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유전성 난청 분야에서 난청 유전자를 이용한 표적 리시퀀싱 방식의 효용성 및 적용

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The efficacy of Targeted resequencing

of deafness genes and its application in the field of hereditary deafness

by

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Abstract

The efficacy of Targeted resequencing of deafness genes and its application in the field of hereditary deafness

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Sensorineural hearing loss (SNHL) is the most common congenital sensorineural disorder affecting 1 of 500 live births, and at least 50% of congenital hearing impairment has a genetic origin. As most genetic hearing loss has a monogenic Mendelian etiology, molecular genetic testing is useful for identifying individuals with hereditary SNHL.

Owing to its easy applicability, the phenotype-driven candidate gene approach has been used widely, especially in molecular diagnosis of deafness in small to mid-sized families with no available linkage data. However,

phenocopies and variable phenotypes can result from changes in a same deafness gene, depending upon the domain/location of the change. In addition, phenotyping may not always be comprehensive and robust in the clinic, making the phenotype-driven candidate gene approach unsuccessful in many cases.

The recent advent of next-generation sequencing (NGS) has rapidly expanded the possibilities of massive genomic analyses, and we established targeted resequencing of 80 deafness genes (TRS-80) to identify candidate gene in SNHL cases without phenotypic markers.

In this study, we performed TRS-80 in cases that showed negative results after candidate gene screening despite the presence of a characteristic phenotypic marker to demonstrate the efficacy and advantages of TRS (**Part 1**). Then we applied TRS-129 or 200 to identify the genotype-phenotype correlation in *COCH* gene related hearing loss (DFNA9) (**Part 2**) and reveal the significant role of *CDH23* to progressive postlingual-onset SNHL in Koreans using a genetic epidemiologic tool (**Part 3**).

Among six probands without detection of a causative variant through a phenotype-driven candidate gene approach, TRS-80 revealed a convincing causative gene in the three probands (SB82-147, SB86-154, and SB128-220),

yielding a 50% molecular diagnosis solve rate in cases that remained undiagnosed after auditory phenotype-driven candidate gene screening, leaving another 50% in need of whole exome sequencing (WES) (**Part 1**). TRS-129 revealed the overall frequency of DFNA9 among autosomal dominant multiplex hearing loss families as 5/39 (12.8 %) in our Korean cohorts. Also, distinct vestibular phenotypes depending on the location of *COCH* mutations were demonstrated, correlating a genotype of p.G38D in *COCH* to the phenotype of bilateral total vestibular loss (**Part 2**). Among 32 Korean adult probands with postlingual non-syndromic SNHL sporadically or in autosomal recessive fashion, the allele frequency of these *CDH23* variants in our postlingual cohort was 12.5%, which was significantly higher than that of the 2040 control chromosomes (5.53%), confirming the contribution of these rare *CDH23* variants to postlingual non-syndromic SNHL (**Part 3**).

Taken together, we demonstrated the advantages of TRS, which had been previously proposed as the strategic pipeline to make a molecular genetic diagnosis of deafness, over the phenotype-driven candidate gene screening. Using TRS, we showed the distinct vestibular phenotypes of DFNA9 depending on the location of *COCH* mutations, and we also demonstrated an important contribution of *CDH23* mutations to poslingual-onset non-

syndromic SNHL, widening phenotypic spectrum of DFNB12. With the rapid development of diagnostic tools for hereditary deafness, therapeutic approaches based on stem cell and gene therapy will be mainstream treatments for the genetic hearing loss.

Key words: Hereditary deafness, Targeted resequencing, *COCH*, *CDH23*

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Introduction

Sensorineural hearing loss (SNHL) is a relatively common congenital sensorineural disorder affecting 1 of 500 live births (1). Profound SNHL mandating early auditory rehabilitation occurs in 4 of 10,000 live births, underscoring the importance of timely detection and etiologic diagnosis. While environmental and multi-factorial features can cause congenital hearing impairment, at least 50% of congenital hearing impairment has a genetic origin (2). As most genetic hearing loss has a monogenic Mendelian etiology, molecular genetic testing is useful for identifying individuals with hereditary SNHL. However, making a convincing diagnosis is not always straightforward due to the high etiologic heterogeneity in terms of the number of causative genes (http://hereditaryhearingloss.org/).

Owing to its easy applicability, the phenotype-driven candidate gene approach has been used widely, especially in molecular diagnosis of deafness in small to mid-sized families with no available linkage data. Using this approach, candidate genes are chosen based on prior knowledge of the gene's biological functional impact and expressed phenotype on the trait or disease. This approach is effective especially for identifying the gene responsible for the fully penetrant and characteristic audiological or radiological phenotype. Indeed, in previous studies, a subset of characteristic inner ear anomaly cases,

such as those with an enlarged vestibular aqueduct (EVA) or incomplete partition type III, showed etiologic homogeneity in a Korean population, thereby serving as an ideal candidate for this approach (3, 4). Several deafness genes with characteristic audiogram configurations have been reported (5-13). However, phenocopies and variable phenotypes can result from changes in a same deafness gene, depending upon the domain/location of the change (14). In addition, phenotyping may not always be comprehensive and robust in the clinic. Correspondingly, this approach in a previous study identified only 40% of the hereditary deaf cases (multiplex cases) in a Korean population (15).

The recent advent of next-generation sequencing (NGS) has rapidly expanded the possibilities of massive genomic analyses that were considered impossible in the past, and has reduced the analysis time and cost of DNA sequencing by over two orders of magnitude (16). Previously, we established targeted resequencing of 80 deafness genes (TRS-80) in SNHL cases without phenotypic markers (15).

Alterations in the *COCH* gene cause autosomal dominant-type hearing loss (DFNA9) and often also vestibular symptoms (17, 18). Recently, a genotype–phenotype correlation was proposed in DFNA9, i.e., that individuals with von Willebrand factor A (vWFA) domain mutations

predominantly exhibit hearing loss, while individuals with Limulus factor C, cochlin, and late gestation lung protein, Lgl1 (LCCL) domain mutations have hearing loss accompanied by vestibular dysfunction (19). Although many *COCH* mutations carriers present self-reported Me´nie`re like symptoms (20), any association with this condition has never been confirmed (21, 22). To date, 21 mutations of the *COCH* gene have been reported (15, 17, 21, 23-36); thus, the *COCH* gene has been determined to be important in terms of an association with hereditary audiovestibular dysfunction.

CDH23 related hearing loss in type 1D Usher Syndrome (USH1D) and non-syndromic hearing loss (DFNB12) has mostly been associated with either congenital or prelingual-onset hearing loss (37). However, some *CDH23* mutations have been reported to be associated with postlingual-onset moderate hearing loss in humans (38, 39). Furthermore, some *Cdh*23 mutant alleles in mice manifested age-related hearing loss, which started as high-frequency hearing loss that eventually progressed to profound impairment with varying degrees of rapidity, as explained by the allelism and modifier gene (40-42). Moreover, *Cdh23* was also found to be susceptible to noise induced hearing loss, which is a different type of SNHL (43, 44). However, the contribution of *CDH23* to the human postlingual-onset hearing loss has not been adequately investigated. Moreover, mechanisms responsible for

different phenotypes of *CDH23* mutations have not been fully elucidated to date.

In this study, we performed TRS-80 in cases that showed negative results after candidate gene screening despite the presence of a characteristic phenotypic marker to demonstrate the efficacy and advantages of TRS (**Part 1**). Then we applied TRS-129 or 200 to identify the genotype-phenotype correlation in *COCH* gene related hearing loss (DFNA9) (**Part 2**) and reveal the significant role of *CDH23* to progressive postlingual-onset SNHL in Koreans (**Part 3**). By addressing these issues, we tried to show the sequentially widening application of TRS in the field of hereditary deafness.

Materials and Methods

Ethical consideration

This study was approved by the Institutional Review Boards at the 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 study participants. In the case of minors, written informed consent was obtained from the parents or guardians.

Audiovestibular Assessment

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).

For the evaluation of vestibular function, results of bithermal caloric test and rotary chair test were analyzed. Bithermal caloric test was composed of alternating stimuli of irrigation for 25 s with 50 ml of cold and hot water (30 and 44-C). Nystagmus was recorded binocularly by videooculography (Bithermal Calroric VNG system; SLMED, Seoul, Korea). Rotatory Chair Test was performed in a dark chamber using a rotatory chair system (System 2000,
Micromedical technologies (MM), Chatham, IL, USA). In addition, electrocochleography (ECOG) was done to aid in the diagnosis of Meniere's disease by recording electrical potentials generated in the inner ear in response to sound stimulation, using an electrode placed in the ear canal (Navigator-Pro system; Bio-logic systems Corp., San
Carlos, CA).

Molecular genetic diagnosis of deafness

Basically, TRS was performed followed by basic filtering steps (**Fig. 1-1**) (15). Detailed filtering steps differed from each study. In general, we selected rare single nucleotide variations (SNV) or indels and we also checked the splice-site sequences, and 5' UTR promoter sites. Then five steps of filtering were followed: basic filtering such as 1) excluding synonymous SNVs and selecting SNVs whose quality score were more than 20 and/ or read depth were more than 20, 2) compatibility with inheritance pattern, 3) confirmation of presence of mutations by Sanger sequencing, 4) control study, and 5) compatibility with clinical features. After filtering steps, we performed an in silico study to predict the pathogenicity of the missense variants using SIFT (http://www.fruitfly.org/seq_tools/splice.html) and Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/). For an estimation of the evolutionary conservation of the amino acid sequence, we referred to the GERP++ score in the UCSC Genome Browser (http://genome.ucsc.edu/).

Statistical analysis

Statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, USA). The level of statistical significance was defined as a p value of <0.05.

Part 1

We recruited six hearing-impaired probands with seemingly non-syndromic features and their family members from six families. They visited ENT clinic for their hearing problems and they were examined thoroughly by an experienced otolaryngologist who was trained in medical genetics. Their entire body and developmental and medical history were also thoroughly evaluated and syndromic features were not detected. This cohort was composed of probands that had been analyzed using an auditory phenotype-driven candidate approach but without detection of a causative variant. All probands showed a characteristic audiogram configuration and one proband had a subtle radiologic abnormality of the inner ear in addition to asymmetrical hearing loss between two ears. Therefore, we applied a sophisticated phenotype-driven candidate approach (**Table 1-1 and Fig. 1-2**) as a first screening. This approach involved direct polymerase chain reaction (PCR)-based Sanger sequencing of *TECTA*, *COL11A2*, *WFS1*, *TMPRSS3*, the p.V37 residue of *GJB2*, and *SLC26A4* corresponding to characteristic phenotypes (4, 8, 10, 11, 45, 46). In detail, for mid-frequency HL in SB82-147 and SB92-176, *TECTA* and *COL11A2* were screened, and for low-frequency HL in SB86-154, *WFS1* sequencing was performed first. For high-

frequency downsloping HL from SB128-220 and SB87-155, *TMPRSS3* or *GJB2* p.V37I was first sequenced according to the onset of hearing loss. For asymmetrical hearing loss between two ears associated with subtle, albeit not definite, enlarged vestibular aqueduct, *SLC26A4* was sequenced (47). As no potential causative variant was detected using the auditory phenotype-based screening in the six probands in this study, we applied TRS-80 and filtered the variants as described previously (15).

Part 2.

We intended to review the vestibular phenotype of our DFNA9 cohort recruited from two tertiary referral centers. Molecular genetic diagnosis of two DFNA9 families (SH-14 and SB-82) was performed previously. The family SH-14 segregated p.G38D of the *COCH* gene and the other family, SB-82, harbored a known pathogenic mutation of p.C162Y (15, 25, 48). To recruit further DFNA9 families, we applied targeted resequencing of 129 known deafness genes (TRS-129) to 22 multiplex Korean families who were segregating bilateral sensorineural hearing loss (SNHL) with varying degrees of hearing loss in an autosomal dominant fashion, and filtered the variants as described previously (15).

TRS-129 included 129 known deafness genes (**Supplemental Table 2-1**). TRS-129 with the extracted gDNA samples of patients was performed by Otogenetics (Norcross, GA, USA). Through the bioinformatic analysis, each raw data was mapped onto the UCSC hg19 reference genome.

Analysis of audiovestibular features of COCH mutations

After reviewing the medical records of the probands and family members, which included history-taking, audiometric data, and caloric and rotary chair test data for the evaluation of vestibular function, they were analyzed according to the pathogenic variant, and possible genotype–phenotype correlations were investigated. Specifically, we tried to delineate the correlation between the vestibular phenotype and the locations of *COCH* variants. Bilateral vestibulopathy was defined by summated slow phase velocity (SPV) of the nystagmus of less than 20 per second during four stimulation conditions (49).

