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일측성 난청의 분자유전학적 원인:
색소 이상 질환과 와덴버그증후군과의 관련성

Molecular Etiology of Hereditary Single Side Deafness: Its Association with Pigmentary Disorders and Waardenburg Syndrome

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Molecular Etiology of Hereditary Single Side Deafness: Its Association with Pigmentary Disorders and Waardenburg Syndrome

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Abstract

Molecular Etiology of Hereditary Single Side Deafness: Its Association with Pigmentary Disorders and Waardenburg Syndrome

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Introduction: Unilateral sensorineural hearing loss (USNHL)/single side deafness (SSD) is a frequently encountered disability in children. The etiology of a substantial portion of USNHL/SSD still remains unknown, and genetic causes have not been clearly elucidated. In this study, the authors evaluated the heritability of USNHL/SSD.

Methods: The authors sequentially recruited 50 unrelated children with SSD. For an etiologic diagnosis, we performed a rigorous review on the phenotypes of family members of all children and conducted, if necessary, molecular genetic tests including targeted exome sequencing of 129 deafness genes and whole exome sequencing.

Results: Among the 50 SSD children cohort, the authors identify four (8%) unrelated SSD probands from four families (SH136, SB173, SB177, and SB199) with another hearing impaired family members. Notably, all four

probands in our cohort with a familial history of SSD also have pigmentary abnormalities such as brown freckles or premature gray hair within 1st degree relatives, which may indicate that genes whose products are involved with pigmentary disorder could be candidates for heritable SSD. Indeed, SH136 and SB199 turned out to segregate a mutation in *MITF* and *PAX3*, respectively, leading to a molecular diagnosis of Waardenburg syndrome (WS).

Conclusion: We report, for the first time in the literature, a significant heritability of pediatric SSD. There is a strong association between the heritability of USNHL/SSD and the pigmentary abnormality, shedding a new light on the understanding of the molecular basis of heritable USNHL/SSD. In case of children with congenital SSD, it would be mandatory to rigorously screen pigmentary abnormalities. WS should also be included in the differential diagnosis of children with USNHL/SSD, especially in a familial form.

Keywords: unilateral sensorineural hearing loss, single side deafness, heritability, pigmentary disorder, Waardenburg syndrome

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Auditory brainstem response (ABR)

Apoptosis inducing factor, mitochondria associated 1 (*AIFM1*)

Auditory steady state response (ASSR)

Endothelin receptor, type B (*EDNRB*)

Genomic evolutionary rate profiling (GERP)

Internal auditory canal magnetic resonance imaging (IAC MRI)

Microphthalmia associated transcription factor (*MITF*)

Narrow bony cochlear nerve canal (nBCNC)

National Health and Nutrition Examination Survey (NHANES)

Paired box 3 (*PAX3*)

Pure tone audiometry (PTA)

Speech audiometry (SA)

Snail homolog 2 (*SNAI2*)

Sensorineural hearing loss (SNHL)

Seoul National University Hospital (SNUH; SH)

Seoul National University Bundang Hospital (SNUBH; SB)

Single-side deafness (SSD)

Solute carrier family 26 member 4 (*SLC26A4*)

SRY (sex-determining region Y) box 10 (*SOX10*)

Temporal bone computed tomography (TBCT)

Targeted exome sequencing (TES)

TES of 129 deafness genes (TES-129)

University of California Santa Cruz (UCSC)

Unilateral sensorineural hearing loss (USNHL)

Whole exome sequencing (WES)

Waardenburg index (WI)

Waardenburg syndrome (WS)

Waardenburg syndrome, type 1 (WS1)

Waardenburg syndrome, type 2 (WS2)

Waardenburg syndrome, type 3 (WS3)

Waardenburg syndrome, type 4 (WS4)

Introduction

Unilateral sensorineural hearing loss (USNHL) is defined as an average pure tone air conduction threshold of more than 20 dB HL at 0.5, 1, and 2 kHz with the good ear less than 15 dB HL (Bess, 2005). Single side deafness (SSD) is an extreme form of USNHL and is defined as sensorineural profound hearing loss (>90 dB HL) in the affected side, while pure tone averages of 0.5, 1, 2, and 3 kHz for the good ear should be better than 20 dB HL (Cire, 2010). Unilateral hearing loss is estimated to occur in 0.83 in 1000 newborn children (Prieve et al., 2000). In the National Health and Nutrition Examination Survey (NHANES) III conducted in USA from 1988 to 1994, 3% of children aged 6 to 19 years suffered from unilateral hearing loss (Niskar et al., 1998).

Recent studies suggest that a significantly increased proportion of children with USNHL/SSD may experience educational and behavioral problems relative to normal-hearing children. Children with USNHL/SSD seem to have delay of speech and language development, increased grade failures, need for additional educational assistance, and perceived behavioral issues in the classroom (Martínez-Cruz et al., 2009). Children with USNHL/SSD may present lower intelligence coefficients than children with bilateral normal hearing (Judith et al., 2004).

The etiology of 35% to 60% of USNHL cases still remains unknown (Kinney, 1953; Everberg, 1960; Brookhauser et al., 1991). The most commonly reported etiologies of USNHL include complication of viral infection, sequelae of bacterial meningitis, head trauma, prenatal or perinatal

problems, and even genetic alterations. Genetic causes accounting for USNHL have not been clearly elucidated.

More than 150 genes for deafness have been mapped to chromosomal regions, and alterations in any of these genes usually resulted in bilateral sensorineural hearing loss (SNHL). Mutations of *SLC26A4* (NM_000441, solute carrier family 26 member 4) can sometimes cause unilateral SNHL; however, the hearing thresholds of better hearing in these cases frequently worsen over time, leading to bilateral SNHL in many cases (Coyle et al., 1996). One of the most frequent etiologies of congenital USNHL is cochlear nerve agenesis/hypoplasia associated with a narrow bony cochlear nerve canal (nBCNC) (Fatterpekar et al., 2000). However, unilateral nBCNC or cochlear nerve agenesis/hypoplasia in the Korean population was not considered to be genetic based upon the very low sibling recurrence rate of the phenotype as opposed to the 20% for bilateral cases (Cho et al., 2013).

Among the syndrome related with hearing loss, Waardenburg syndrome (WS) patients showed mostly profound bilateral SNHL. USNHL/SSD was anecdotally reported in some of Waardenburg syndrome (WS) patients, but there have not been many reports that rigorously describe the audiological phenotypes of WS (Newton, 1990). The aim of this study was to calculate the proportion of definite hereditary cases among the total USNHL/SSD subjects in Koreans. Through this, we tried to identify association between the heritability of USNHL/SSD and pigmentary disorders including WS.

