02). This variant was predicted to be ''probably damaging'' by Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) based on *in silico* analyses. Furthermore, this variant was not detected among the 544 control chromosomes from normal hearing Korean subjects. Similarly, SH170-377 carrying the p.V193E mutation in *GJB2* also contained a previously reported homozygous p.Glu396Argfs*36 mutant allele in *Myosin XVA (MYO15A)* (NM_016239) (Table 1).[29]

Although no other causative deafness mutation was detected in the initial analysis of TES data, Sanger sequencing for the low coverage area (<10×) in TES (see Table S2, Supplemental Content, http://links.lww.com/MD/A877, which illustrates regions showing significantly low depth of coverage in TES: *OTOF*, *STRC*, and *OTOA*) revealed the two known pathogenic *STRC* mutations as a compound heterozygous configuration in SB175-334 (Table 1).[30] To sum up, SH166-367, SH170-377, and SB175-334 which would have been considered DFNB1 without TES were found to be DFNB7/11, DFNB3, and DFNB16, respectively.

Finally, a subject with the heterozygous p.R143W mutation in *GJB2* (SH60-136) carried a p.D771N variant in *Wolfram syndrome 1 (WFS1)* (NM_001145853) according to TES. However, neither p.R143W in *GJB2* nor p.D771N in *WFS1* was predicted to contribute to SNHL of SH60-136 based on rigorous segregation analysis of the phenotype and the variants (Figure 3). As a result, DFNB1 was excluded for SH60-136.

Single Heterozygous *GJB2* Mutant Allele Possibly Contributing to Deafness via Digenic Inheritance: Double Heterozygosity with Additional Mutation in Other Deafness Genes (Group II)

Interestingly, two subjects (SH107-225 and SH175-389) showed double heterozygosity for a *GJB2* mutation and another likely pathogenic mutation in another deafness gene. We detected a *de novo Microphthalmia-associated transcription factor (MITF)* (NM_000248) variant, p.R341C, in one of the c.235delC carriers (SH107-225) (Figure 4A). She inherited c.235delC of *GJB2* from her father and did not have any known large genomic deletions within the DFNB1 locus (Figure 4B). The p.R341 residue of *MITF* is a well-conserved sequence among species, including zebrafish and tunicates (Figure 4C). Moreover, this *MITF* variant was not detected in the 666 control chromosomes from normal hearing Korean subjects, supporting the pathogenic potential of p.R341C in *MITF* in SH107-225. However, symptoms and signs suggesting Waardenburg syndrome type2 (WS2) including retinal abnormalities and pigmentation abnormalities could not be determined due of the patients' young ages.

By screening other gap junction genes, another subject (SH175-389) carrying a single heterozygous p.V193E in *GJB2* allele harbored a single heterozygous p.A194T mutant allele of *GJB3* (NM_001005752) (SH175-389) with known pathogenicity (Figure 4D).[31] This 2-year-old female showed severe autosomal recessive SNHL with a mean hearing threshold of 87.5 dB HL.

Single Heterozygous *GJB2* Mutant Allele with Unknown Contribution to SNHL in Our Cohort (Group III)

A 39-year-old female subject (SH94-208) showed the p.T123N variant of *GJB2*. The pathogenic potential of the p.T123N variant is controversial. Three variants of *USH2A*

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TABLE 1. *GJB2* Single Heterozygotes where DFNB1 was Excluded as a Final Molecular Diagnosis (Groups I and II)

Subjects	Sex	Age (year)	Onset of Hearing Loss	Average Hearing Thresholds (dB)	*GJB2* Mutation	Other Mutations	Final Diagnosis
Group I							
SH166-367	F	1.7	Prelingual	100	c.235delC	p.R34X/p.W482R (*de novo*) of *TMC1*	DFNB7/11
SH170-377	F	2.7	Prelingual	118.3	p.V193E	p.Glu396Argfs*36/p.Glu396Argfs*36 of MYO15A	DFNB3
SB175-334	F	7	Prelingual	45	p.V37I	c.2303delGp.Lys769Serfs*15/c.5125A>G, p.T1709A of STRC	DFNB16
SH60-136	M	13	Prelingual	93.8	p.R143W	p.D771N of *WFS1*	*No causative deafness mutation*
Group II							
SH107-225	F	5	Prelingual	81.7	c.235delC	p.R341C (*de novo*) of *MITF*	*MITF/GJB2*
SH175-389	F	2.8	Prelingual	87.5	p.V193E	p.A194T of *GJB3*	*GJB2/GJB3*