Part 3

Patients with postlingual adult-onset SNHL, segregated in either a sporadic or AR fashion, were selected from our Korean cohorts, and

published data from a previous study on pediatric cohorts were retrieved for analysis (50). Additionally, our adult cohort fulfilled the following criteria: 1) bilateral non-syndromic hearing loss, 2) moderate hearing loss with progressive nature, and 3) onset of hearing loss at the age of 15 or older, excluding the possibility of later development of USH1D. Also, when possible, family members were invited to participate in the study. Clinical data were obtained for this study population, including gender, age, medical history, physical examination, and audiological test results.

Molecular genetic diagnosis of postlingual SNHL

Genomic DNA was extracted from the peripheral blood samples or buccal cells, using the standard protocols (Gentra Puregene Blood Kit, Qiagen, cat. 158389; Venlo, Limburg, Netherlands). After *GJB2* sequencing, we performed targeted resequencing of the known 129,200 deafness genes (TRS-129 and TRS-200), as previously described (51, 52). TRS-129 and TRS-200 were performed by Otogenetics (http://www.otogenetics.com/) and SGI (Samsung Genome Institute, http://www.samsunghospital.com/dept/main/index.do?DP_CODE=BP7), respectively. The obtained reads were aligned to the UCSC hg19 reference

genome (http://genome.ucsc.edu/index.html) and variants were filtered. Further bioinformatics analyses were performed as previously described (53). If the results of these steps were not convincing, WES was performed. Thereafter, the final candidate variants in these families were verified by Sanger sequencing and validated by ethnic-specific MAF filtering in 200 unrelated Korean control chromosomes from 100 normal hearing control subjects.

We considered the *CDH23* variants as potentially pathogenic when they satisfied the following criteria:

1) *CDH23* variants were not detected in the 200 normal control chromosomes (< 0.005) from our institute, which was a proposed ethnicity-specific MAF with a cut-off threshold (0.005) for autosomal recessive pathogenic variants (54, 55).

2) They were designated as 'damaging or probably damaging' by either SIFT or Polyphen-2, or a GERP++ score of higher than 3.

3) The residues of these variants were also conserved among several species.

A 'probable DFNB12' was defined as when we detected two potentially pathogenic *CDH23* variants in a *trans* configuration that fully satisfied the

above criteria. In contrast, 'possible DFNB12' was defined as when we were able to identify only one *CDH23* variant whose pathogenic potential was previously documented.

We have deposited our whole sequencing data in our private SNUH-SNUBH sequencing database and have submitted the novel variants of *CDH23,* which were detected by next generation sequencing, to the Leiden Open Variation Database (LOVD) (http://databases.lovd.nl/shared/genes/CDH23).

Comparison of ethnicity-specific MAF of the rare CDH23 variants among the SNHL cohort and the normal control cohort

To further evaluate the MAF of potentially pathogenic, rare *CDH23* variants in a larger size of the normal control population, a composite control cohort—which was comprised of up to 2726 normal Korean subjects (5452 alleles)—was used. The composite control cohort included 622 Korean Reference Genome (KRG) database (http://152.99.75.168/KRGDB), 700 Korean in-house exome data from the Korean National Institute of Health (KNIH), 1020 Korean control data from SGI, and 384 control individuals using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City,

CA) **(Supplementary Fig S 3-1)**.

Phase I: First, to evaluate the contribution of potentially pathogenic, rare *CDH23* variants to the Korean postlingual adult SNHL cohort, we compared the total frequency of potentially pathogenic, rare *CDH23* variants with MAF of less than 0.005 between our postlingual adult SNHL cohort and the control cohort from SGI (2040 alleles). Next, we genotyped the specific *CDH23* variants identified from our postlingual adult-onset SNHL cohort among the 1020 Korean control data from SGI (2040 alleles). We compared the MAF between the two groups using a Chi-square test.

Phase II: We tried to further calculate the MAF of *CDH23* variants detected from our postlingual adult SNHL cohort in this study and our previously reported pediatric SNHL cohort (50) in the ethnicity-matched composite control cohort (5452 alleles). Then we compared the MAF of *CDH23* variants from two cohorts among the composite control cohort using Fischer's exact test and evaluated to see if there was any correlation between the MAF of *CDH23* variants and the clinical phenotype.

Results

Part 1

For five probands, we applied candidate gene screening based on the genes previously reported to correspond to the characteristic audiogram configurations and inheritance patterns displayed. We regarded their inheritance patterns as sporadic or autosomal recessive if a child showed the trait but neither of the parents showed the trait. However, this approach was unsuccessful in these families (**Table 1-1, Fig. 1-3**). For the remaining proband (SB100-192) with asymmetric hearing loss associated with subtle EVA, *SLC26A4* screening detected no potential candidate variant of the gene, indicating that this approach was unsuccessful in this case (**Table 1-1, Fig. 1-3**). Therefore, TRS-80 was applied to the six probands. The list of candidate variants remaining after filtering the basic bioinformatic data are shown in **Table 1-2**. In probands SB100-192, SB92-176, and SB87-155, none of the variants that underwent the initial basic filtering step survived additional filtering steps, leaving the molecular genetic etiology undefined even after performing TRS-80 in these probands (**Tables 1-2** and **1-3**). Intriguingly, we detected a novel truncation variant (c.501delT; p.F167fsX22) in the *MYO1A* gene in proband SB92-176. However, we detected this variant in 2 of 276 chromosomes from

normal hearing controls, arguing against its pathogenic potential, which was in accordance with a recently published paper arguing the non-pathogenic potential of *MYO1A* variants (56).

In contrast, TRS-80 revealed a convincing causative gene in the remaining three probands (**Table 1-3, Fig. 1-4**). These variants from the three probands included two reported mutations (p.C162Y in *COCH* [SB82-147] (57) and c.235delC [rs80338943] in *GJB2* [SB128-220]) with known pathogenicity and one novel truncation variant (p.T297Lfs*84 in *PAX3* [SB86-154]) with probable pathogenicity. The p.T297Lfs*84 variant of *PAX3* cosegregated perfectly with the hearing loss phenotype in the SB86 family. This truncation variant of *PAX3* was undetected in 276 normal hearing control chromosomes, supporting its pathogenicity.

Hence, the TRS-80 strategy yielded a 50% molecular diagnosis solve rate in cases that remained undiagnosed after auditory phenotype-driven candidate gene screening, leaving another 50% in need of whole exome sequencing (WES).

Part 2

Targeted resequencing data analysis and the frequency of DFNA9 among

autosomal

dominant hearing loss

Among the 22 newly recruited multiplex families segregating hearing loss in an autosomal dominant fashion, we recently identified two DFNA9 families (SH140-294 and SB200-388) carrying a *COCH* mutation (**Fig. 2-1**). A proband (SH140-294) and her affected brother and mother in SH-140 were recruited for the molecular genetic diagnosis, and for SB-200, a proband (SB200-388) was analyzed (**Fig. 2-2**). Including the three other DFNA9 families among 17 autosomal dominant multiplex hearing loss families in our previous series (15, 48), the overall frequency of DFNA9 among such cases was calculated to be 5/39 (12.8 %) in our Korean cohorts.

Specifically, we found a p.G38D variant of the *COCH* gene from both of the new DFNA9 families through targeted resequencing and subsequent filtering steps (**Figs. 2-1, 2-2**), making the total number of Korean families segregating p.G38D to be three (60 %) of five total DFNA9 families. This suggests that p.G38D is the most frequent variant in our Korean DFNA9 cohorts. Genotypes and audiovestibular phenotypes of the four families in this study were summarized in **Table 2-1**. In other 20 families segregating hearing loss in an autosomal dominant fashion, *COCH* variants were not detected and two variants including *EYA4* and *MITF* were reported

separately (58, 59). Convincing variants were not verified in remaining families, and molecular genetic diagnosis process using WES is ongoing.

Analysis of vestibular phenotypes of COCH mutations

Regarding the detailed clinical phenotype of the four DFNA9 families with clearly documented vestibular phenotype, we were able to classify our cohorts into two groups: one (SB82) with an MD-like phenotype and the other (SH140, SB200 and SH14) with significant bilateral vestibular loss without any diagnostic MD symptoms. Specifically, a 56-year-old female (SH140-294) carrying p.G38D in the LCCL domain of *COCH* complained of sudden hearing loss on the right side and progressive hearing loss on the left side that started in her early 40 s (**Fig. 2-3a**). She had a family history of progressive hearing loss that started in the 40s and became prominent in the 50s, eventually requiring bilateral hearing aids. Detailed history-taking from the proband and her family members revealed that her maternal grandmother, her mother, two uncles on her mother's side, and her younger brother showed a similar pattern of progressive hearing loss with middle-age onset. She sometimes complained of mild lightheadedness, but it is notable that classical whirling type vertigo was not reported. Interestingly, the bithermal

caloric test and rotatory chair test revealed complete bilateral vestibular loss (**Fig. 2-3b, c**). No specific findings were noted in physical examination including spontaneous nystagmus, gaze evoked nystagmus but head impulse test revealed catch-up saccades. A 56-year-old male (SB200-388) from the other family that was referred from the other tertiary hospital manifested an autosomal dominant hearing loss (**Fig. 2-2**). His hearing loss began in his-forties and he started using bilateral hearing aids 7 years ago (**Fig. 2-3a**). He also complained of oscillopsia without definite vertigo attack and the bithermal caloric test documented complete bilateral vestibular loss (**Fig. 2-3b**). A 51-year-old male (SH14–37) from another family (SH14) also carrying p.G38D was previously reported to have bilateral profound hearing loss without any definite vestibular symptoms, including whirling type vertigo, oscillopsia, or disequilibrium, and he eventually received a CI (**Fig. 2-3a**) (15). Further detailed history-taking revealed that his sister (SH14–38) had a similar pattern of hearing loss with intermittent lightheadedness type of dizziness (**Fig. 2-3a**). An additional caloric test and rotatory chair test were performed; the results also indicated complete bilateral vestibular loss (**Fig. 2-3b, c**).

In contrast, a 30-year-old male (SB82–147) carrying p.C162Y in the vWFA 1 domain showed a remarkably different phenotype: he manifested

asymmetrical bilateral SNHL with left-sided tinnitus and aural fullness (**Fig. 2-4a**). In addition to this, he also complained of recurrent devastating whirling-type vertigo attacks, which were well controlled by diuretics (isosorbide dinitrate). Caloric testing showed reduced response on the right side with canal paresis of 56 %, which might imply an irritative phase of MD on the left side, and the rotary chair test also demonstrated subtly reduced gain and phase lead features, which are usually evident in patients with vestibular hypofunction (**Fig. 2-4b, c**). On the physical examination which was performed on the day of caloric testing, persistent left beating nystagmus was observed. Additionally ECOG was later performed—results favored the diagnosis of bilateral MD with Summating Potential/Action Potential ratios of 0.45 and 0.46 on the right and left sides, respectively (**Fig. 2-4d**). His mother and one of affected sisters also had MD-like symptoms, including hearing loss and recurrent vertigo attacks, similar to those of SB82-147. His affected brother and another sister presented with hearing loss and headache, which did not meet the diagnostic criteria of MD. According to the current diagnostic criteria for MD (60), three out of five affected members met the criteria of definite MD, and this family fulfilled criteria for familial MD.

Part 3

Molecular genetic diagnosis and Clinical features of probands

Among the 32 families of postlingual adult-onset non-syndromic SNHL (NS-SNHL), with segregation in either a sporadic or AR fashion, we have identified four (12.5%) potential DFNB12 families: three probable DFNB12 families (SH62, SH151, and SB210) and one possible DFNB12 family (SB116). Notably, three of the four potential DFNB12 families segregated one definitely pathogenic DFNB12 variant in *trans* with a rare *CDH23* variant with unknown pathogenicity (SH62, SH151) or with a *CDH23* allele harboring a series of neighboring variants that were presumably minimally pathogenic when alone (SB116). The remaining family, SB210, co-segregated two rare *CDH23* variants of unknown pathogenicity with postlingual NS-SNHL **(Fig 3-1)**. The fifth family, SB172, carried only one potentially pathogenic *CDH23* variant (p.R1916H), precluding any conclusive molecular diagnosis of SB172 **(Fig 3-1)**.

Three families (SH62, SH151, and SB210) carrying two *CDH23* variants in a *trans* configuration manifested a rapidly progressive SNHL that started in their mid-teens to early twenties (**Table 3-1** and **Fig 3-1**). All of the affected subjects in these families underwent cochlear implantation (CI) or were scheduled to have CI in their twenties or early thirties.