Materials and methods

Subjects and ethical statements

This study was approved by the institutional review boards at Seoul National University Bundang Hospital (IRB–B–1007–105–402) and Seoul National University Hospital (IRBY–H–0905–041–281). First, we have recruited 50 unrelated children (<15 years of age) with SSD as documented by the audiological examination in Seoul National University Hospital (SNUH; SH) or Seoul National University Bundang Hospital (SNUBH; SB), from January 2012 through July 2014. The audiological and neurotological examinations were composed of pure tone audiometry (PTA), speech audiometry (SA), auditory brainstem response (ABR), or auditory steady-state response (ASSR).

Clinical evaluation

A comprehensive clinical history taking and audiological, neurotological, ophthalmological, and dermatological examinations were performed on all 50 children with SSD. The audiological and neurotological examinations consisted of otoscopy, PTA, SA, ABR, and ASSR. All the 50 children were asked whether they had any family members within first degree who manifested any prominent syndromic feature, such as ophthalmologic abnormality, lateral displacement of eyes, depigmentation of skin, freckled face, or early graying of the hair. In addition, we investigated whether there were either siblings or parents with bilateral or unilateral hearing loss from the

50 children to address the potential hereditary component of SSD. The association between pigmentary abnormality and heritable USNHL/SSD was estimated by Fisher's exact test, and the *P* values less than 0.05 were considered significant.

To make a clinical diagnosis of WS, we relied on the criteria proposed by the WS consortium (Farrer et al., 1992). The presence of a lateral displacement of the inner canthi of eyes was a differential point between the WS type 1 (WS1; OMIM 193500) and WS type 2 (WS2; OMIM 193510). The Waardenburg index (WI) was calculated as below, and the WI value of greater than 2.07 (or 1.95 with a *PAX 3* mutation) meant WS1 in this study (Milunsky, 2001).

$$WI = X + Y + a/b$$

$$X = (2a - (0.2119c + 3.909))/c$$

$$Y = (2a - (0.2479b + 3.909))/b$$

a: inner canthal distance, b: interpupillary distance, c: outer canthal distance

We also performed a temporal bone computed tomography (TBCT) or internal auditory canal magnetic resonance imaging (IAC MRI) to identify, whether any, nBCNC, or cochlear nerve agenesis/hypoplasia, or enlarged vestibular aqueduct from our cohort with SSD.

DNA preparation

Informed consent and blood samples were obtained from the 4 probands with at least 1 additional affected first-degree relative and also from their family members. Genomic DNA was extracted from probands and their family members' peripheral blood using the Gentra Puregene Blood Kit (Quiagen, CA, USA) (Choi et al., 2013). The Cytology Brush (MPC, CA, USA) was sent to the family members who could not visit the outpatient clinic, and the genomic DNA was extracted from their family members' buccal cell using the Gentra Buccal Cell Kit (Quiagen, CA, USA).

Mutational analysis

To make a molecular genetic diagnosis, direct Sanger sequencing, targeted exome sequencing (TES), and/or whole exome sequencing (WES) were implemented. Direct Sanger sequencing of *PAX3* (NM_181457, paired box 3) was performed on SB199 family with suspicion of WS1 (Bondurand et al., 2000). TES of 129 deafness genes (TES–129) was performed (Otogenetics, GA, USA) on SH136 family, and subsequent Sanger sequencing of *MITF* (NM_000248, microphthalmia associated transcription factor) was performed to verify the suspicious variant of *MITF*.

Direct Sanger sequencing of *MITF*, *SOX10* (NM_006941, *SRY* [sex-determining region Y] box 10), *EDNRB* (NM_277580, endothelin receptor type B), and *SNAI2* (NM_602150, snail homolog 2) were performed on SB173 and SB177 families with suspicion of WS2 (Tassabehji et al., 1994;

Attie et al., 1995; Sanchez-Martin et al., 2002; Bondurand et al., 2007). WES was subsequently performed (LAS, Gimpo, Korea) on SB173 and SB177 families to find any mutations in other genes.

If a variant was found, each gene was sequenced and compared with previously reported sequence. To estimate the evolutionary conservation of amino acid sequence, we refer to Genomic Evolutionary Rate Profiling (GERP) score in University of California Santa Cruz (UCSC) genome browser [http://genome.ucsc.edu]. Cosegregation of the detected mutation among family members was validated by Sanger sequencing. The 160 unrelated Korean control chromosomes were checked to see whether the variant was common or not (Fig. 1).

Results

Multiplex families segregating SSD in children

Among the 50 SSD children, we were able to identify 4 (8%) unrelated young probands (SH136–282, SB173–329, SB177–336, and SB199–386) with at least 1 additional first-degree relative manifesting SSD through comprehensive history taking and multidisciplinary physical examination. Affected members in SH136 and SB199 family showed a significant intra-familial variability in terms of the auditory phenotype (Fig. 2A,B). The proband (SH136–282), his mother (SH136–284), and another proband (SB199–386) showed SSD; however, SH136–285, SH136–283, and SB199–387 manifested USNHL (Fig. 2A,B). However, all the affected siblings of SB173 and SB177 consistently showed SSD (Fig. 3). Parents (SB177–338 and father of SB173–329) of the affected siblings with SSD show premature gray hair and/or freckled face which might manifest as a sign of WS.

Imaging studies from the 4 probands revealed heterogeneous findings. Most of the 50 SSD children except some cases that were detected through newborn hearing screening noticed their hearing loss in their elementary school age (7–12 years old) even though their USNHL/SSD was presumed to be congenital. SH136–284 and SB199–386 had a normal cochleovestibular nerve, while SB173–329 and SB177–337 showed nBCNC and cochlear nerve agenesis/hypoplasia on TBCT and IAC MRI, respectively (Fig. 4).

Signs suggesting WS and pigmentary disturbances

Interestingly, heritable SSD was significantly associated with pigmentary abnormalities, that is, the presence of brown freckles or premature gray hair within the first-degree relative of the probands. In detail, when we expanded our investigation into the siblings and parents of 50 children with SSD, all 4 families with at least 2 members with documented USNHL/SSD (SH136, SB199, SB173, and SB177) manifested pigmentary abnormalities with varying degrees (Table 1).

In the other unrelated 46 children and their first-degree relatives, there were no syndromic features such as brown freckles or premature gray hair. In this study, an observation of the early onset of brown freckles and premature gray hair from a proband or first-degree relative of the proband was much more frequent from familial USNHL/SSD cases than those without the family history (Fisher exact test, P=0.000119). Indeed, the pigmentary disturbances were exclusively from the probands with a family history of USNHL/SSD.

SH136 family segregated brown freckles and/or premature gray hair, as well as SNHL, which satisfies the previously proposed diagnostic criteria of WS2 (Fig. 2A and Fig. 5A,B) (Liu et al., 1995). The brown freckles observed in SH136, SB199, and SB177 characteristically started in their first decade (Fig. 5A–C,I,J), which differentiated this lesion from a simple dyschromia. The freckles were characteristically limited to the face and the extremities, not involving the trunk. In addition to a sib pair with SSD, SB177 also segregated brown freckles and/or premature gray hair (Fig. 3 and Fig. 5K,L). Graying of

the hair typically started in their third or fourth decades in these four families, and also observed as early as at the age of 12 (Fig. 5G,H).