DFNB = nonsyndromic hearing loss and deafness, F = female, GJB2 = gap junction protein beta 2, GJB3 = gap junction protein beta 3, M = male, MITF = microphthalmia-associated transcription factor, MYO15A = myosin XVA, STRC = stereocilin, TMC1 = transmembrane channel-like 1, WFS1 = wolfram syndrome 1.
*Average of 500, 1000, 2000, and 4000 Hz.

TABLE 2. GJB2 Single Heterozygotes Where DFNB1 Could Not be Excluded (Group III)

Subjects	Sex	Age (year)	Onset of Hearing Loss	Average Hearing Thresholds* (dB)	*GJB2* Mutation	Other Mutations or Variants (TES, GJC3)	Carrier Rate (%) (allele frequency)	References
GJB2 subjects with a clearly pathogenic heterozygous *GJB2* mutation								
SB201-389	M	10	Prelingual	41.25	p.R143W	None	0.02% (1/2072) About 1% (In African)	Han et al 2008 Meyer et al 2002
SH118-242	M	2.7	Prelingual	63.3	p.V193E	None	N/A	
SH153-332	F	4	Prelingual	87.5	c.235delC	None	0.44% (11/1256) 1.25% (26/2072)	Kim et al 2011 Han et al 2008
Subject with a *GJB2* variant with a controversial pathogenicity								
SH94-208	F	39	Prelingual	75	p.T123N†	R5143C, C4870F and G805A of *USH2A* G1748S *of ANK1*	0.12% (5/2072) 0.09% (1/1176) (In China)	Han et al 2008 Tang et al 2006

DFNB1 = nonsyndromic hearing loss and deafness 1, F = female, GJB2 = gap junction protein beta 2, M = male.
*Average of 500, 1000, 2000, and 4000 Hz.
†*GJB2* variant with unknown pathogenic potential.

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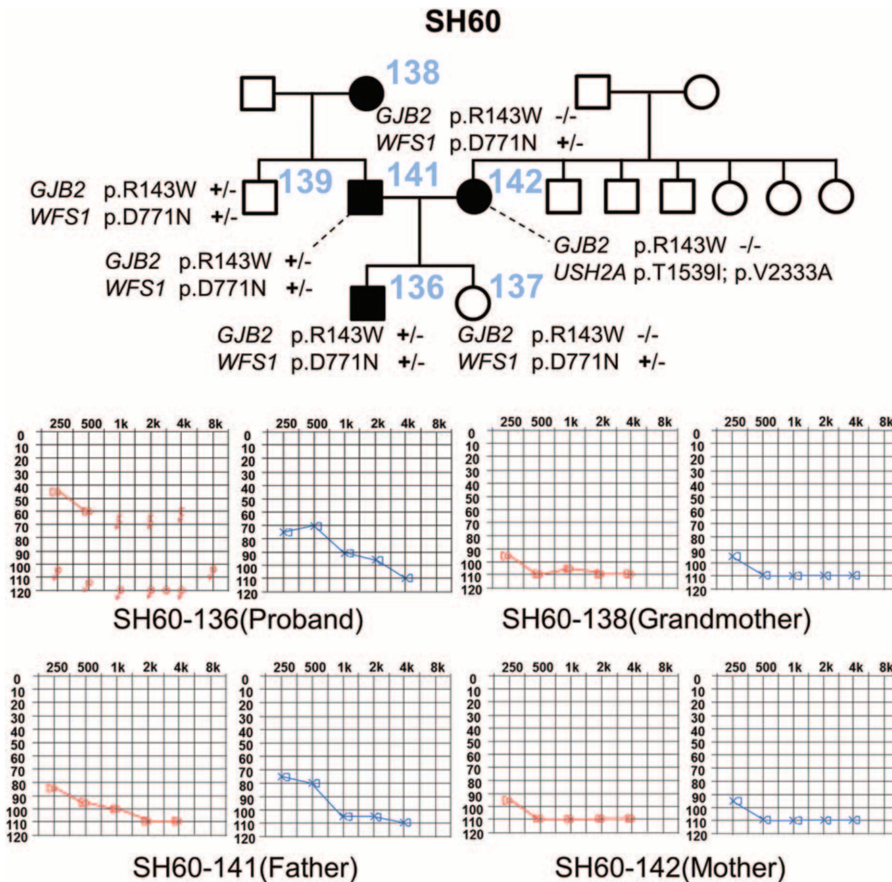