In contrast, SB116-208, 280, and 293 showed a progressive, moderate SNHL that started in their sixties, much later than in three aforementioned probable DFNB12 families. In this family, WES was performed on all affected family members in SB-116 to exclude other causative genes. In this family, the pathogenic p.P240L variant of *CDH23* perfectly co-segregated with hearing loss phenotype, increasing the chance of *CDH23* compound heterozygote being the probable candidate etiology. However, a *CDH23* haplotype in *trans* with the p.P240L allele from one affected member (SB116-280) was different from those in the other two affected siblings (allele (II) vs (I) in **Fig 3-1**). In this family, the contribution of *CDH23* was not confirmed. We checked whether any variant from either *ATP2B2* or *PCDH15* can contribute to hearing loss in this family in *trans* with the p.P240L of *CDH23* as a modifier or in a digenic fashion, as previously suggested (39, 42, 61, 62). However, none of the variants of *ATPB2* or *PCDH15* co-segregated with the hearing loss phenotype in this family (**Supplementary tables S 3-1 and S 3-2**).

Lastly, one 28-year-old female (SB172-326) carrying only one potentially pathogenic *CDH23* variant (p.R1916H) also complained of bilateral progressive NS-SNHL which had started 2 years ago, and had started wearing bilateral hearing aids 1.5 years ago. Her recent audiogram

demonstrated progressive, moderate SNHL on both sides, in which the hearing loss pattern was similar with SH151.

Phase I: Contribution of CDH23 variants to postlingual adult-onset sporadic or arSNHL

Potentially pathogenic *CDH23* variants were screened, both in our adult study and adult control cohorts. Eight alleles from six types of variants were detected: two p.P240L, one p.P402L, one p.R1588W, one p.A1713D, two p.R1916H, and one p.D2202N; the allele frequency of potentially pathogenic *CDH23* variants was determined to be 12.5% (95% CI, 4.4% - 20.6%)(8/64) in our postlingual adult-onset sporadic or arSNHL cohort. However, the allele frequency of all potentially pathogenic *CDH23* variants in the 1020 ethnically-matched control WES data from Samsung Genome Institute (SGI) was calculated to be 5.53% (95% CI, 4.54% - 6.52%) (113/2040) (**Supplementary table S 3-3**), which was significantly lower than that in our adult study cohort (p=0.037 by Chi-square test) (**Fig S 3-1**).

Next, we focused on specific *CDH23* variants, which were identified from our postlingual adult-onset SNHL cohort among the 2040 alleles from the Korean control data provided by SGI. One of the control subjects carried a p.P240L variant, and the other control subject carried a p.R1588W variant,

and there were no control subjects carrying any rare *CDH23* variants, such as p.R1916H, p.A1713D, p.P402L, or p.D2202N (described in **Table 3-2** and highlighted in **supplementary table S 3-3**). Therefore, the allele frequency of these *CDH23* variants associated with postlingual adult-onset SNHL was calculated to be 0.098% (95% CI, 0% - 0.23%)(2/2040) among the 1020 ethnicity-matched control subjects from SGI, which was significantly lower than that of our adult study cohort ($p<0.0001$ by Chi-square test) (**Fig S 3-1**).

Phase II: Comparison of MAF between CDH23 variants detected in adult study cohort and pediatric study cohort among ethnicity-matched controls

Three adult patients (SH62-147, SH151-324, and SB210-412) carried two potentially pathogenic *CDH23* variants as a compound heterozygote, whereas four pediatric patients (SH59-133, SH97-211, SH164-359, and SB56-103) carried two *CDH23* variants either in homozygous or compound heterozygous state (**Table 3-1**) (50). We focused on the pathogenicity of each *CDH23* allele, which were determined by their ethnic-specific MAF in our composite cohort (adult control cohort) **(Fig S 3-1)** (54, 55).

Ethnic-specific MAF of seven variants of *CDH23* in our adult and pediatric study cohorts was displayed **(Table 3-2)**. MAF of a missense

variant, p.T1618K, of *CDH23*, which was detected from a pediatric cohort in *trans* configuration to p.P240L in SH59-133, was 0% (0/4208), while that of p.R1588W and p.R1916H, which were in *trans* to p.P240L and p.D2202N from adult patients, was 0.15% (95% CI, 0.05% - 0.25%) (8/5452) and 0.11% (95% CI, 0.02% - 0.2%) (5/4722), respectively, in our composite adult cohort (**Table 3-2**). The ethnic-specific MAF of p.T1618K detected from the pediatric SNHL population was significantly lower than that of p.R1588W and p.R1916H detected from the postlingual-onset adult SNHL population (p = 0.03, and 0.05 respectively by Fischer's exact test). None of the control individuals carried p.D2202N, p.A1703D, and p.P402L.

Discussion

Part 1

Identifying a causative gene responsible for a certain phenotype can be laborious and costly. The phenotype-driven candidate gene approach especially based on prior knowledge of the auditory phenotype and function of a gene is an intuitive method for identifying causative genetic mutations. Such findings are relatively easy to interpret, and therefore this method is used widely in the field of medical genetics. However, this method has

limited utility for identifying novel genes, with little statistical power or reproducibility (63, 64). In addition, the phenotype is not always fully penetrant and might demonstrate variable expressivity. Consequently, some phenotype-causing genes remain undetected with this method. In the present study, we used TRS-80 to make a molecular diagnosis in three of six families with sensorineural hearing loss, in which the initial auditory phenotype-driven candidate gene screening was unsuccessful.

Our results demonstrate the underestimated potential of targeted resequencing and show that this strategy would be an unbiased powerful tool to efficiently screen 80 genes associated with deafness. First, it can complement incomplete physical examinations or history taking of family members. Genetic hearing loss can be classified into non-syndromic and syndromic hearing loss based on the presence of systemic manifestations other than SNHL (65). Non-syndromic hearing loss (NSHL) constitutes ~75% of genetic hearing loss; however, a certain portion of seemingly non-syndromic deafness may subsequently be determined to be syndromic after comprehensive clinical evaluations. In the present study, proband SB86-154 initially presented with profound right-sided and subtle left-sided low-frequency SNHL loss with no noticeable syndromic feature. Driven by the audiogram profile and autosomal-dominant inheritance of hearing loss, the

WFS1 gene was initially sequenced, but no convincing variant in the gene was identified. Using TRS-80, the *PAX3* frameshift mutation was detected (c.885*del*T: p.T297Lfs*84) in exon 6. The *PAX3* gene is responsible for type 1 Waardenburg syndrome (WS1) (66). Therefore, we reviewed the patient's medical record, contacted the patient, and eventually noticed that her prominent nasal root, synophrys, and premature graying of the hair had been camouflaged with makeup during her physical examination. In addition, sapphire blue eyes (which did not exist in proband SB86-154) segregated in the SB86 family with variable penetrance. Segregation analyses of three additional members (two affected and one unaffected) from this family further confirmed that the proband's hearing loss was due to the *PAX3* frameshift mutation. The clinical features of WS1 can show incomplete penetrance and highly variable phenotype expressivity, which may sometime make the diagnosis challenging (67). Although we initially regarded hearing loss in this patient as non-syndromic, the application of TRS-80 facilitated the diagnosis of WS1. Currently, prenatal testing for *PAX3* is possible, and folic acid supplementation in pregnancy is recommended for individuals with increased risk of having a child with WS1 (68). In this way, families can receive counseling and management with appropriate measures.

Secondly, using the NGS technique, TRS-80 can lead a clinician

directly to a molecular diagnosis, without the influence of audiogram configurations. We identified a previously reported missense variant (c.889G>A; p.C162Y) of *COCH* (57) in proband SB82-147, who presented with bilateral mid-frequency hearing loss in an autosomal-dominant manner. In this case, the audiogram configuration initially led us to sequence the *TECTA* (OMIM 602574) and *COL11A2* (OMIM 120290) genes. The *COCH* gene encodes the cochlin protein, which is one of the most abundant proteins in the inner ear. It is expressed predominantly in the spiral ligament, spiral limbus, and osseous spiral lamina of the cochlea and in the stromal fibrocyte and ampullary wall of the vestibular labyrinth and cristae (6, 69). To date, 21 identified *COCH* mutations are known to reside within functional domains in cochlin, limulus factor C, cochlin, late gestation lung protein (LCCL), and two von Willebrand factor A proteins (vWFA1 and vWFA2) (19, 23). The detected mutation (c.889G>A; p.C162Y) is located within the first intervening domain (ivd1) and is the first reported *COCH* mutation outside the LCCL and vWFA2 domains (19, 57). The advantage of TRS-80 in this regard was demonstrated by the molecular diagnosis of proband SB128-20. Bilateral, progressive, mild-to-moderate degree of downsloping SNHL segregating either in a sporadic or autosomal recessive inheritance form detected in a 5-year-old male (**Fig. 1-2**) prompted us to screen the *TMPRSS3*

gene (13) and the first amplicon of the *GJB2* gene, focusing on the p.V37 residue (46). However, no convincing variant was detected in the initial screening. During the next step, TRS-80 detected a homozygous mutation of c.235delC (p.L79Cfs*3) in the *GJB2* gene, which was quite unexpected considering the degree of residual hearing from this proband (**Fig. 1-2**). The mutation in *GJB2* was not suspected initially because most of the frequent mutant alleles of this gene in the Korean deaf population were associated with more severe hearing loss and were found mainly in the patient group diagnosed during the first 3 years (70). SB128-220 passed the newborn hearing screening and showed progressive downsloping high-frequency hearing loss, which is not typical for subjects with the homozygous c.235*del*C. However, the audiogram profile of SNHL due to *GJB2* mutations varies according to the involved allele and among subjects (70). The presence of genetic modifiers might account for this extreme degree of residual hearing. Our results highlight the role of unbiased screening of targeted deafness genes.

Lastly, utilization of TRS-80 in this study inadvertently cast doubt on the contribution of a known deafness gene to SNHL. The myosin superfamily was one of the first proteins found to be associated with hearing loss and is known to play a major role in hair-cell function (71). The *MYO1A*

gene located within the DFNA48 locus is a member of the myosin superfamily and was reported to cause autosomal-dominant NSHL (72, 73). Using TRS-80 in the analysis of SB92-176, we detected a novel truncation mutation of *MYO1A* (p.F167fsX22), in which transcripts may undergo 'nonsense-mediated decay.' However, this variant was detected in 2 of 276 normal control chromosomes. In addition, it was detected in two unrelated families with a completely different degree of hearing loss. Interestingly, this variant did not co-segregate with the hearing loss phenotype in one of the two unrelated families, strongly arguing against its pathogenic potential by a loss of function mechanism. While we were submitting this manuscript, this observation was published by a separate group (56).

Taken together, this study suggests widened applicability of the TRS-80 strategy into *GJB2* negative Korean familial SNHL cases even with the characteristic audiologic phenotypic markers, especially when the prevalence of corresponding candidate gene in the Korean population is low.

Part 2

Mutations in *COCH* cause DFNA9, which is characterized by an autosomal dominant, non-syndromic, progressive SNHL that is frequently

associated with vestibular dysfunction (17). Auditory impairment is usually a late-onset progressive hearing loss, and it often begins with high-frequency hearing loss. Self-reported and/or predominant vestibular symptoms may or may not be present (19).

The *COCH* gene encodes an extracellular protein, cochlin, which is composed of an N-terminal secretory signal peptide, LCCL domain, and two vWFA domains (**Fig. 2-5**) (28). Cochlin has been reported to be expressed most abundantly in the inner ear, especially in the spiral ligament, spiral limbus, and osseous spiral lamina of the cochlea, and in the stromal fibrocytes and ampullary wall of the vestibular labyrinth and cristae (69, 74). Currently, a total of 14 mutations of the *COCH* gene have been found in the LCCL domain, while seven *COCH* mutations were found outside the LCCL domain (19). Previously, an interesting genotype–phenotype correlation was hypothesized based on the observation, albeit from a small cohort, that those patients with mutations in the LCCL domain of *COCH* were more likely to have vestibular dysfunction than those with mutations in the vWFA2 domain (29). This hypothesis has been revisited by a recent similar observation (19, 26). Furthermore, the studies postulated a different subcellular localization based on the position of the mutation in the *COCH* gene as a possible cause of variable phenotypes (19, 26). In detail, cochlins containing mutations in

the LCCL domain showed localization in the endoplasmic reticulum (ER) and Golgi complex, while cochlins containing mutations in the vWFA domains also localized to the ER but not the Golgi complex, indicating a failure to transport mutant cochlins associated with the vWFA domain from the ER to the Golgi complex. These mutant proteins associated with the vWFA domain characteristically formed higher-molecular-weight aggregates in cells due to accumulation of the aggregates in cells, this causes earlier cell damage, and thus earlier onset of hearing loss (19, 26).