The constellation of the signs from SB177 possibly suggests WS2. However, the phenotypes did not satisfy the previously proposed diagnostic criteria for WS2, making a clinical diagnosis of this family elusive. A phenotype of lesser degree, but possibly suggesting WS2 was also observed from SB173, which had a father (father of SB173–329 and SB173–329–1) with premature gray hair starting in their twenties (Fig. 3).

SB199 segregated dystopia canthorum (Fig. 5D–F), confirmed by a higher WI than 2.07, in addition to USNHL/SSD, freckled face, and/or premature gray hair (Fig. 2B and Table 1). Moreover, SB199–392 showed heterochromia iridium, which means 2 different colored eyes (Fig. 5F), which made clinical diagnosis of SB199 as WS1. Heterochromia iridium was not observed in any of the family members of SH136, SB173, and SB177.

Molecular genetic test

We searched for a molecular genetic etiology of USNHL/SSD in these 4 families. As they had pigmentary disturbances as well as inherited USNHL/SSD and some of them had signs suggesting either WS1 or WS2, we performed direct Sanger sequencing of *PAX3* (SB199), TES–129 (SH136), or WES (SB173 and SB177). Sanger sequencing of *PAX3* from SB199 identified a previously reported pathogenic missense variant of c.668G>A (p.R223Q) of *PAX3* (Fig. 6) (DeStefano et al., 1998). This variant perfectly cosegregated

with the WS1 phenotype, confirming a pathogenic role of this variant. TES-129 from SH136 identified a previously reported pathogenic mutation of *MITF*, c.763C>T (p.Arg255X), to cosegregate with the WS2 phenotype (Fig. 6).

In contrast, Sanger sequencing of *MITF*, *SOX10*, *EDNRB*, and *SNAI2* could not identified any convincing mutations in SB173 and SB177 families. For the next, WES was performed, and mutations of *EDNRB*, c.257C>G (p.Ser86Tyr), were found in both SB173 and SB177 families. However, subsequent Sanger sequencing of *EDNRB* in SB173–329, SB177–336, SB177–337, and SB177–338 did not reveal any *EDNRB* variant.

Discussion

This is the first study that addresses the SSD heritability from a homogenous cohort. We identified that at least 8% (4 of 50) of SSD probands in our cohort had a genetic etiology as suggested by the presence of USNHL/SSD in additional first-degree relative. Among the 4 families, SB199 and SH136 turned out to segregate WS1 and WS2, respectively, as confirmed by detection of the causative mutation. Therefore, about 4% of SSD probands were calculated to have a genetically proven WS.

WS has an incidence of approximately 1/40,000 births and is responsible for 1% to 3% of cases of congenital deafness (Pingault et al., 2010). WS is a neural crest cell disorder associated with dystopia canthorum, pigmentary abnormalities of the skin, hair, and iris, and sensorineural deafness (Yang et al., 2013). There are 4 subtypes of WS. WS1 and WS2 are the most common types of the syndrome, whereas WS type 3 (WS3; OMIM 1448820) and WS type 4 (WS4; OMIM 277580) are rare. All of 4 subtypes have been characterized by deafness and pigmentary disturbance.

WS1 and WS2 are distinguished by whether dystopia canthorum presents or not (Bondurand et al., 2000). Dystopia canthorum, which describes the lateral displacement of inner canthi of the eyes, is a pathognomonic finding of WS1. This may not be easily noticeable without the measurement of WI, especially in East Asian children with epicanthal folds and broad flat nose. WS2 is characterized by a normally placed medial canthi and the most common autosomal dominantly inherited syndrome with hearing loss among

the WS. WS3 and WS4 are characterized by the presence of limb abnormalities and aganglionic megacolon, respectively.

There were reports that sensorineural deafness and heterochromia iridium are the most common findings in Chinese WS2 patients (Chen et al., 2010; Yang et al., 2013). However, none of SH136 showed heterochromia iridium. Instead, in family SH136, premature gray hair was observed in all 10 adult subjects with SNHL (10/10, 100%), except for 2 young subjects. Freckled face was also detected in a significant proportion (9/12, 75%) (Fig. 2). Only 1 (SB199–392) of the 3 WS1 subjects from SB199 showed blue iris, even though it was reported that heterochromia iridium was more frequent in WS2 than in WS1 (Read et al., 1997).

It is worth noting that freckled face could orientate the genetic screening to specific genes (eg, the genes involved in WS1 and WS2) in young subjects from 2 families with USNHL/SSD (SH136 and SB199). For example, freckled face was the only syndromic feature of SH136–282 who manifested SSD (Fig. 2A). This could also be the case for young East Asian children with WS1 (eg, SB199–386) (Fig. 2B), in whom dystopia canthorum could be mistakenly overlooked due to commonly occurring epicanthal folds and broad flat nose.

WS1 is due to mutations in the *PAX3* gene, whereas some WS2 cases are usually associated with mutations in the *MITF* gene (Yan et al., 2011). The *PAX3* gene is known to directly regulate the *MITF* gene expression (Watanabe et al., 1998; Bondurand et al., 2000). While the mutation of *MITF* detected in WS2 appears to specifically affect survival, proliferation, and differentiation

of melanocytes, *PAX3* defects affect other neural crest cell derivatives, resulting in additional features of craniofacial malformations such as dystopia canthorum. To date, alterations of 4 genes have been reported to be associated with the development of WS2: *MITF*, *SOX10*, *EDNRB*, and *SNAI2*. Nevertheless, mutations of these 4 genes are considered to account for only about 30% of total WS2, *MITF* (dominant transmission) and *SOX10* (dominant with neurological features) each being responsible for 15% of WS2, and *EDNRB* (dominant with incomplete penetrance) in a small percentage and *SNAI2* (recessive) in another small percentage (Read et al., 1997; Pingault et al., 2010; Yang et al., 2013).

The hearing loss in WS is sensorineural and congenital (Newton, 1990). As for the degree and the laterality of SNHL, the most common type is profound bilateral SNHL (>100 dB) (Milunsky, 2001). In detail, laterality and degree of SNHL also showed a significant inter-familial and intra-familial variability. With regard to this variable audiological phenotype, stochastic variation does not seem to solely account for the differences in the penetrance of deafness in WS families.

Genetic backgrounds in combination with certain *PAX3* alleles is known to be important factors with comparing the probabilities for deafness in affected subjects from 24 WS1 families having *PAX3* mutations (Morell et al., 1997). WS2 also has been noted to display a broad spectrum of SNHL in terms of degree and pattern (Chen et al., 2010; Haddad et al., 2011; Yan et al., 2011; Yang et al., 2013). Most of the *MITF* mutations reported in the literature cause truncation likely as our reported p.Arg255X variant (Chen et al., 2010;

Wildhardt et al., 2013). This Arg residue is located in the helix-loop-helix leucine zipper (b-HLH-Zip) domain, the basic region of which would bind to a sequence-specific DNA in promoters and mediate the interactions required for DNA binding (Takeda et al., 2000).