FIGURE 3. Pedigree and audiograms of SH60 and segregation of variations of *GJB2* and *WFS1* in this family: two subjects with SNHL, SH60-138 and SH60-142, showed a discrepancy in the *GJB2* genotype. Two unaffected subjects, SH60-137 and SH60-139, also carried p.D771N in *WFS1*. This indicates that neither p.R143W in *GJB2* nor p.D771N in *WFS1* contributed to SNHL in SH60-136 and that p.R143W in *GJB2* was an incidentally detected variant in this subject. GJB2 = gap junction protein beta 2, SNHL = sensorineural hearing loss, WFS1 = wolfram syndrome 1.

(NM_007123), *R5143C*, *C4870F*, and *G805A* with unknown pathogenic potential were identified using TES (see Table S3, Supplemental Content, http://links.lww.com/MD/A877, which illustrates variants or mutations of *Usher syndrome type 2A (USH2A)* and *Ankyrin 1 (ANK1)* identified in SH 94-208). However, this subject showed no retinal abnormalities and only manifested severe SNHL with a mean hearing threshold of 75 dB HL, which was not compatible with type II Usher syndrome. Therefore, these variants of *USH2A* were excluded as causative deafness mutations. SH94-208 also showed the G1748S variant of *ANK1* (NM_000037). Structural variations such as large genomic deletions involving *ANK1* at chromosome 8p11.2p12 can lead to contiguous syndrome, with SNHL as one of the symptoms.[32] However, the G1748S variant of *ANK1* was a point mutation, not a structural variation (see Table S4, Supplemental Content, http://links.lww.com/MD/A877, which illustrates depth of coverage of TES). Moreover, SH94-208 showed no phenotypic markers for syndromic SNHL, such as craniometaphyseal dysplasia. Thus, this *ANK1* variant was not likely to be a causative mutation. The remaining three subjects showed no other convincingly pathogenic mutations other than the detected *GJB2* mutation (Table 2).

Detection Rates of Single Heterozygous *GJB2* Mutation in our SNHL Cohort and a Normal Hearing Control Cohort From the Literature

Based on previously published Korean *GJB2* normal carrier rates, we compared the rates of *GJB2* single heterozygotes in the hearing-impaired cohort with that of normal hearing controls.[4,26] Total rates (5.63% [9/160]) of *GJB2* single heterozygotes (N = 9 from group I + II + III excluding a *GJB2* variant with controversial pathogenicity [SH94-208]) among all *GJB2*-sequenced hearing-impaired subjects (N = 160) was significantly higher than that (2.58%) of normal controls ($P = 0.03$). When we excluded two cases (Group II) with the *GJB2* mutation contributing to SNHL possibly through digenic inheritance, we still observed a slightly higher rate of *GJB2* single heterozygotes (Group I + III) than in the normal controls (4.38% vs. 2.58%, $P = 0.20$), although the difference was not statistically significant.