Our DFNA9 families also revealed distinct audiovestibular phenotypes. In families SH-14, SB-200 and SH-140, which carry a mutation (p.G38D) in the LCCL domain, clinical manifestations, including the onset and progression of symptoms, were very similar, resulting in bilateral profound hearing loss and bilateral vestibular loss without definite acute vestibular symptoms. Specifically, all affected members appeared to have rapidly progressive hearing loss that started in their 40 s and then progressed into profound hearing loss within less than a decade, mandating bilateral hearing aids or a cochlear implant. Audiograms showed bilateral profound hearing loss involving all frequencies in three affected members in their 50s and a high-frequency hearing loss pattern in one member in her mid-40s (**Fig. 2-3a**). Notably, although one affected member (SH14-38) experienced

occasional intermittent dizziness, she did not experience definite vertigo spells. Also, vestibular function tests performed on three affected members commonly showed bilateral vestibular loss (**Fig. 2-3b, c**). If the rigorous vestibular examination and tests had not been performed, their bilateral vestibular loss might not have been detected due to the absence of acute vestibular symptoms. On the other hand, SB82-147 in our cohort, who had a missense mutation (c.G485A; p.C162Y) in the vWFA1 domain of the *COCH* gene (**Fig. 2-5**), complained of bilateral asymmetrical sensorineural hearing loss, tinnitus, and aural fullness on the left side, accompanied by recurrent devastating vertigo attacks.

The phenotype of SB82-147 does not fit perfectly into the proposed genotype–phenotype correlation. In the previous study that first reported p.C162Y as a deafness-causing variant, no vestibular symptoms were described (57), and a subsequent study also reported that individuals with vWFA domain mutations predominantly exhibit hearing loss, while LCCL domain mutations cause hearing defects that are accompanied by vestibular dysfunction, based upon analyses of reported *COCH* mutations and phenotypes. However, the mechanism by which mutants in the LCCL domain result in vestibular dysfunction has not been fully elucidated (19). SB82-147 and his affected members in this study manifested MD-like

vestibular features, against the initially proposed hypothesis of genotype–phenotype correlation. This might be in part because vestibular function tests were not performed in the previous study (57). Although probands in their study did not complain of vestibular symptoms, vestibular phenotypes might have existed in a subclinical or mild form. Simply put, p.C162Y could manifest a wide range of vestibular phenotypes ranging from no symptoms to MD-like features. However, the fact that no vestibular phenotype was documented among 28 hearing-impaired family members with the p.C162Y variant in their study (57) is still questionable. Nevertheless, we identified a clear distinction in terms of the vestibular phenotypes between three families carrying p.G38D (SH14, SB200, and SH140) and one family with p.C162Y (SB82). This might be a beginning of proof against the already published hypothesis of differences in vestibular phenotypes according to location of mutation location in vWFA against LCCL location.

In this study, the p.G38D variant of the *COCH* gene accounted for three Korean multiplex families segregating hearing loss as well as vestibular symptoms. Considering the extreme heterogeneity of the molecular genetic etiology of progressive SNHL in Koreans (15), detection of this variant in three families manifesting progressive audiovestibular dysfunction suggests it to be a frequent cause of such a phenotype in this population.

A progressive hearing loss that starts in the 30 or 40 s with the need for bilateral hearing aids and eventually CI in the 50 or 60 s and very slowly progressive bilateral vestibular loss not accompanied by a definite vertigo attack may be a hallmark phenotype of DFNA9 with alterations in the LCCL domain, specifically p.G38D variant. Moreover, MD-like features may be related to only a subset of DFNA9 subjects with mutations in the vWFA domains. However, evaluation of a small number of cohorts in this study and the conflicting reports regarding the same genotype in the literature preclude drawing of a convincing correlation. A study involving a larger series of DFNA9 subjects is required to identify a stronger genotype–phenotype correlation.

This study shows a correlation between p.G38D in *COCH* and complete bilateral vestibular loss, suggesting that significant bilateral vestibular loss may also accompany DFNA9 hearing loss especially when the LCCL domain is mutated, despite the apparent absence of dizziness episodes. Collectively, our results expand the vestibular phenotypic spectrum of DFNA9 to range from bilateral vestibular loss without episodic vertigo to MD like features with devastating episodic vertigo.

Part 3

Some *CDH23* mutations in humans have previously been reported to be associated with adult-onset postlingual progressive SNHL, in both Caucasians and Japanese (38, 39). However, systematic documentation of the contribution of *CDH23* mutations to this late-onset postlingual progressive SNHL was not a main concern in these two reports. Instead, the role of a modifier gene, *ATP2B2*, was elucidated to account for the phenotypic differences among siblings (39). Miyagawa et al. (2012) did not rigorously investigate the causal relationship between the *CDH23* genotype and its phenotype (38). In contrast, our current study, which employs a genetic epidemiologic approach, clearly demonstrated that an alteration of the *CDH23* gene contributes to adult-onset postlingual progressive NS-SNHL. The rare *CDH23* alleles that satisfy our criteria for a potential pathogenicity were more frequently detected in Korean adult-onset postlingual progressive SNHL than in normal hearing controls, with statistical significance. In fact, this result is not surprising since the association of age-related progressive SNHL and some *Cdh23* alleles, such as *Cdh23*^{ahl} and *Cdh23*^{erl}, has already been documented in mouse models with certain genetic backgrounds (42, 43, 75). The replacement of a single nucleotide (A to G) in a *Cdh23* gene on progressive SNHL had been shown

to prevent age-related SNHL phenotype (38, 42, 76, 77).

Based on our result, we could think that the major form of *CDH23*-related SNHL might be adult-onset progressive SNHL, rather than prelingual-onset severe-to-profound SNHL (DFNB12), at least in Koreans or East Asians. In fact, *CDH23* mutations accounted for 9.4% (3/32)—or possibly up to 12.5% (4/32)—of postlingual adult-onset SNHL, while the genetic load of *CDH23* mutations was 3.1% (4/128) in our pediatric cohort with prelingual-onset severe-to-profound SNHL (50). A statistical analysis was performed to compare the frequency of *CDH23* mutation between the adult and pediatric hearing loss cohorts; a difference was shown with marginal significance (p=0.051 by Fischer's exact test). From this observation, we can assert that *CDH23* variants might contribute more to adult-onset progressive SNHL than to prelingual-onset severe-to-profound SNHL. However, we need to be cautious to draw a firm conclusion from these results due to the relatively small number of subjects in this study.

One important finding from our study is that the p.P240L allele of *CDH23*, which turned out to be the founder allele among the prelingual DFNB12 Korean subjects, was revisited in our adult-onset postlingual SNHL cohort. Two (SH151 and SB116) of the five families with co-segregating adult-onset progressive SNHL with at least one potentially pathogenic

CDH23 variant turned out to have the p.P240L allele. A contribution of the p.P240L allele to our adult-onset postlingual SNHL was confirmed by a higher frequency of this allele (2/64) in our adult SNHL cohort than in normal controls (1/2040) (p<0.0001 by Fischer's exact test). The p.P240L homozygotes were previously reported to cause prelingual severe-to-profound SNHL in a majority of cases (38, 50, 78). According to our study, as well as previous Japanese studies, audiological phenotypes of *CDH23* compound heterozygotes that carry one p.P240L allele seem to be highly variable (38, 78). Indeed, SH151-324 carrying p.P240L/p.R1588W manifested progressive SNHL, which started from mid to high frequencies at the age of 20. In contrast, SB116-208 showed progressive SNHL that became noticeable after the age of 60 years in the present study. Given this, it can be postulated that auditory phenotypes of these families depend on the pathogenic potential or residual CDH23 protein dosage from the *trans* *CDH23* allele to p.P240L. In our previous study with a pediatric pre-lingual SNHL population, the *trans* allele of p.P240L was p.P240L itself, p.D2858E*fs*X8, which was a truncation mutation, and p.T1618K, respectively. It is easily conceivable that p.D2858E*fs*X8 leads to a serious deleterious effect on proteins. The MAF of this missense variant, p.T1618K, was extremely low as indicated by zero detection among 4208 control alleles,

implying a strong pathogenic potential (79). Among the two postlingual SNHL families (SH151 and SB116) carrying p.P240L, the *trans* allele of p.P240L of *CDH23* from SH151-324 was p.R1588W; however, none of the potential polymorphisms of *CDH23* in *trans* with p.P240L was compatible with the segregation of SNHL in SB116. The pathogenic potential of p.R1588W of *CDH23* was previously disputed due to the presence of normal hearing from one homozygous carrier of p.R1588W (38). However, it is likely that p.R1588W, presumably with a mild pathogenicity, can exert its pathogenic effect leading to progressive postlingual SNHL when in *trans* with a strongly pathogenic p.P240L.

The role of *CDH23* mutations in SB116 is enigmatic. The *CDH23* haplotype of *trans* allele to p.P240L was not shared by all the siblings with SNHL in this family. This may suggest that mutations in *CDH23* do not account for SNHL in SB116 and that the detection of p.P240L was fortuitous. However, some of the frequent neighboring single nucleotide polymorphisms of *CDH23* were indeed shared en bloc by all three siblings with SNHL **(Fig 3-1)**. The collective effects of these SNPs in *CDH23* might exert a pathogenic effect, albeit not severe, in a *trans* configuration with p.P240L, leading to progressive SNHL prominent after the 60's. A perfect co-segregation of p.P240L of *CDH23* with the SNHL phenotype in SB116

supported this hypothesis. Significantly late onset age of SNHL in SB116 might also increase the possibility of a relationship between *CDH23* and presbycusis in an older population.

Differential auditory phenotype, depending on the combination of the two *Cdh23* alleles in mice, showed a striking resemblance to our observation. Homozygous mice carrying the functionally null mutations of *Cdh23*, such as $Cdh23^{v}$ or $Cdh23^{v\text{-}ngt}$, manifest congenital profound SNHL and a severe vestibular phenotype (80, 81). In contrast, homozygosity with respect to $Cdh23^{753A}$ (hypomorphic Ahl allele) leads to increased susceptibility to age-related SNHL, thereby showing severe hearing loss by 9-12 months of age, under certain genetic backgrounds (43). Interestingly, the compound heterozygous mice with one null allele of $Cdh23^{v\text{-}ngt}$ and one hypomorphic allele ($Cdh23^{ahl}$) under C57BL/6J showed an intermediate phenotype: hearing impairment that started from the age of 4 months which increased in severity in an age-dependent manner (82). Taken together, the combination of two *Cdh23* alleles with a different pathogenic potential decided the fate of auditory phenotype in mice.

From this perspective, we looked into the human *CDH23* variants that we detected from our present study, as well as from our previous study by Kim et al. (2015) (50). First, we made a comparison of the ethnic-specific MAF

between the missense variants detected from our adult SNHL cohort and the missense variants from our pediatric SNHL cohort. We observed significantly higher MAF of p.R1588W and p.R1916H from the postlingual-onset adult SNHL population than that of p.T1618K, which was in *trans* with p.P240L (50) in a pre-lingual profound SNHL. The most likely explanation would be that the difference in the pathogenic toxicity of the two alleles (p.R1588W vs p.T1618K) in *trans* with the known pathogenic allele, p.P240L, decided the fate of auditory phenotype in two subjects. The milder pathogenic potential of p.R1588W as compared with p.T1618K could be indirectly supported by normal hearing from the p.R1588W homozygous carrier (38) and also by the higher Korean MAF (79). Another two missense variants, p.D2202N (rs121908349 flagged) and p.A1713D, in postlingual adult-onset SNHL, which were detected in *trans* with p.R1916H and p.P402L, respectively, may exert just as strong pathogenic potential as p.P240L, especially considering the GERP score of more than 5, in *silico* prediction results and extremely low MAF **(Table 3-2).** Additionally, the pathogenic potential of p.D2202N was previously described (83). In contrast, p.R1916H and p.P402L (rs373168635) may also serve as milder pathogenic alleles, considering their relatively low GERP score of <5, MAF, and in *sili*co prediction results **(Table 3-2)**. From this explanation, it is possible to

suggest MAF as a predictor of the clinical fate of DFNB12.

In a previous genotype-phenotype correlation study, it was suggested that the phenotypic consequence of the compound heterozygosity for a DFNB12 allele in *trans* configuration with a predicted USH1D allele of *CDH23* was determined, mostly by the DFNB12 allele (37). In this present study, we tried to extend this hypothesis to include the phenotypic consequence of the compound heterozygosity of a known pathogenic DFNB12 allele in *trans* configuration with *CDH23* allele with an unknown pathogenicity, or to even include the compound heterozygosity of two *CDH23* alleles with an unknown pathogenicity **(Fig 3-2)**.