On the previous study that reported 3 Chinese affected subjects carrying the same c.763C>T variant of *MITF*, 2 had moderate or profound hearing loss on both ears, while the other manifested SSD. The 2 subjects had brown freckle and heterochromia iridium, but the other had normal color of skin and iris (Yang et al., 2013). The variable clinical and auditory phenotypes could be mediated by genetic background or specific modifiers, as most patients with *MITF* mutations show variable penetrance of WS2 associated phenotypes, even within families segregating the same mutation.

It is reported that hearing loss in WS is typically nonprogressive (Milunsky, 2001). This stability of hearing status in the contralateral side based on molecular diagnosis for WS is of paramount importance when we consider a bone conduction implantable hearing aid for these patients with SSD. In this study, the Sophono® Alpha 2 MPO™ bone conduction hearing device (Medtronic, CO, USA) was implanted in SB199–386, based on this stable feature of hearing loss in WS.

To further address the etiologic mechanism of USNHL/SSD in the 4 families with heritable SSD, we also performed TBCT and IAC MRI. SH136–282 and SB199–386 did not carry any noticeable abnormality of inner ear and cochlear nerve. Inner ear deformities do not appear to be a characteristic for all types of documented WS (Kontorinis et al., 2014). Therefore, intact inner

ear and cochlear nerve from SH136 and SB199–386 cosegregating a mutation in *MITF* and *PAX3*, respectively, is compatible with previous reports. Interestingly, all of the affected family members from SB173 and SB177 without any mutation in *MITF*, *SOX10*, *EDNRB*, and *SNAI2* showed nBCNC and cochlear nerve agenesis/hypoplasia at the affected side (Fig. 4).

No convincing variant in *MITF*, *SOX10*, *EDNRB*, and *SNAI2* was detected from SB173 and SB177, making molecular genetic etiology and final diagnosis of these 2 families elusive. Considering the brown freckles and premature gray hair observed in SB173 and SB177, the nBCNC and cochlear nerve agenesis/hypoplasia detected from these 2 families can be a novel subtype of WS2 or different disease entity related to pigmentary disturbance. The possibility that inner ear deformities could be related to certain subtypes of WS has already been raised (Madden et al., 2003; Elmaleh-Berges et al., 2014). For example, there has been a report that *SOX10* mutations causing approximately 15% of WS2 and > 50% of WS4 were associated with agenesis or hypoplasia of the semicircular canals, enlarged vestibules, and cochlear deformity (Elmaleh-Berges et al., 2014). Of 14 patients with WS and *SOX10* mutations, 2 showed bilateral cochlear nerve agenesis/hypoplasia, and 1 showed unilateral cochlear nerve agenesis/hypoplasia.

Alternatively, SSD in SB173 and SB177 families could be completely unrelated to WS, resulting from an alteration of other recessive deafness genes, and brown freckles and premature gray hair detected from these families may have been coincidental. In these two families, there was no detected mutation such as c.1352G>A (p.R451Q) in *AIFM1* (NM_004208, apoptosis inducing

factor, mitochondria associated 1) which known to be related with cochlear nerve agenesis/hypoplasia on WES (Zong et al, 2015). Our previous study showed that a unilateral nBCNC without any syndromic feature is least likely to have a genetic etiology based on a very low sibling recurrence risk (close to zero) (Cho et al., 2013). However, on the basis of a statistical association between family history of SSD and pigmentary disturbances, such as brown freckles and premature gray hair in our Korean cohort, SSD that is related to nBCNC and cochlear nerve agenesis/hypoplasia may have a genetic etiology if it is accompanied by a pigmentary disorder.

The deficiency of melanocytes in WS, which are neural crest derivatives, is responsible not only for the observed pigmentation defects but also for high incidence of deafness. The migration of melanocytes from the neural crest during the 18^{th} gestational week gives rise to the intermediate cells of the stria vascularis. The failure of melanocyte migration in WS causes the absence of the intermediate cells. This failure is associated with atrophy of the stria vascularis and cochleosaccular degeneration, and presumed to be related to the hearing loss (Steel et al., 1989). According to the previous study about temporal bone abnormalities associated with hearing loss in WS, computed tomography measurements disclosed statistically significant widening of the upper vestibule, narrowing of the IAC porus, and decreased modiolus size in the WS patients (Madden et al., 2003). These findings coexist with the effects of melanocyte depletion on the otic vesicle. These results suggest that WS and cochlear nerve agenesis/hypoplasia are not mutually exclusive. Further studies are needed to fully delineate the effect of WS on temporal bone development

and melanocyte migration.

All subjects in this cohort with a family history of SSD also have pigmentary disorders, such as brown freckles and premature gray hair. This may indicate that genes, whose products are involved in the development and migration of melanocytes from the neural crest, could be candidates for inherited SSD. Our observation may contribute to understanding the molecular basis of heritable USNHL/SSD.

Here, we provide an approach to children with congenital USNHL/SSD (Fig. 7). The majority of pediatric subjects with USNHL/SSD does not have a family history of hearing loss and usually displays the finding of cochlear nerve agenesis/hypoplasia on IAC MRI. Presence of family history of USNHL/SSD, coupled with a freckled face and/or premature gray hair, suggests WS of which confirmatory diagnosis is facilitated by the molecular genetic test. Genetically documented WS subjects with mutations in *PAX3* or *MITF* tend to show normal TBCT and IAC MRI findings. Note that there is a subset of pediatric SSD subjects with signs that suggest WS and a positive family history of both SSD and cochlear nerve agenesis/hypoplasia, however, without any detectable mutation in previously reported WS genes. This group may imply presence of another type of WS or of a different disease entity.

Conclusion

On the basis of our results, we identified a strong association between the heritability of USNHL/SSD and the pigmentary abnormality. WS should be included in the important differential diagnosis of children with USNHL/SSD especially in a familial form. Multidisciplinary physical examination that includes neurotological, ophthalmological, and dermatological examinations is mandatory.

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Table1. Our entire single side deafness cohort and summary of clinical phenotypes.