Analysis of Missing Regions of TES Data From *GJB2* Single Heterozygotes with Unknown Molecular Etiology

The average depth of coverage for these six subjects, including group III and SH60-136, were 225.73 (see Table S4,

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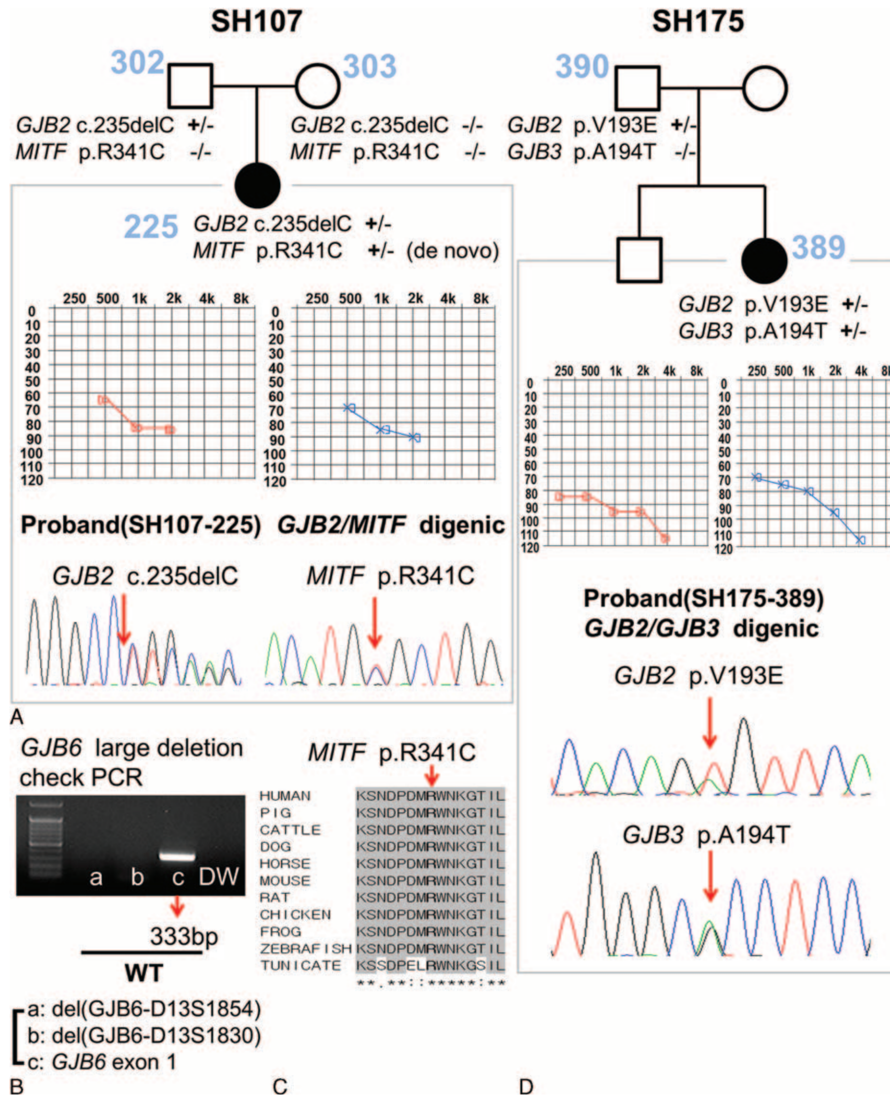

FIGURE 4. Digenic inheritances of *GJB2*/*MITF* and *GJB2*/*GJB3* (group II). (A) In addition to c.235delC in *GJB2*, the *de novo* variant of *MITF*, p.R341C was identified in SH107-225. (B) There was no *GJB6* large deletion within the DFNB1 locus. (C) The sequence of the p.R341C variant is well-conserved from humans to tunicates. (D) SH175-389 harbored a monoallelic p.V193E variant of *GJB2 and a monoallelic p.A194T variant of GJB3.* DFNB1 = nonsyndromic hearing loss and deafness 1, GJB2 = gap junction protein beta 2, GJB3 = gap junction protein beta 3, GJB6 = gap junction protein beta 6, MITF = microphthalmia-associated transcription factor.