In our study, analysis of copy number variants (CNVs) was not performed. CNVs are genomic variants that alter the diploid state of a portion of the genome, either by increasing or decreasing the number of alleles (84), and it could exert pathogenic potential. It is a relatively new field in genomics, and its application in the field of genetic deafness is currently emerging (84-86). To date, CNVs related with non-syndromic HL were identified in 18 genes, and most of CNVs were distributed in *STRC* and *OTOA* gene (84, 87). Related phenotypes include mostly prelingual onset, severe SNHL with or without infertility in males. The fact that CNVs were not included in the analysis could be a limitation in this study, however,

CNVs were not considered as a likely cause of deafness in our probands, with its relatively milder phenotypes being considered.

Future prospects of research

As molecular genetic tool for diagnosis of genetic hearing loss has developed, our focus has moved to the development of therapy for hereditary hearing loss. Below is the potential treatment strategies for genetic hearing loss.

Gene therapy

Nuclease-based technologies have been developed that enable targeting of specific DNA sequences directly in the zygote. These technologies include zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and CRISPR/Cas9 system. These approaches could provide an opportunity to modify the genomes of inbred mice, and allow the removal of strain-specific mutations that may confound phenotypic assessment such as *Cdh23*^{ahl} allele (76). A homology directed repair (HDR) is one way of correcting the mutation at the targeted locus.

Regenerative therapy

One of the way to regenerate the damaged hair cell is to introduce stem cells into the damaged ear. Stem cells could be derived from embryonic stem cells, induced pluripotent stem cells or isolated from the inner ear itself (88). Recently combination of induced pluripotent stem cell (iPSC) technology with genome-editing technology was introduced (89).

Pharmacological approaches

Although pharmacological approaches would be an ideal treatment for hearing loss, no drug has been approved for directly treating hearing loss by Food and Drug Administration (FDA) (34). Patients who have hearing loss with limited pathological changes or slowly progressive nature such as presbycusis could be potential candidates for pharmacological approaches.

Conclusion

Taken together, we proposed the strategic pipeline (TRS) to make a molecular genetic diagnosis of deafness and widened its applicability to

cases with failed phenotype-driven candidate gene screening. Then we applied TRS to show the distinct vestibular phenotypes of DFNA9 depending on the location of *COCH* mutations, and this strategy was also used combined with a genetic epidemiologic approach to demonstrate an important contribution of *CDH23* mutations to poslingual-onset NS-SNHL, widening phenotypic spectrum of DFNB12. Coupled with the rapid development of these molecular genetic diagnostic tools, approaches based on stem cells and gene therapy for the restoration of hearing are also rigorously investigated.

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Table 1-1. Cases where initial candidate gene approach by Sanger sequencing failed

Patient	Characteristic phenotype	Inheritance pattern	Initial Approach
SB82-147	Mid-frequency HL*	AD**	*TECTA, COL11A2* sequencing
SB86-154	Asymmetric low-frequency HL	AD	*WFS1* sequencing
SB87-155	Postlingual progressive HL	AR***	*TMPRSS3 sequencing*
SB92-176	Mid-frequency HL	AD	*TECTA, COL11A2sequencing*
SB100-192	Asymmetric hearing loss & subtle EVA	AD	*SLC26A4 sequencing*
SB128-220	Prelingual or postlingual high-frequency HL	AR or sporadic	*GJB2* p.V37I and *TMPRSS3 sequencing*

*HL: hearing loss, **AD: autosomal dominant, ***AR: autosomal recessive

Table 1-2. Characteristics of candidate genes through basic bioinformatic filtering

Patient	Gene	Variant annotation	Inheritance mode of the variant	Chr	Ref	Var	Coverage		Quality score	1000g
							Ref	Var		
SB82-147 (AD)	SLC26A4	NM_000441:exon2:c.C147G:p.S49R	AR	7	C	G	55	68	99	-
	PCDH15	NM_001142765:exon25:c.A3342G:p.I1114M	AR	10	T	C	117	132	99	-
	COCH[a]	**NM_001135058:exon7:c.G485A:p.C162Y**	**AD**	**14**	**G**	**A**	**105**	**103**	**99**	-
	MYH9	NM_002473:exon36:c.G5090A:p.R1697H	AD	22	C	T	61	77	99	-
SB86-154 (AD)	**PAX3[a]**	**NM_181457:exon6:c.885delT: p.T297Lfs*84**	**AD**	**2**	**A**	-	**143**	**106**	**99**	-
SB92-176 (AD)	DSPP	NM_014208:exon5:c.G3337A:p.D1113N	AD	4	G	A	34	15	99	-
	MYO1A	NM_005379:exon7:c.501delT:p.F167LfsX22	AD	12	A	-	101	93	99	-
	ESRRB	NM_004452:exon4:c.C156G:p.H52Q	AR	14	C	G	109	62	99	-
SB100-192 (AD)	TRIOBP	NM_001039141:exon7:c.G1591A:p.A531T	AR	22	G	A	115	128	99	-
	TRIOBP	NM_001039141:exon7:c.C1719G:p.D573E	AR	22	C	G	193	56	99	-
SB87-155 (AR)	PCDH15	NM_001142765:exon32:c.5080_5085del:p.1694_1695del	AR	10	AGGAGC	-	29	21	99	-
	PCDH15	NM_001142765:exon20:c.C2671T:p.R891C	AR	10	G	A	74	87	99	0.0014
	MYH14	NM_024729:exon2:c.219_220insGGC:p.E73delinsEG	AD	19	-	GGC	138	75	99	-
SB128-220 (AR or sporadic)	PDZD7	NM_001195263: exon10:c.G1526A:p.G509E	AR	10	G	A	109	140	99	-
	GJB2[a]	**NM_004004:exon 2:c.235delC: p.L79Cfs*3[b]**	**AR**	**13**	**C**	-	**0**	**140**	**99**	

All variants listed here are not found in dbSNP135, [a]Final causative variants in bold, determined by targeted exome sequencing, [b]rs80338943 flagged SNP

Table 1-3. Five filtering steps for detection of candidate variants in 3 sensorineural hearing loss families

Patient	1) Basic filtering	2) Inheritance pattern	3)Sanger sequencing	4) Control study	5) Segregation study	Final
ADNSHL[a]						
SB82-147	4	2	1	1	-[c]	1
SB86-154	1	1	1	-	1	1
SB92-176	3	2	1	0	-	0
SB100-192	2	0	-	-	-	0
Sporadic or ARNSHL						
SB87-155	3	2	2	-	0	0
SB128-220	2	2	1	1	1	1[b]

[a]Cases that are classified as nonsyndromic at initial evaluation not by molecular genetic testing
[b]Homozygous variant
[c]Not performed

Table 2-1. Mutations of the *COCH* gene identified and audiovestibular phenotypes of the families in this study

Patient	Gene	Variant annotation	Vestibular Testing tool	Vestibular testing result	Audiometric Result	Ref	Var	Coverage	Quality score	GERP score	Found by
SH14-37 (M/51)	*COCH*	exon3:c.G113A: p.G38D	Bithermal Caloric test and Rotary chair test	Bilateral vestibulopathy	Profound SNHL	G	A	162	60(Q call)	5.67	TRS80 (15)
SH14-38		Right High Frequency, Left moderate SNHL				G	A				Sanger sequencing
SB82-147 (M/30)	*COCH*	exon8:c.G485A: p.C162Y	Bithermal Caloric test and Rotary chair test	Unilateral vestibular hypofunction	Asymmetric SNHL	G	A	150	99	-	TRS80 (48)
SB82-171		HL, recurrent vertigo (>2), aural fullness				G	A				Sanger sequencing
SB82-172		HL, persistent headache and dizziness				G	A				Sanger sequencing
SB82-173		HL, recurrent vertigo (>2), aural fullness, vomiting				G	A				Sanger sequencing
SB82-174		HL, persistent headache, dizziness				G	A				Sanger sequencing
SH140-294 (F/56)	*COCH*	exon3:c.G113A: p.G38D	Bithermal Caloric test and Rotary chair test	Bilateral vestibulopathy	Moderate SNHL	G	A	41	60(Q call)	5.67	TRS129
SH140-362		Bilateral severe SNHL				G	A				Sanger sequencing
SH140-380		Bilateral severe SNHL				G	A				Sanger sequencing
SB200-388 (M/56)	*COCH*	exon3:c.G113A: p.G38D	Bithermal Caloric test	Bilateral vestibulopathy	Severe SNHL	G	A	21	46.029(Q call)	5.67	TRS129

Table 3-1. Genotype of individuals segregating homozygous or heterozygous mutations of *CDH23* identified by TRS or WES, and from the previous study by Kim et al. (50).

Patient	Sex/ Age	Variant annotation	Location	Ref	Var	Coverage	Quality score	Found by
SH62-147	F/32	exon42:c.G5747A:p.R1916H	Chr10:73545422	G	A	178	99	TRS200
		exon46:c.G6604A:p.D2202N	Chr10:73553289	G	A	237	99	
SH151-324, 383	F/26	exon8:c.C719T:p.P240L	Chr10:73330641	C	T	193	60(Qcall)	TRS129
	M/23	exon37:c.C4762T:p.R1588W	Chr10:73501595	C	T	71	60(Qcall)	
SB210-412	F/21	exon12:c.C1205T:p.P402L	Chr10:73405652	C	T	59	60(Qcall)	TRS129
		exon38:c.C5138A:p.A1713D	Chr10:73538016	C	A	21	60(Qcall)	
SB116-208	F/71	exon8:c.C719T:p.P240L	Chr10:73330641	C	T	50	60(Qcall)	TRS129, WES
		Not determined						
SH59-133	F/3	exon8:c.C719T:p.P240L	Chr10:73330641	C	T	238	99	Kim et al. (50)
		exon37:c.C4853A:p.T1618K	Chr10:73537445	C	A	29	99	
SH97-211	F/1	exon8:c.C719T:p.P240L	Chr10:73330641	C	T	102	60(Qcall)	Kim et al. (50)
		exon8:c.C719T:p.P240L	Chr10:73330641	C	T	102	60(Qcall)	
SH164-359	F/1	exon8:c.C719T:p.P240L	Chr10:73330641	C	T	218	99	Kim et al. (50)
		exon8:c.C719T:p.P240L	Chr10:73330641	C	T	218	99	
SB56-103	F/4	exon8:c.C719T:p.P240L	Chr10:73330641	C	T	238	60(Qcall)	Kim et al. (50)
		exon58:c.8574delC:p.D2858EfsX8	Chr10:73567616	C	-	95	60(Qcall)	

Table 3-2. Ethnic-specific MAF and *in silico* pathogenicity prediction of *CDH23* variants in our study

Variant	Patient	dbSNP	In-house exome from KNIH (n=700)	KRG database (n=622)	Genotyping (n=384)	SGI (n=1020)	Score in Pph2/ SIFT/ GERP	MAF in composite cohort	ExAC	1000 Genomes
p.T1618K (c.C4853A)	SH59-133 (Pediatric)	No data	0/1400	Not detected in unknown number	0/768	0/2040	Probably Damaging/ Damaging / 5.9	0% (0/4208)	N/A	N/A
p.P240L (c.C719T)	SH59-133 SH151-324 SB116-208 (Pediatric & Adult)	rs121908354 (flagged)				1/2040	Possibly Damaging/ Damaging/ 5.19	0.05% (1/2040)	0.00009	0.0002
p.D2202N (c.G6604A)	SH62-147 (Adult)	rs121908349 (flagged)	0/1400	Not detected in unknown number	0/768	0/2040	Probably Damaging/ Damaging/ 5.06	0% (0/4208)	0.000008	N/A
p.A1713D (c.C5138A)	SB210-412 (Adult)	No data				0/2040	Probably Damaging/ Damaging/ 5.4	0% (0/2040)	N/A	N/A
p.P402L (c.C1205T)	SB210-412 (Adult)	rs373168635 (Non-flagged)				0/2040	Probably Damaging/ Tolerated/ 4.91	0% (0/2040)	0.00003	N/A
p.R1588W (c.C4762T)	SH151-324 (Adult)	rs137937502	4/1400	2/1244	1/768	1/2040	Probably Damaging/ Damaging/ 3.24	0.15% (8/5452)	0.0002	0.0008
p.R1916H (c.G5747A)	SH62-147 (Adult)	**rs746971522**	3/1400	2/518	0/764	0/2040	Probably Damaging/ Damaging/ 4.28	0.11% (5/4722)	0.00006	N/A

KRG, Korean Reference Genome (http://152.99.75.168/KRGDB); ExAc, Exome Aggregation Consortium (http://exac.broadinstitute.org/); 1000 Genomes (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/); N/A, not applicable