	Family members	**Syndromic features**	**WI[§]**	**Normal or WS[δ]**
SSD*1	Proband 1 (M/2Y)	None	< 2.07	Normal
SSD2	Proband 2 (F/1Y)	None	< 2.07	Normal
SSD3	Proband 3 (M/6Y)	None	< 2.07	Normal
SSD4	Proband 4 (M/8Y)	None	< 2.07	Normal
SSD5	Proband 5 (F/1Y)	None	< 2.07	Normal
SSD6	Proband 6 (M/9Y)	None	< 2.07	Normal
SSD7	Proband 7 (F/12Y)	None	< 2.07	Normal
SSD8	Proband 8 (M/9Y)	None	< 2.07	Normal
SSD9	Proband 9 (M/7Y)	None	< 2.07	Normal
SSD10	Proband 10 (M/6Y)	None	< 2.07	Normal
SSD11	Proband 11 (F/7Y)	None	< 2.07	Normal
SSD12	Proband 12 (M/8Y)	None	< 2.07	Normal
SSD13	Proband 13 (F/13Y)	None	< 2.07	Normal
SSD14	Proband 14 (M/10Y)	None	< 2.07	Normal
SSD15	Proband 15 (F/12Y)	None	< 2.07	Normal
SSD16	Proband 16 (M/9Y)	None	< 2.07	Normal
SSD17	Proband 17 (M/7Y)	None	< 2.07	Normal
SSD18	Proband 18 (M/2Y)	None	< 2.07	Normal
SSD19	Proband 19 (M/13Y)	None	< 2.07	Normal
SSD20	Proband 20 (M/2Y)	None	< 2.07	Normal
SSD21	Proband 21 (F/2Y)	None	< 2.07	Normal
SSD22	Proband 22 (M/12Y)	None	< 2.07	Normal
SSD23	Proband 23 (M/13Y)	None	< 2.07	Normal
SSD24	Proband 24 (F/12Y)	None	< 2.07	Normal
SH136 (SSD25)**	Maternal grandfather (SH136-330)	None	< 2.07	Normal
	Maternal grandmother (SH136-283, F/62Y)	**Hearing loss, Freckles, Gray hair**	< 2.07	**WS2**

	Maternal uncle (SH136-331)	None	< 2.07	Normal
	Father	None	< 2.07	Normal
	Mother (SH136-284, F/35Y)	**Hearing loss, Freckles, Gray hair**	< 2.07	**WS2**
	Twin sister (SB199-285, F/4Y)	**Hearing loss, Freckles**	< 2.07	**WS2**
	Proband (SB199-282, M/4Y)	**Hearing loss, Freckles**	< 2.07	**WS2**
SSD26	Proband 26 (F/8M)	None	< 2.07	Normal
SSD27	Proband 27 (M/10M)	None	< 2.07	Normal
SSD28	Proband 28 (M/12Y)	None	< 2.07	Normal
SSD29	Proband 29 (M/1Y)	None	< 2.07	Normal
SSD30	Proband 30 (M/1Y)	None	< 2.07	Normal
SSD31	Proband 31 (F/10M)	None	< 2.07	Normal
SSD32	Proband 32 (M/12Y)	None	< 2.07	Normal
SSD33	Proband 33 (M/14Y)	None	< 2.07	Normal
SSD34	Proband 34 (F/6Y)	None	< 2.07	Normal
SSD35	Proband 35 (F/8Y)	None	< 2.07	Normal
SSD36	Proband 36 (F/10Y)	None	< 2.07	Normal
SSD37	Proband 37 (M/7Y)	None	< 2.07	Normal
SSD38	Proband 38 (M/7Y)	None	< 2.07	Normal
SSD39	Proband 39 (M/9Y)	None	< 2.07	Normal
	Maternal grandfather	Freckle, Gray hair	< 2.07	Normal
	Maternal grandmother	None	< 2.07	Normal
	Maternal uncle1	Freckle, Gray hair	< 2.07	Normal
	Maternal uncle2	Freckle, Gray hair	< 2.07	Normal
SB177	Father	None	< 2.07	Normal
(SSD40)	Mother (SB177-338, F/40Y)	**Freckles, Gray hair**	< 2.07	**Possible WS2**
	Sister (SB177-337, F/14Y)	**Hearing loss, Freckles**	< 2.07	**Possible WS2**
	Proband (SB177-	**Hearing loss only**	< 2.07	**Possible**

	336, F/10Y)			**WS2**
	Brother	None	< 2.07	Normal
SSD41	Proband 41 (M/7Y)	None	< 2.07	Normal
SSD42	Proband 42 (F/5Y)	None	< 2.07	Normal
SB173 (SSD43)	Father	Gray hair	< 2.07	Normal
	Mother	None	< 2.07	Normal
	Sister (SB173-329-1, F/15Y)	**Hearing loss**	< 2.07	**Possible WS2**
	Proband (SB173-329-2, M/13Y)	**Hearing loss**	< 2.07	**Possible WS2**
SSD44	Proband 44 (M/12Y)	None	< 2.07	Normal
SSD45	Proband 45 (M/10Y)	None	< 2.07	Normal
SSD46	Proband 46 (F/7M)	None	< 2.07	Normal
SSD47	Proband 47 (M/6Y)	None	< 2.07	Normal
SH199 (SSD48)	Father	None	< 2.07	Normal
	Mother (SB199-387, F/42Y)	**Freckles, Gray hair, Dystopia canthorum**	2.81	**WS1**
	Sister (SB199-392, F/13Y)	**Hearing loss, Freckles, Gray hair, Dystopia canthorum, Heterochromia iridium**	2.70	**WS1**
	Proband (SB199-386, F/12Y)	**Hearing loss, Freckles, Gray hair, Dystopia canthorum**	3.04	**WS1**
	Brother	None	< 2.07	Normal
SSD49	Proband 49 (M/6Y)	None	< 2.07	Normal
SSD50	Proband 50 (F/8M)	None	< 2.07	Normal

*SSD: Single side deafness, **SSD probands with additional family members with SSD or unilateral sensorineural hearing loss in bold, [§]WI: Waardenburg index, WS[δ]: Waardenburg syndrome

Fig.1. Schematic flow chart of filtering variants obtained from targeted exome sequencing in this study: A p.Arg255X variant of *MITF* is selected as the single strongest candidate in the analysis.