Supplemental Content, http://links.lww.com/MD/A877, which illustrates depth of coverage of TES), with more than 97.09% of the target bases covered >20×. Among the 1737 regions that were targeted in this study, 2.17% were covered <20×, including several regions of *OTOF* (NM_001287489), *STRC* (NM_153700), and *OTOA* (NM_144672) (see Table S2, Supplemental Content, http://links.lww.com/MD/A877, which illustrates regions showing significantly low depth of coverage in TES: *OTOF*, *STRC*, and *OTOA*). We confirmed that the 48th exon of *OTOF* (NM_001287489) showed very high GC content and that *STRC* and *OTOA* had pseudogenes (*STRCP1* and *LOC6537686*, respectively), which generated missing regions of the TES data. Sanger sequencing of these exons with significant poor coverage by TES revealed two compound heterozygous *STRC* mutations in SB175-334 (Table 1). However, no pathogenic variants was detected in the remaining five subjects (SH 153-332, SB201-389, SH94-208, SH60-136, and SH118-242) (data not shown).

DISCUSSION

In the first phase analysis of TES data, we were able to exclude DFNB1 from 4 subjects (SH166-367, SH170-377, SH60-136, and SH175-389) and possibly from 1 subject (SH107-225) among a total of 10 single heterozygotes of *GJB2* in a Korean SNHL population. The second phase analysis of TES data which double-checked the exons with poor coverage in *OTOA*, *STRC*, and *OTOF* by Sanger sequencing enable us to reveal DFNB16 from one subject (SB175-334). Detection of the *GJB2* mutation in four subjects (SH166-367, SH170-377, SB175-334, and SH60-136) (Group I) was found to be incidental, independently of the phenotype. This is expected based on the similarity of the incidence

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rates of detection of c.235delC of *GJB2* between the one (0.63% (1 [SH166-367]/160 [our total SNHL cohort]) calculated from group I and reported figures from normal hearing Korean subjects (0.44%–1.25%) (Tables 1 and 2).[4,27]

Our first-line screening of known large genomic deletions involving *GJB6* or upstream regions of *GJB2* yielded no convincing structural variations in our population, which was in sharp contrast with previous reports of a Spanish population.[33] In the Spanish population, 66% of the affected subjects with a monoallelic *GJB2* mutation carried the del (GJB6-D13S1830) in *GJB6*, which was also frequently observed in subjects from France and Israel.[33] A completely different molecular etiology was clearly observed or strongly suggested in four of the five subjects in groups I and II, except SH60-136 in the present study, indicating that TES covered the known prevalent deafness genes, including gap junction genes and genes known to cause syndromic deafness such as Usher syndrome and Waardenburg syndrome for the *GJB2* single heterozygotes in Korean SNHL subjects.

For the most common *GJB2* variant in Koreans, c.235delC, suspicion of other molecular etiologies should be examined, particularly when detected as a single heterozygous state from a child with no residual hearing in Koreans, such as for SH166-367. c.235delC was recently reported to manifest a dynamic range of SNHL and a slightly milder audiologic phenotype compared with other *GJB2* variants in Koreans.[34] Detection of mutations in *MYO15A* and *TMC1* in group I are relatively common in East Asian populations, including Koreans,[2,35–38] indicating that application of panel sequencing covering the genes prioritized based on the ethnicity-specific prevalence would be effective for identifying *GJB2* single heterozygotes with severe to profound SNHL in Koreans.

For the family SH60 with a most likely genetic etiology but without a clear result after TES, whole exome sequencing can be used for definitive molecular diagnosis. This family SH60 segregates prelingual or perilingual severe to profound SNHL, likely in an autosomal dominant fashion, although prelingual SNHL of SH60-136 was caused by autosomal recessive mutations in other deafness genes (Figure 3). Further segregation analyses of the two variants (p.R143W and p.D771N) among the six family members of SH60 as well as clinical evaluations including audiograms excluded both p.R143W of *GJB2* and p.D771N of *WFS1* as a molecular etiology of SH60-136. Specifically, SH60-138 (grandmother of SH60-136) complained of perilingual deafness but did not carry p.R143W of *GJB2*, eliminating the contribution of this allele to prelingual profound SNHL if it segregated dominantly from SH60-138 to SH60-136 (Figure 3). Alternatively, SNHL of SH60-136 may result from autosomal recessive mutations in the same gene with SH60-142 (mother of SH60-136) if SNHL of SH60-142 was because of autosomal recessive mutations in a certain deafness gene other than *GJB2* and if the father (SH60-141) is a carrier of a mutation in the gene. In any case, p.R143W in *GJB2* does not contribute to the SNHL of SH60-136 (Figure 3). Therefore, a completely different deafness gene yet to be identified would account for the SNHL of SH60-136, warranting whole exome sequencing in this subject.