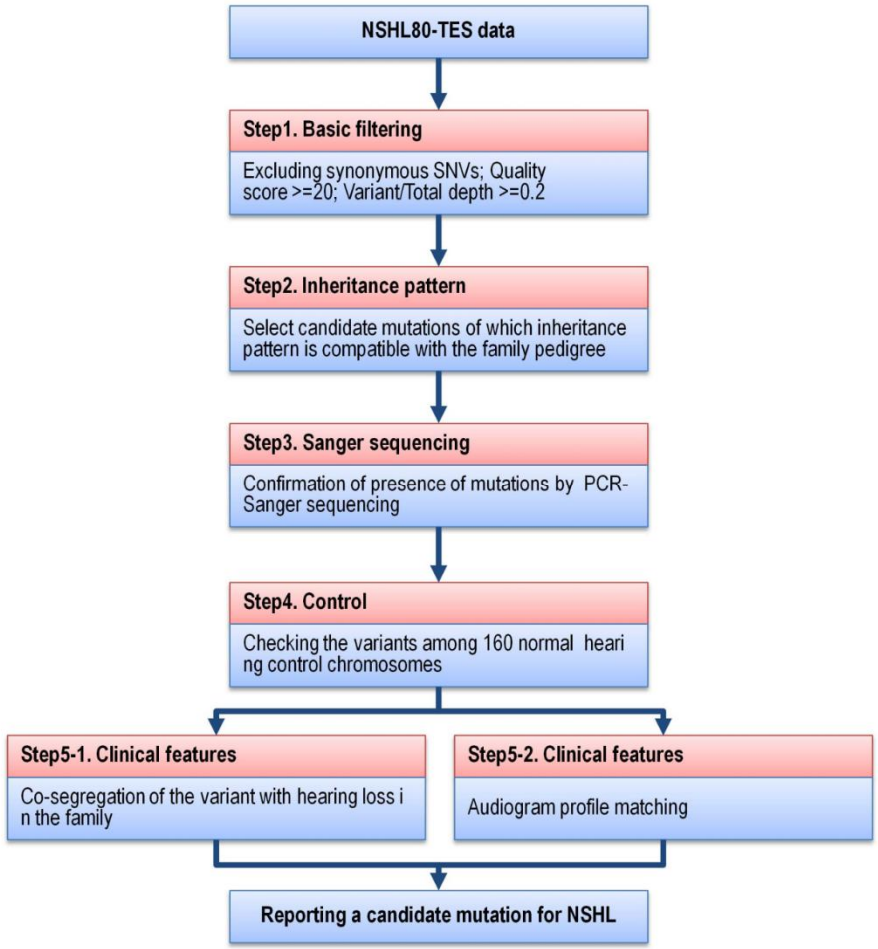


Figure 1-1.

Analysis flow of targeted resequencing (TRS) on familial non-syndromic hearing loss (NSHL). Targeted resequencing data from familial NSHL cases were filtered through five steps to select candidate SNVs in NSHL genes.

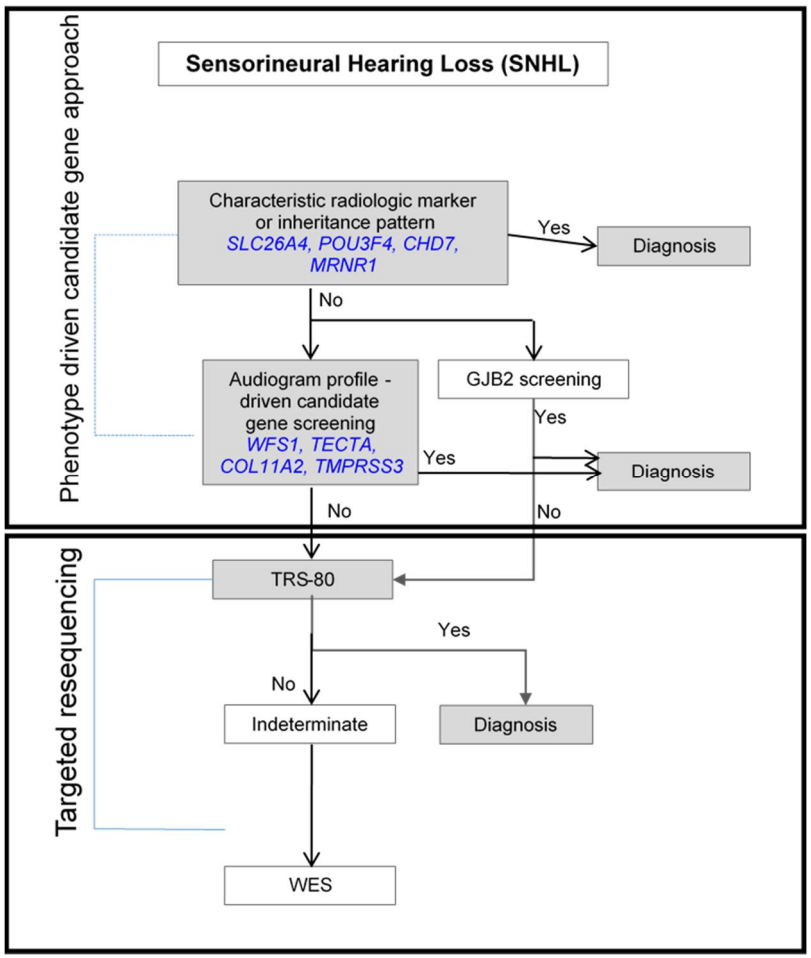


Figure 1-2.

Overview of the molecular genetic diagnosis strategy in patients with SNHL. Flow analysis of the combined diagnostic approaches using phenotype-driven candidate gene analysis and targeted resequencing (TRS) of deafness genes.

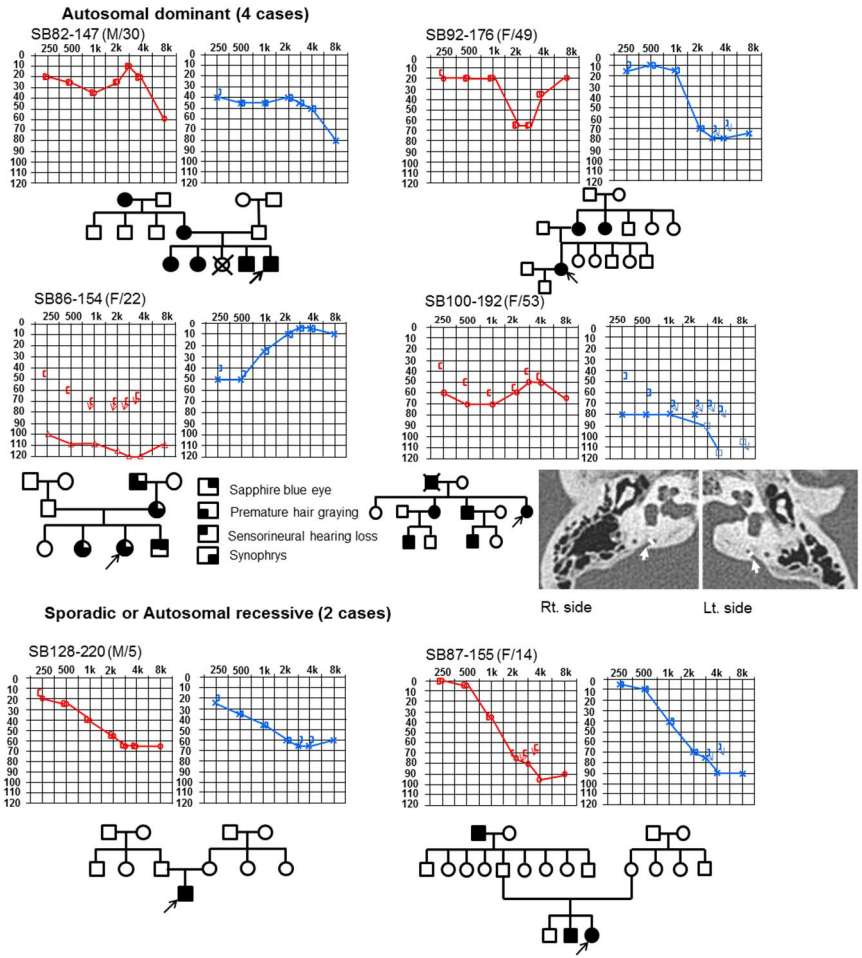


Figure 1-3.

Audiogram and pedigree analysis in six families with sensorineural hearing loss, including four with autosomal-dominant and two
with sporadic or autosomal-recessive inheritance patterns.

Right (red in online version) and left (blue in online version) ear hearing thresholds. Filled symbols represent hearing-impaired individuals, and clear symbols denote those with normal hearing. Black arrows indicate the probands. Two white arrows indicate a subtle bilateral enlarged vestibular aqueduct (EVA) with a diameter equal to 1.3 mm (Rt.) and 1.4 mm (Lt.) at the midpoint (two white bars).

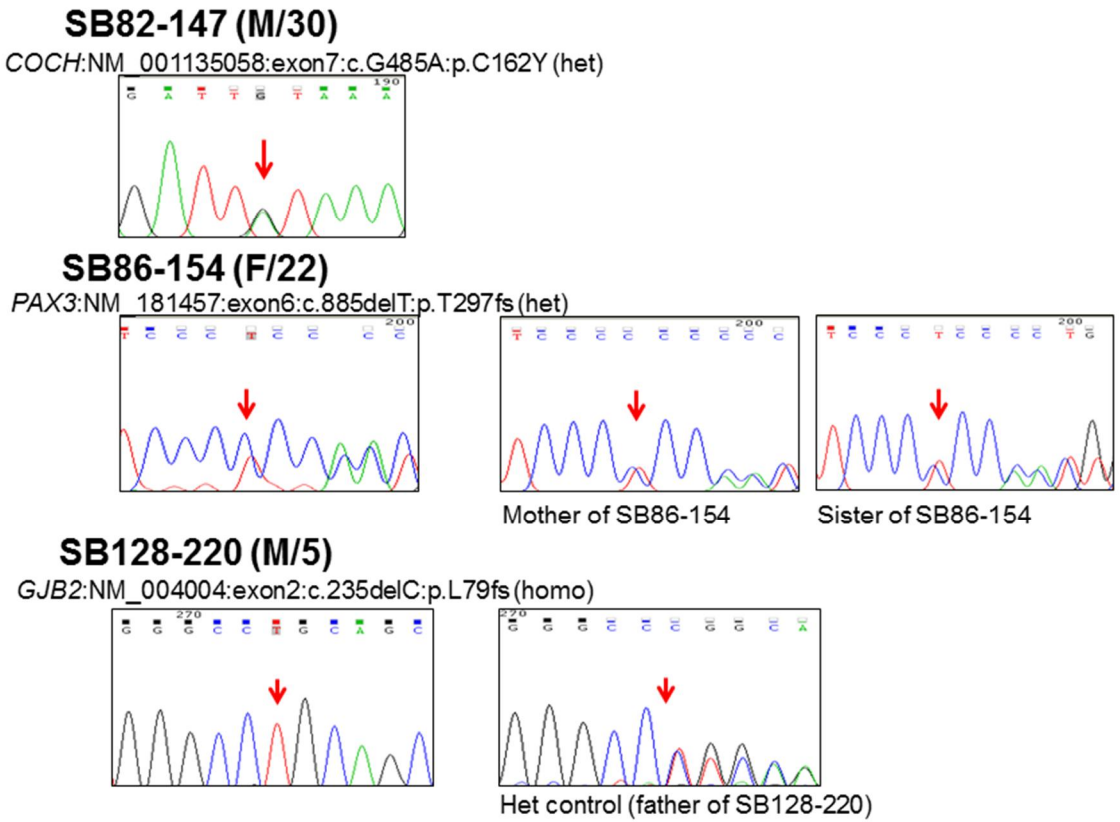


Figure 1-4.

Validation of candidate mutations by PCR-Sanger sequencing.

Candidate mutations of *COCH*, *PAX3*, and *GJB2* in three subjects and two affected family Members (SB86) with sensorineural hearing loss are shown in Sanger sequencing chromatograms.