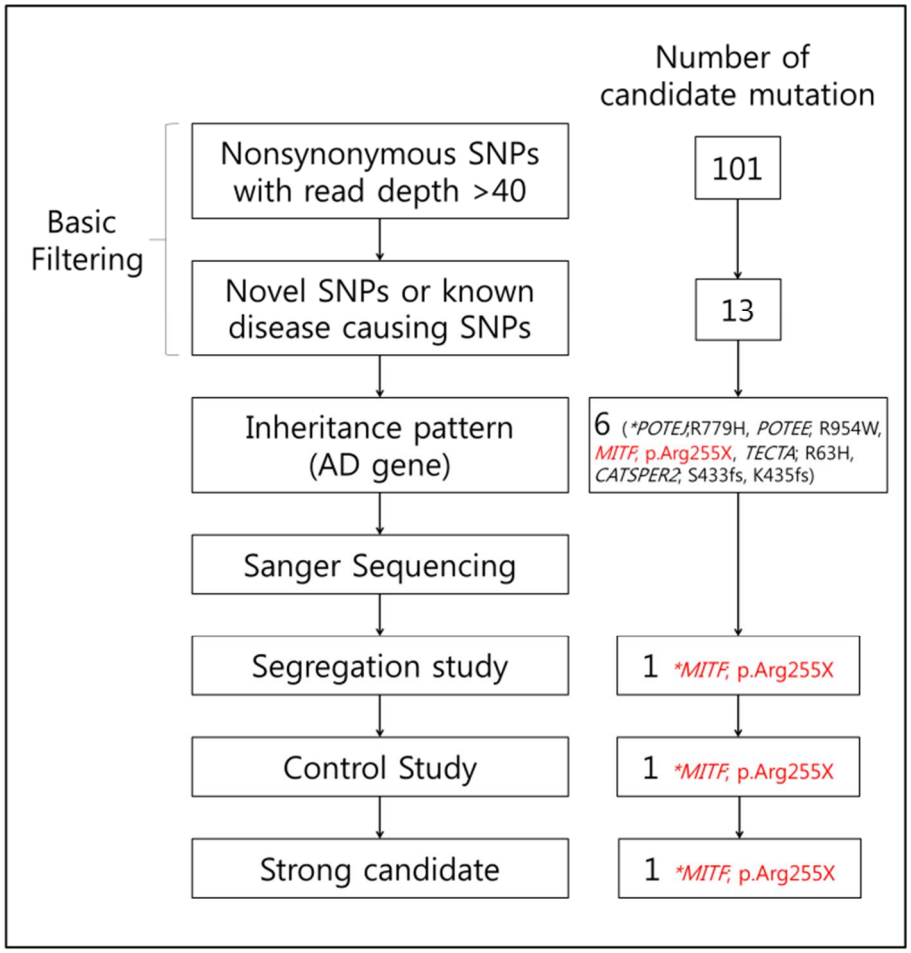
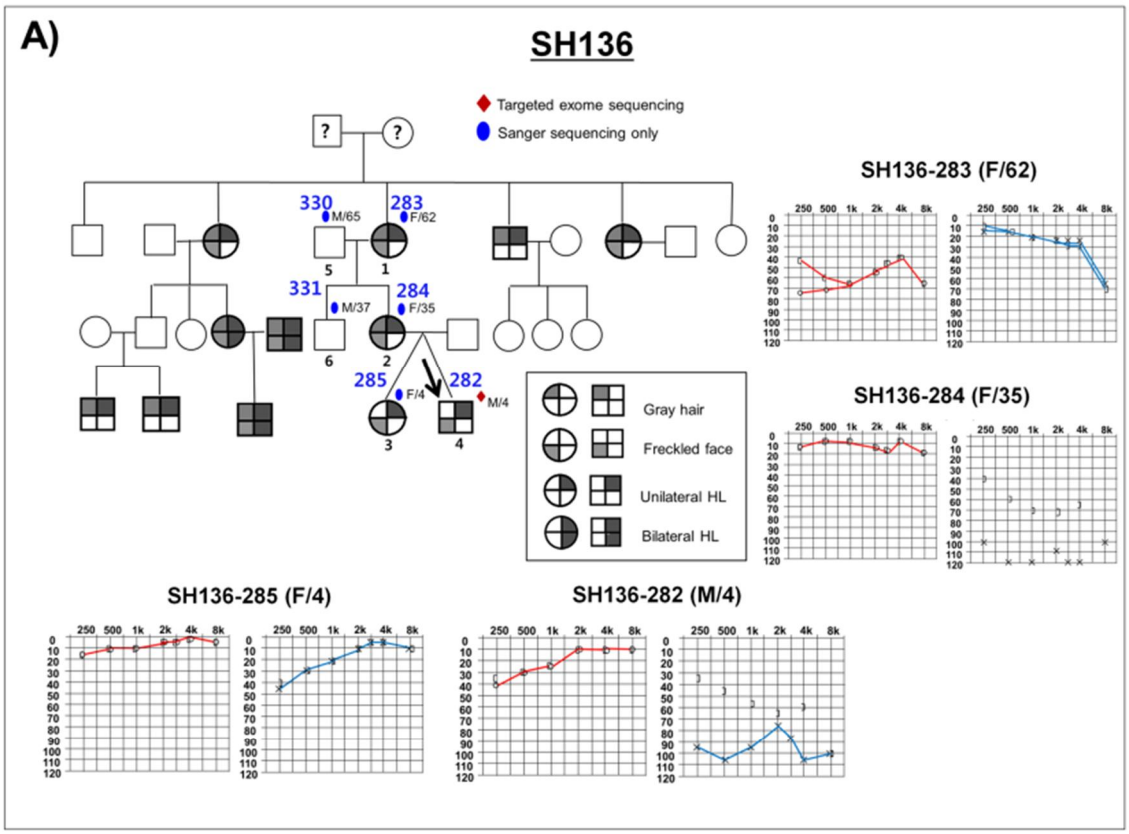


Fig.2. Pedigrees of SH136 (A) and SB199 (B) family: These two multiplex families segregate single side deafness or unilateral sensorineural hearing loss and brown freckles or gray hair, which strongly suggests Waardenburg syndrome.



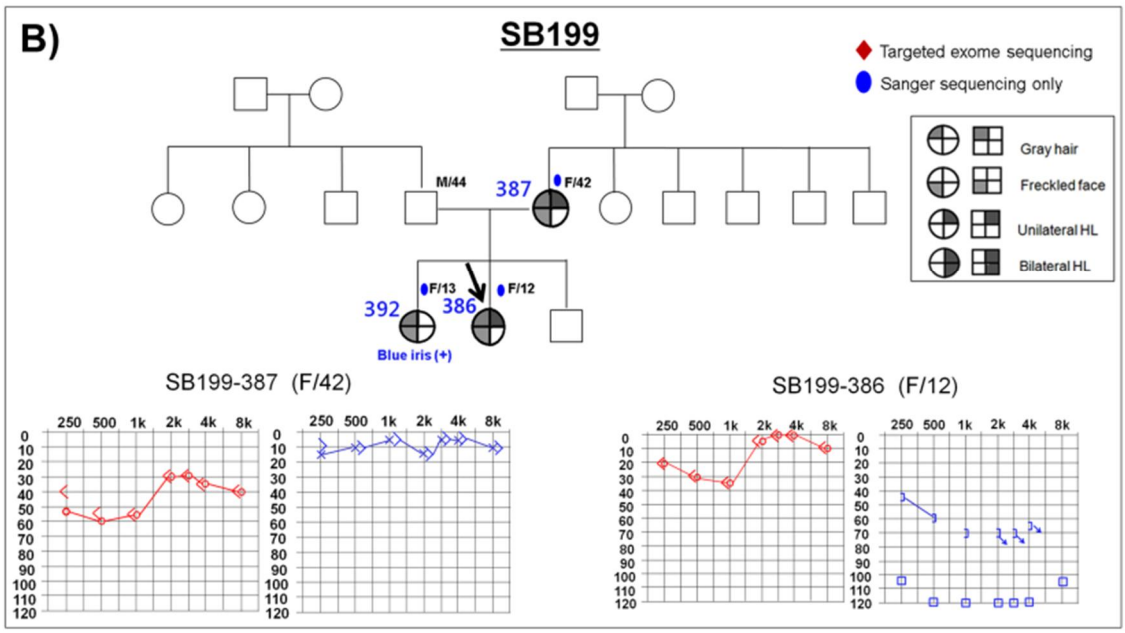


Fig.3. Pedigrees of SB173 and SB177 family: Both families have a sibling pair with single side deafness, showing a definite heritability.