The p.V193E variant in *GJB2* occurring in complex heterozygosity with a pathogenic *GJB3* variant, p.A194T from SH175-389, suggests a possible digenic etiology of SNHL involving two different gap junction proteins, Cx26 and Cx31. Large deletions in *GJB6* (del [GJB6-D13S1830] and del [GJB6-D13S1854]) are frequently detected in a *trans* configuration with a monoallelic *GJB2* mutation in certain populations.[33,35,39] Based on these findings, it was previously hypothesized that variations in *GJB2* and *GJB6 in trans* can cause SNHL through digenic inheritance.[33,40] However, subsequent studies revealed that *GJB6* deletions result in an allele-specific lack of GJB2 mRNA expression, contributing to SNHL in a manner not resulting from digenic inheritance.[40] Nevertheless, a digenic etiology involving mutations in several gap junction genes has been proposed.[18,41] Structurally, the gap junction is composed of two connexons (Cxs), which are formed by the oligomerization of six Cx subunits. Several Cx genes, including Cx26, Cx29, Cx30, Cx31, Cx32, Cx30.3, and Cx43, can induce SNHL.[42] These Cx gene families are known to be able to form heteromeric gap junction assemblies.[43] In this context, digenic inheritance of SNHL involving *GJB2* (Cx26) and *GJB3* (Cx31) has been strongly supported by functional studies conducted by Liu et al (2009), which demonstrated a direct physical interaction between Cx26 and Cx31, the presence of heteromeric Cx26/Cx31 connexons, and finally co-assembly of two transfected proteins in the same junction plaques *in vitro*.[31] In their study, two different *GJB3* mutations (p.N166S and p.A194T) were identified in three unrelated families among 108 Chinese families with a single pathogenic *GJB2* mutation.[31] This strongly corroborated a possible digenic etiology of SNHL involving *GJB2* and *GJB3* in SH175-389. Thus, we excluded DFNB1 as a molecular etiology of SNHL from SH175-389.

Although no direct physical interaction occurs in the heterotypic Cx formation, two different genes can functionally interact with each other, which may result in functional deficit if these interactions are disrupted. *MITF* is an important gene in the development and regulation of melanocytes and is expressed in melanoblast-derived intermediate cells of the stria vascularis.[25] Interestingly, *MITF* also regulates potassium ion circulation of endolymphatics in the inner ear.[44,45] Although the signaling networks between *GJB2* and *MITF* remain unclear, their common final pathway in regulating potassium ion circulation in the inner ear can be significantly disrupted by the digenic effect of *MITF* and *GJB2* mutations. A subject with Waardenburg syndrome type II (WS2) in a large Chinese population had both *MITF* and *GJB2* mutations in a compound heterozygous state.[17] The profound SNHL in the subject may have been caused by the digenic effect of *GJB2* and *MITF* mutations, although the WS2 phenotype was caused by the *MITF* mutation.[17] In our studied family, SH107-225 with profound SNHL carried c.235delC in *GJB2* and a *de novo* variant, p.R341C in *MITF*. DFNB1 as a molecular etiology was excluded from this subject, while digenic inheritance of SNHL can be proposed for this subject because the pathogenic potential of p.R341C was strongly supported by significant conservation of the p.R341 residue among various species and by the absence of this variant among the 666 control chromosomes from normal hearing control subjects. Based on the varying degrees of audiologic phenotypes of *MITF*-related WS2, including single side deafness,[37] bilateral profound SNHL of SH107-225 may have resulted from the additive effect of single heterozygous c.235delC of *GJB2*, and the *MITF* mutation through digenic inheritance. Thus, panel sequencing should be conducted to examine syndromic deafness genes as well as gap junction genes for definitive genetic diagnosis of *GJB2* single heterozygotes in seemingly nonsyndromic SNHL in Koreans. However, final documentation of digenic inheritance of deafness in our two subjects (SH175-389 and SH107-225) warrants a rigorous functional study.