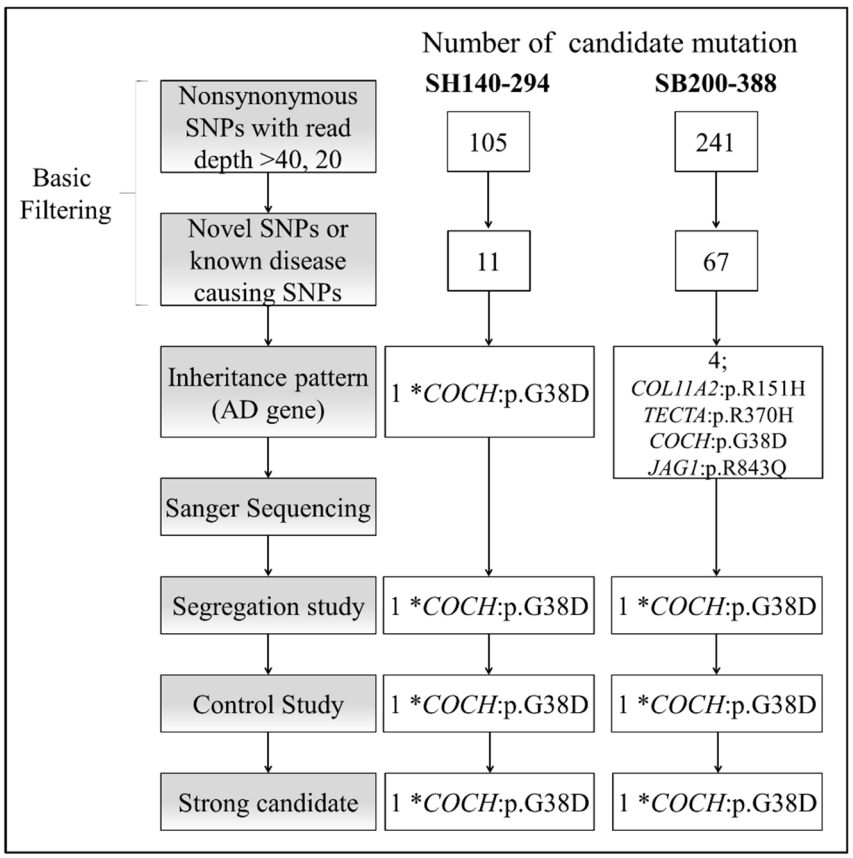


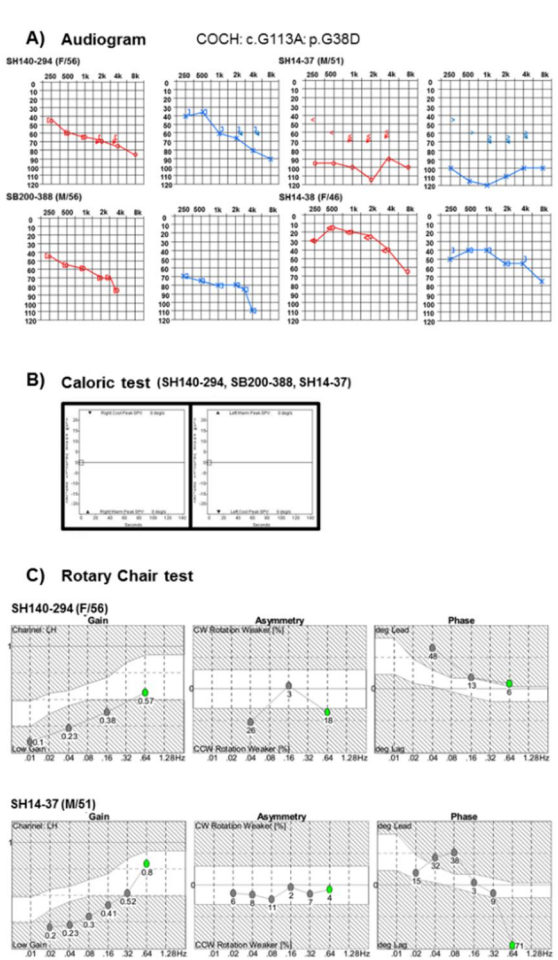
Fig 2-1.

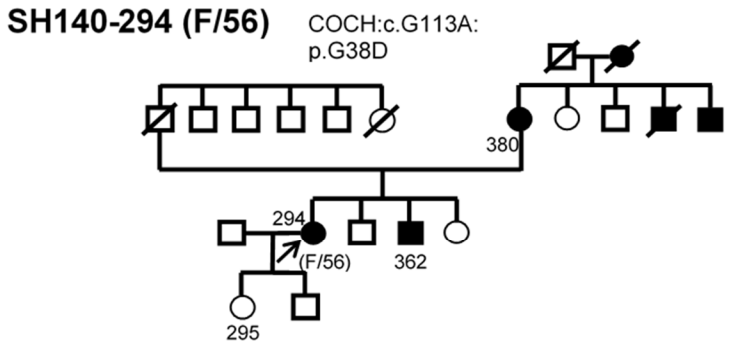
Process of filtering COCH:

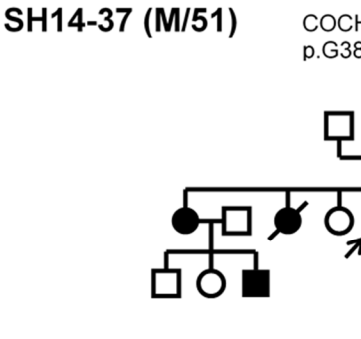
p.G38D from 105 to 241 candidate mutations to one strong, responsible candidate in SH140–294 and SB200–388, respectively

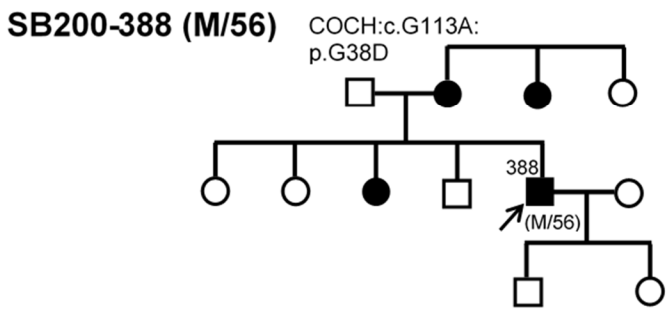
Figure 2-2.

Pedigree analysis in SH-140, SH-14, SB-200, and SB-82 with sensorineural hearing loss:
pedigrees showing the autosomal-dominant inheritance pattern: filled symbols represent hearing-impaired individuals, and clear symbols denote those with normal hearing. The black arrow indicates the proband









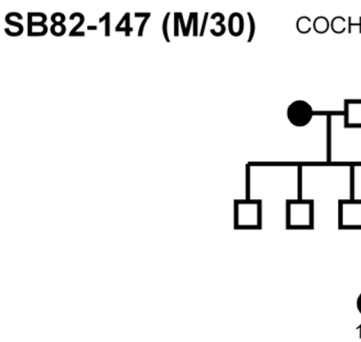
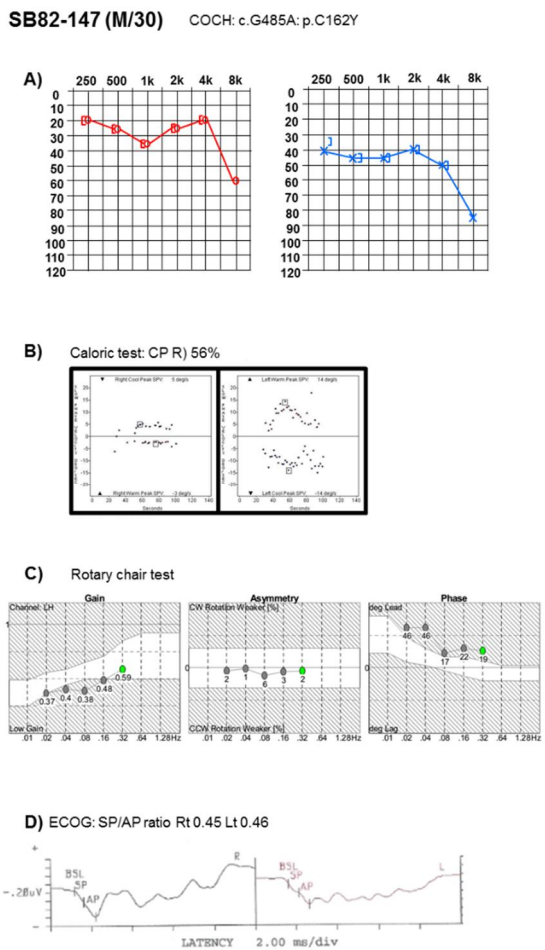


Figure 2-3.

Audiogram and vestibular function test results in SH-140, SB-200, and



SH-14 with sensorineural hearing loss:

A) right (red) and left (blue) ear hearing thresholds.

B, C) Bithermal caloric test shows no response bilaterally, and rotatory responses exhibit reduced gain at all frequencies except 0.64 Hz and increased phase leads without asymmetry, which is compatible with the diagnosis of bilateral vestibulopathy.

Figure 2-4.

Audiogram, and vestibular function test results in SB-82 with sensorineural hearing loss:

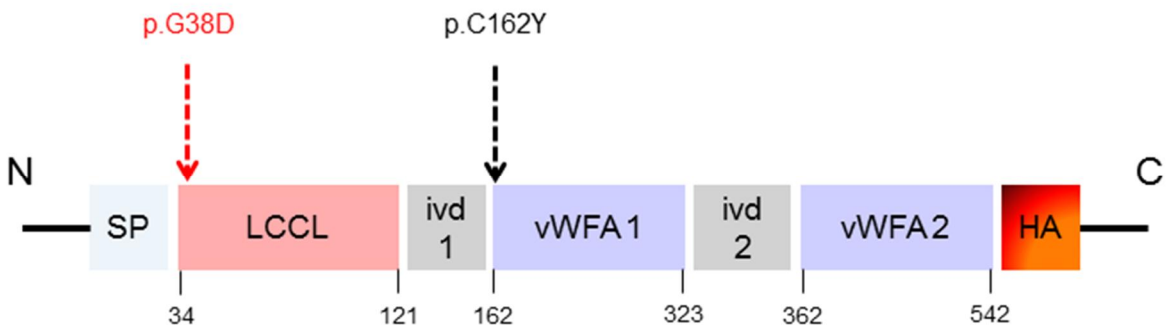
A) right (red) and left (blue) ear hearing thresholds.

B–D) Bithermal caloric test shows reduced response on the right side with canal paresis of 56 %, which might be interpreted as an irritative phase on the left side.

The rotary chair test result also favors the diagnosis of vestibular hypofunction, and the ECOG result shows a bilateral enlarged summating potential (SP): action potential (AP) ratio of 0.45 and 0.46 on the right and left sides, respectively, suggesting bilateral MD.

Figure 2-5.

Schematic view of cochlin domain structure showing the location of mutations in this study (p.G38D with the red dotted arrow and p.C162Y with the black dotted arrow)