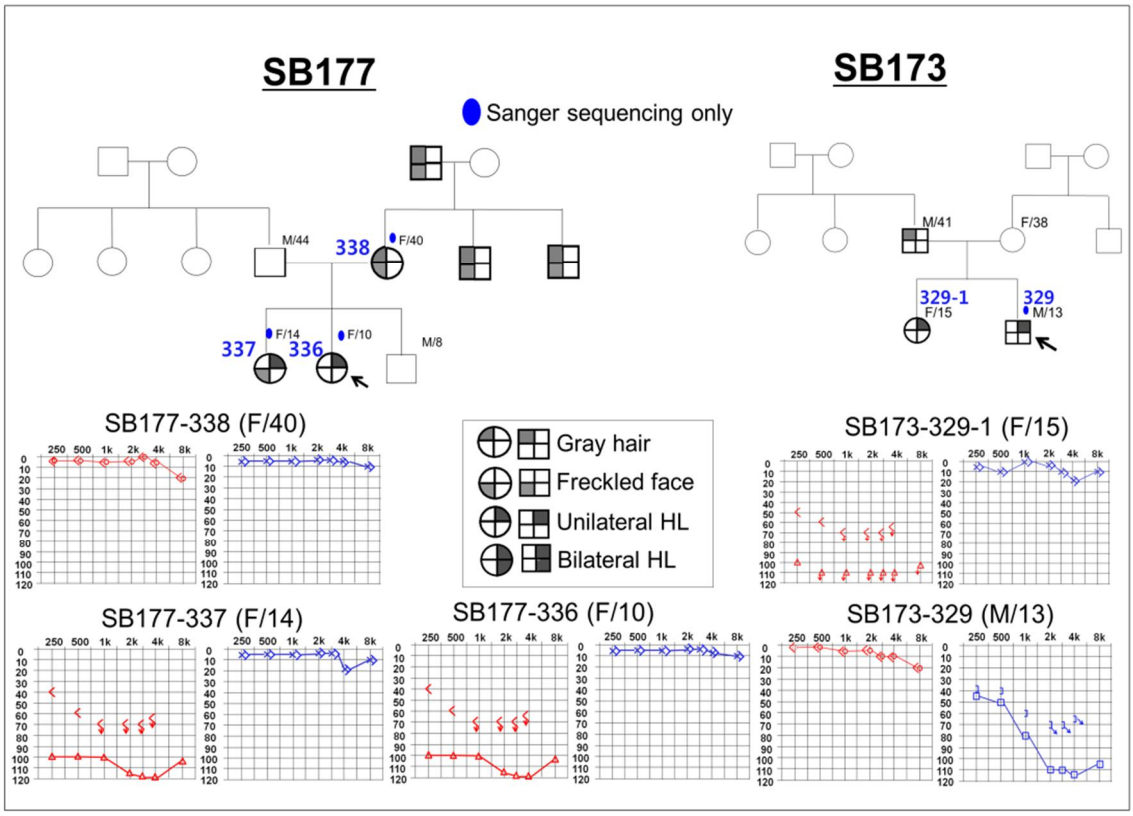


Fig.4. Computed tomography and magnetic resonance imaging images of the inner ear and internal auditory canal from the four probands with single side deafness: SH136-282 and SB199-386 display normal bony cochlear nerve canal (white arrow) and cochlear nerve (white arrow head). However, SB173-329 and SB177-336 show narrow bony cochlear nerve canal (black arrow) and cochlea nerve agenesis (dotted arrow).

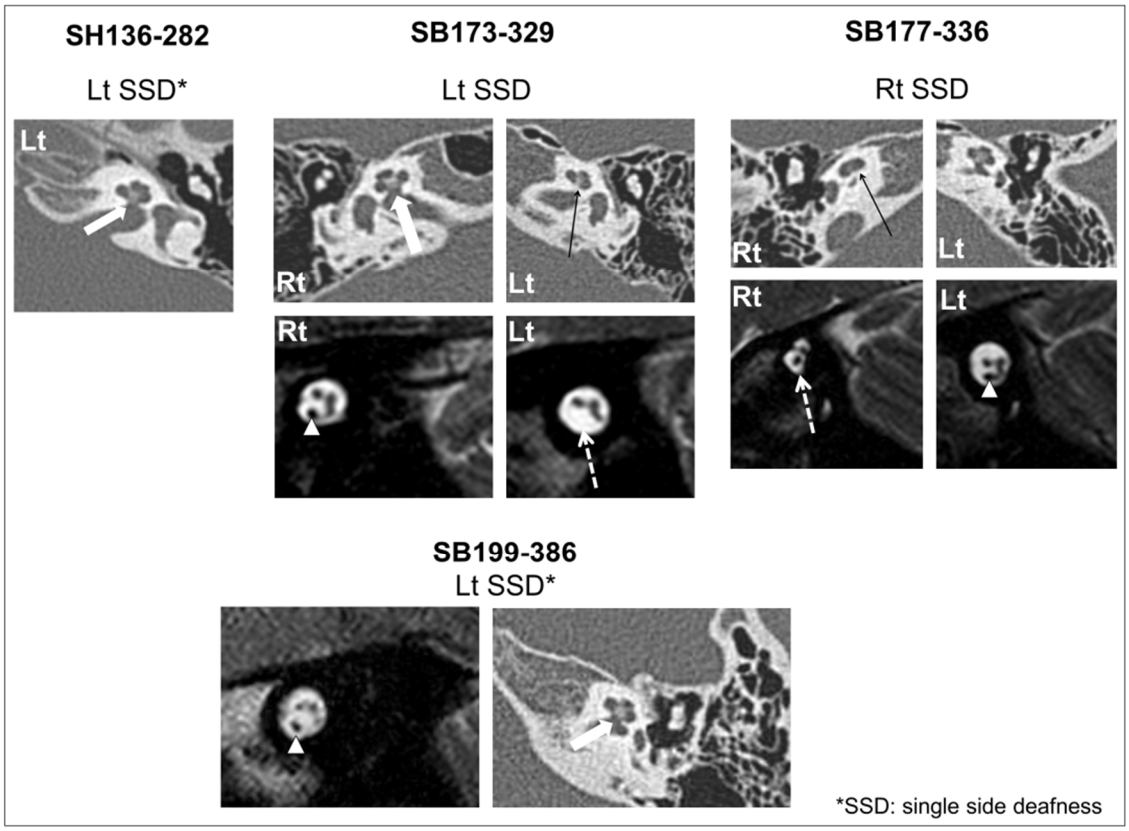


Fig.5. Phenotypes suggesting Waardenburg syndrome in three families: Freckles starts as early as 4 years of age (A–C,I,J). Premature gray hair is also observed as early as 12 years of age of 12 (G,H). SB199–387, SB199–386, and SB199–392 show dystopia canthorum (D-F). SB177–338 manifests freckles (K) and premature gray hair (L).