Several exons in *OTOF*, *STRC*, and *OTOA* were not fully covered (see Table S2, Supplemental Content, http://links.lww.com/

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MD/A877, which illustrates regions showing significantly low depth of coverage in TES: *OTOF, STRC, and OTOA*) by our TES data. We tried to minimize the possibility that pathogenic mutations reside in these regions by performing Sanger sequencing of all exons showing poor coverage (<10 × coverage). Considering that *STRC*-related SNHL (DFNB16) is generally presented as mild to moderate SNHL,[30] two moderate SNHL subjects (SB175-334 and SB201-389) in our cohort were initially hypothesized to have either occult structural variations or point mutations in the poorly covered regions of *STRC*. In accordance with our assumption, one moderate SNHL subject (SB175-334) with a single heterozygous p.V37I turned out to carry two mutant alleles of *STRC*. However, it is still possible that the remaining moderate SNHL subject (SB201-389) has pathogenic structural variations in the *STRC* gene. Copy number variations of the *STRC* gene were reported to be common among deafness genes.[46] Designing probes with different or multiple tiling on the probes may improve the capture efficiency; arrayCGH may be another option if a subject is strongly suspected to have STRC-related structural variations.

The contribution of the detected monoallelic *GJB2* mutations in four subjects in group III is unclear in the present study. The rate of *GJB2* single heterozygotes in our hearing-impaired cohort was significantly higher than that in normal hearing controls, suggesting that a substantial portion of the detected monoallelic *GJB2* mutations contributes to SNHL. Moreover, the detected *GJB2* mutation from five subjects in group I were not found to contribute to their SNHL, and thus the slightly higher rate of *GJB2* single heterozygotes in groups I + III compared with that of controls (4.38% vs 2.58%) suggests that at least some of the monoallelic *GJB2* mutations in group III contribute to SNHL.

Genetic approaches such as targeted resequencing of whole noncoding regions of *GJB2* in group III may reveal occult mutations in these regions. RNA-seq or microarray mainly focusing on gap junction genes using patient-derived lymphoblastoid cell lines may detect low or absent expression of gap junction genes either because of novel occult large deletions in these genes or disruption of regulatory elements by occult mutations in some *GJB2* single heterozygotes in group III.

At least 40% of *GJB2* single heterozygotes in Korean SNHL subjects appear to be nonDFNB1. Digenic etiology involving the *GJB2* mutation may increase the proportion. TES to cover prevalent autosomal recessive deafness genes should be employed to elucidate these non-DFNB1 *GJB2* single heterozygotes in Koreans. Similarly, a recent study also showed that ∼33.3% (4/12) of the 12 Han Chinese subjects with the monoallelic *GJB2* mutations were identified as the non-DFNB1; 2 patients with *MYO15A* mutations, 1 patient with *Potassium voltage-gated channel subfamily KQT member 4 (KCNQ4)* mutation, and 1 patient with *SLC26A4* mutations (under minor revision from ''Medicine'' by personal communications). Therefore, our results can be probably extrapolated to at least whole East Asian populations and our diagnostic approaches for the *GJB2* single heterozygotes can be applied to broader populations beyond Koreans. Because there have been only a small number of study subjects of *GJB2* single heterozygotes thus far, further studies with various ethnic groups and more number of subjects are warranted to generalize our diagnostic strategies. Our stepwise molecular genetic approaches presented in the present study serve as an example for understanding single heterozygotes of other autosomal recessive genes as well as of *GJB2*.

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