p.G38D
p.C162Y
N
C
SP
LCCL
ivd 1
vWFA1
ivd 2
vWFA2
HA
34
121
162
323
362
542

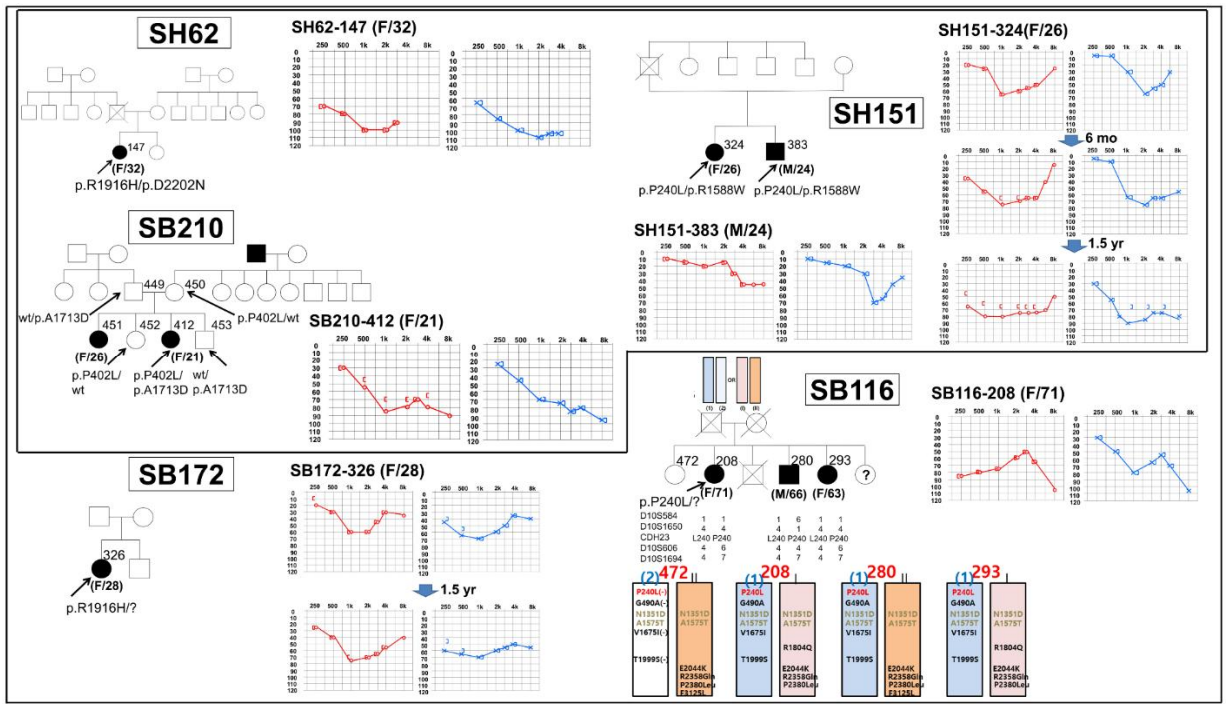
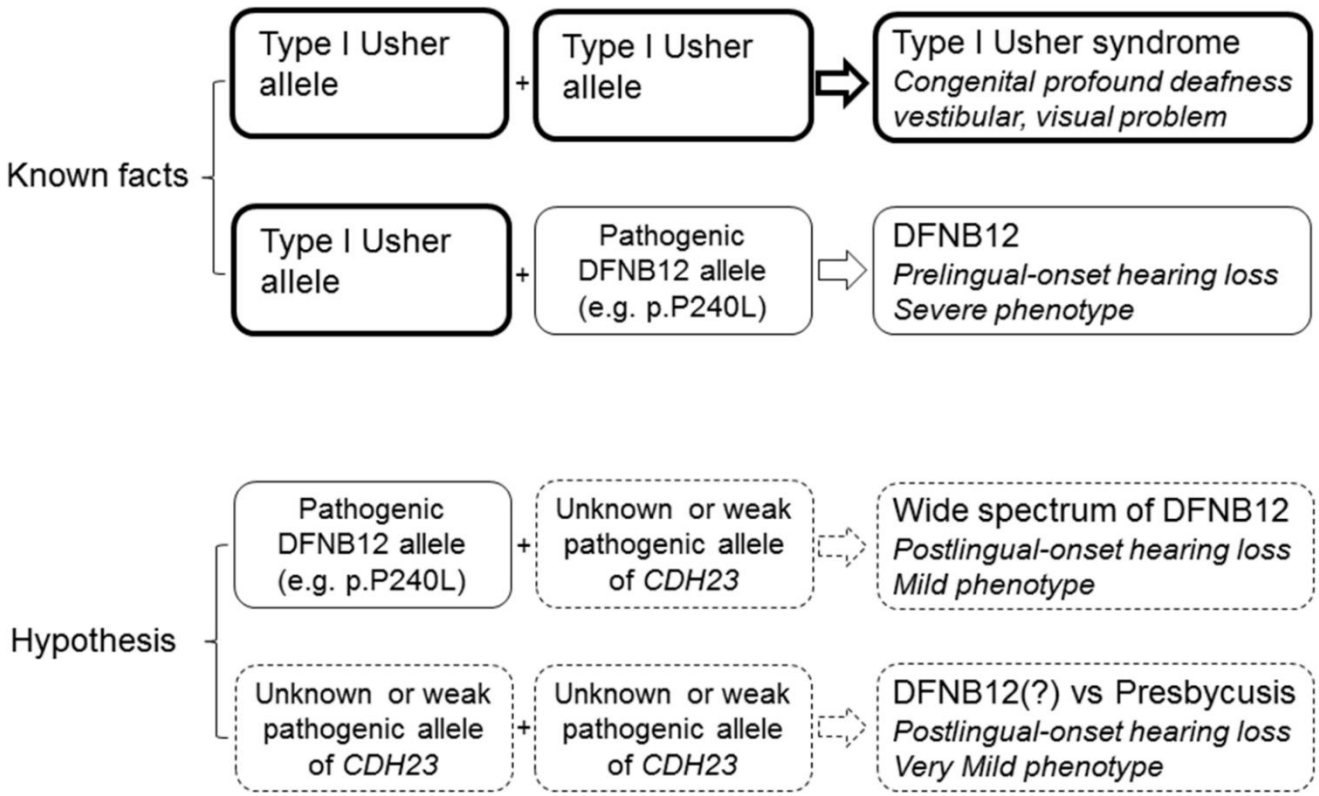


Figure 3-1. Pedigrees and audiograms of subjects from 5 families possibly carrying compound heterozygous *CDH23* mutations

Audiogram: Right (red) and left (blue) ear hearing thresholds. Pedigree: Filled symbols represent hearing-impaired individuals, and clear symbols denote those with normal hearing. Black arrow indicates the proband. Possible arrangement of *CDH23* variants in SB116 family based on haplotype and segregation study.

Figure 3-2.



Suggestion of Genotypic Hierarchy of *CDH23* mutations deciding the fate of USH1D and DFNB12.

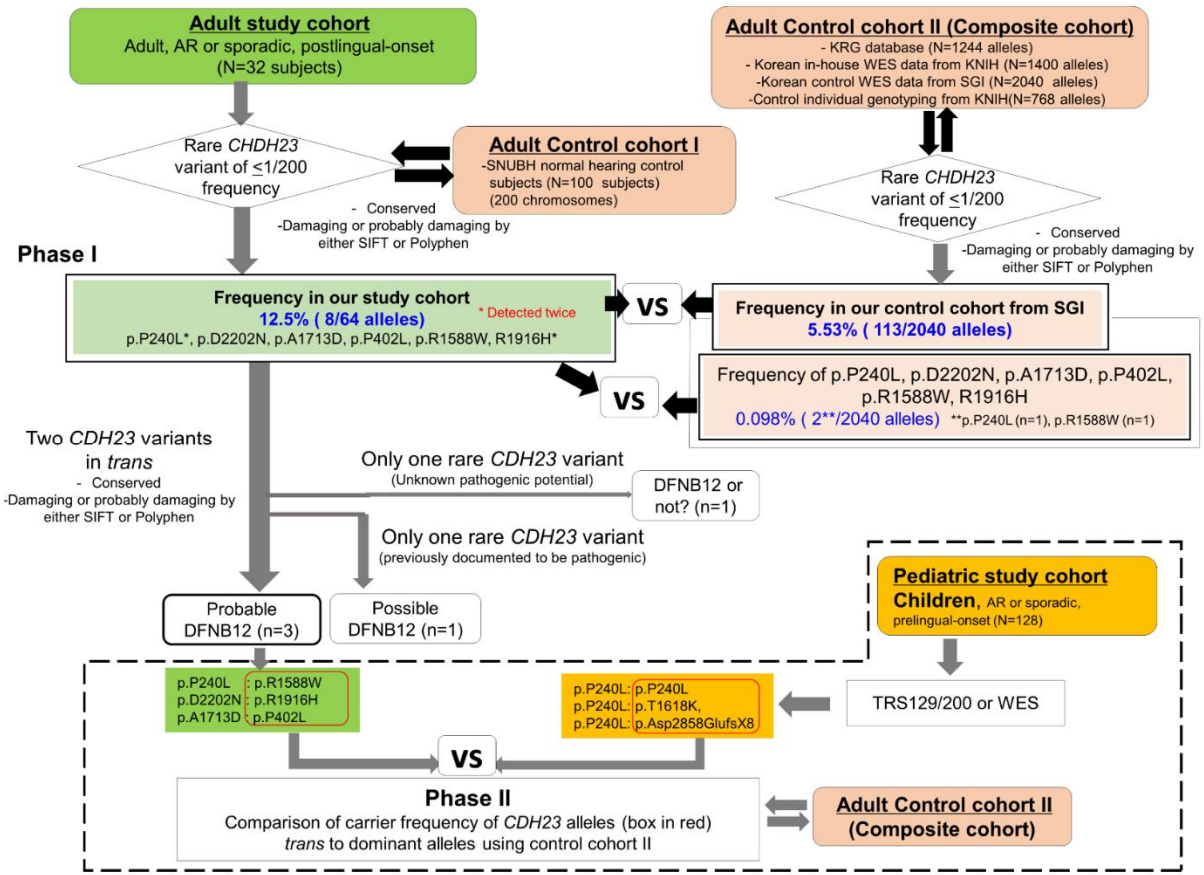


Figure S 3-1 Flow chart of the study combining Phase I and II:
Phase I: Comparison of carrier frequency of potentially pathogenic *CDH23* alleles between our postlingual adult SNHL cohort and the control cohort.
Phase II: Comparison

of MAF between *CDH23* variants detected in postlingual adult SNHL cohort and prelingual pediatric SNHL cohort among the composite control cohort.

국문 초록

감각신경성 난청은 가장 흔한 감각신경성 질환이며 신생아 500명당 약 1명에서 발생한다. 이 중 유전성 난청이 적어도 약 50%에서 그 원인을 차지한다. 대부분의 유전성 난청이 멘델리안 유전양식을 따르는 일유전자성 원인을 가지므로, 유전성 감각신경성 난청을 가진 환자를 진단하는데 분자유전학적 진단이 유용하다.

적용하기 쉽다는 점 때문에, 표현형 중심 후보 유전자 접근법이 널리 사용되어왔고, 특히 연관 자료가 없는 작은 또는 중간 크기의 가족에서 분자유전학적으로 난청을 진단하는데 유용했다. 그러나, 같은 유전자의 변이라도 표현형 모사 또는 다양한 표현형이 변이의 위치에 따라 나타날 수 있다. 또한 표현형이 외래에서 환자를 볼 때 항상 명확하게 나타나는 것은 아니라서 표현형 중심 후보 유전자 접근법이 유용하지 않은 경우가 많다.

차세대 염기서열 분석이 도입되면서 유전자의 대량 분석이 가능해졌고, 연구팀은 이를 이용하여 특별한 표현형이 없는 감각신경성 난청에서 원인 유전자를 찾기 위한 80개의 난청유전자를 이용한 표적 리시퀀싱 방식을 확립했다.

본 연구에서는 특징적인 표현형을 가짐에도 표현형 중심 후보 유전자 접근법으로 진단되지 않은 경우, 표적 리시퀀싱을 시행함으로써 이 방식의 효용 및 장점을 확인하였다 (Part 1). 다음으로 DFNA9 난청과 연관된 *COCH* 유전자의 유전형-표현형 상관성을 129개 또는 200개의 유전자를 이용한 표적 리시퀀싱 방법으로 확인하였다 (Part 2). 마지막으로 표적 리시퀀싱 방식으로 분자유전학적 진단을 하고, 유전 역학적인 방법을 분석

에 사용함으로써 한국인 언어습득 이후 진행성 난청에서 *CDH23* 유전자의 중요성을 규명하였다.

표현형 중심 후보 유전자 접근법으로 원인 변이를 밝히지 못한 6명의 환자 중 세 명의 환자 (SB82-147, SB86-154, SB128-220)에서 그 원인 변이를 밝힘으로써 표적 리시퀀싱 방법은 50%의 진단율을 보였고, 진단이 되지 않은 나머지 환자들은 전체 엑솜 시퀀싱을 시행하였다 (Part 1). 표적 리시퀀싱 방식으로 우성 유전양식 한국인 난청 코호트에서 DFNA9의 비율이 12.8%에 이르는 것을 밝혀냈고, *COCH* 유전자 변이의 위치에 따라 어지럼 표현형이 달라지고, p.G38D 변이가 양측성 완전 전정기능 소실과 관계됨을 밝혀냈다 (Part 2). 열성 유전양식 또는 산발적으로 발생하는 언어습득 이후 발생한 난청을 가진 32명의 한국인 코호트에서, *CDH23* 유전자 변이의 빈도는 12.5%로 2040개의 정상 유전자에서의 빈도인 5.53%보다 의미 있게 높았고, 이는 언어습득 이후 비증후군성 난청에서 *CDH23* 유전자의 기여도를 보여준다 (Part 3).

본 연구에서는 유전성 난청이 명확한 다중 가족에서 표현형 중심 후보 유전자 접근법에 비해 표적 리시퀀싱 방식의 우수성을 보여주었고, 또한 표적 리시퀀싱 방법을 이용해 *COCH* 유전자 관련 난청에서 유전형-표현형 상관관계를 규명할 수 있었다. 더 나아가 과거에는 유전성 난청으로 진단이 어려웠던 열성 유전양식이나 산발성으로 주로 성인 시기에 발생하는 *CDH23* 유전자 관련 비증후군성 난청에서도 분자유전학적 진단 뿐 아니라, 그 기여도도 유전역학적인 방식을 이용해 보여줌으로써 표적 리시퀀싱 방식의 적용 범위를 넓힐 수 있었다. 이러한 유전성 난청의 진단 방법 발전과 더불어, 유전자 치료, 줄기세포 치료에 기반을 둔 유전성 난청의 치료법도 활발히 연구되고 있다.

중심 단어: 유전성 난청, 표적 리시퀀싱, *COCH*, *CDH23*

학번: 2012-31140