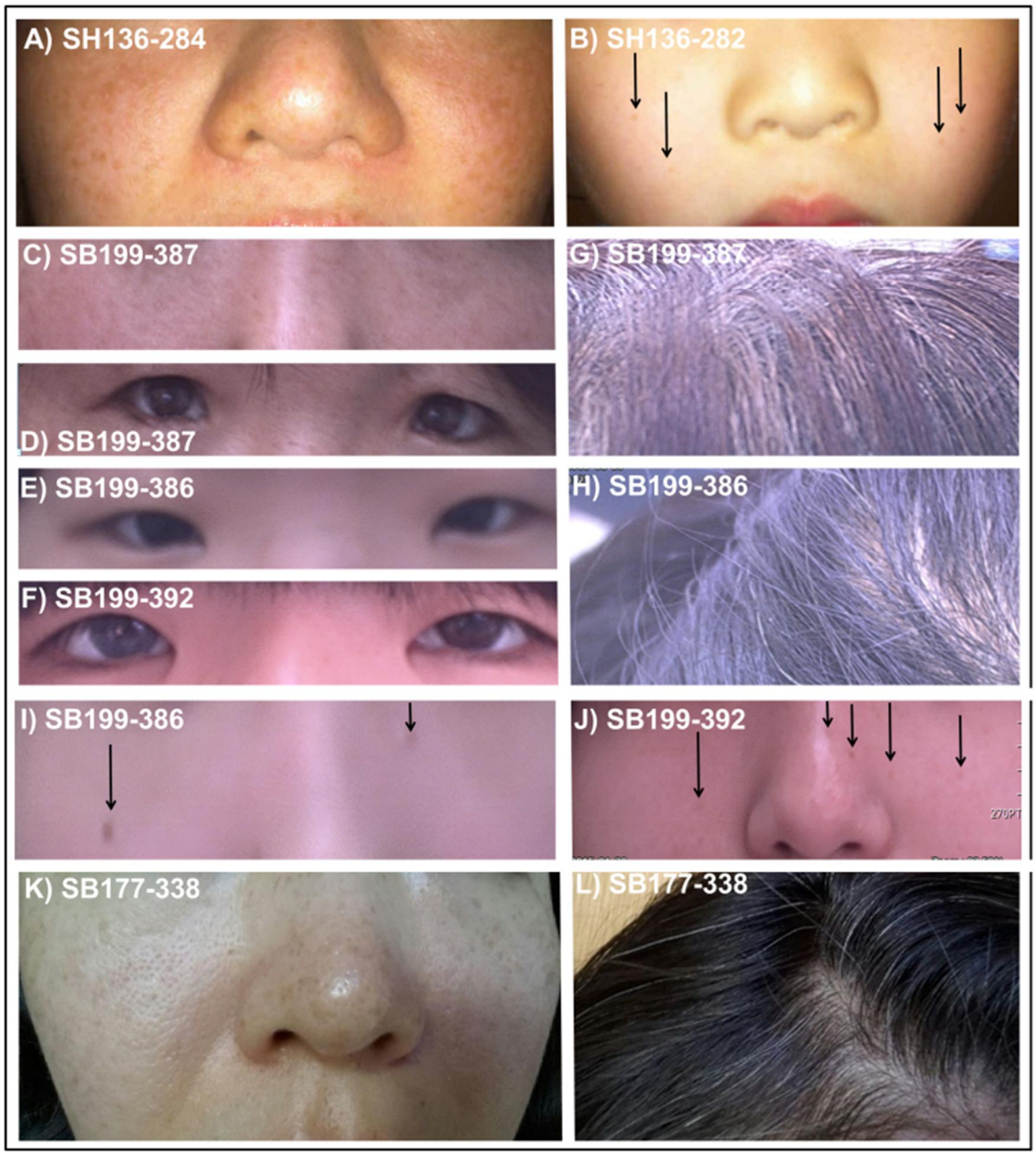


Fig.6. Sanger sequencing trace of c.763C>T (p.Arg255X) of *MITF* and c.668G>A (p.R223Q) of *PAX3* from SH136 and SB199, respectively: These two variants perfectly co-segregate with the Waardenburg syndrome features within the two families. In SH136, two subjects (SH136-330 and SH136-331) without any feature of Waardenburg syndrome do not carry c.763C>T (p.Arg255X) of *MITF*.

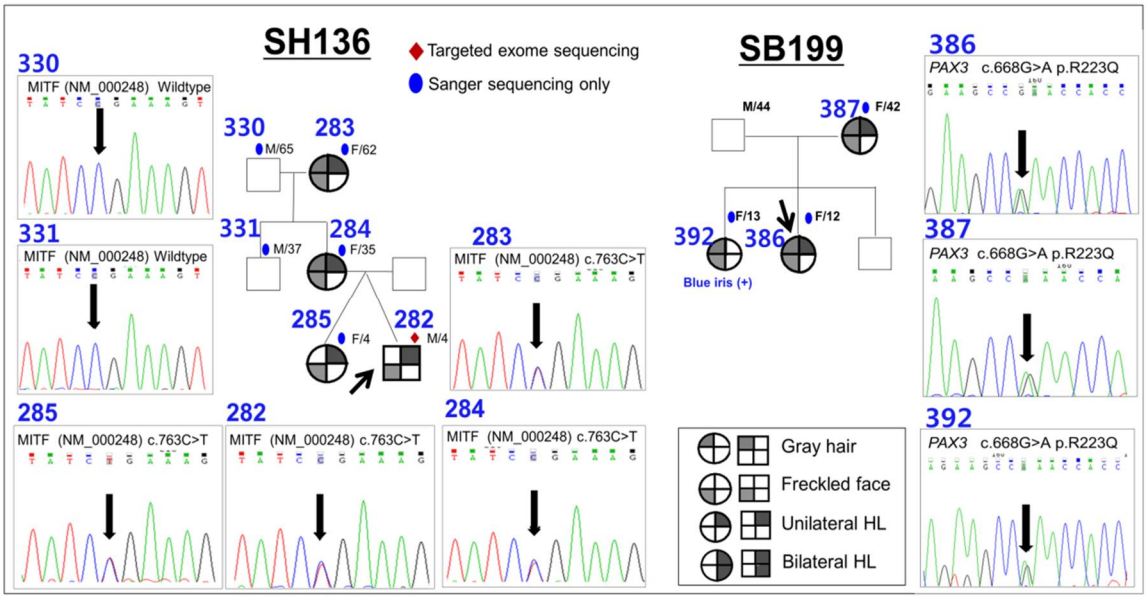
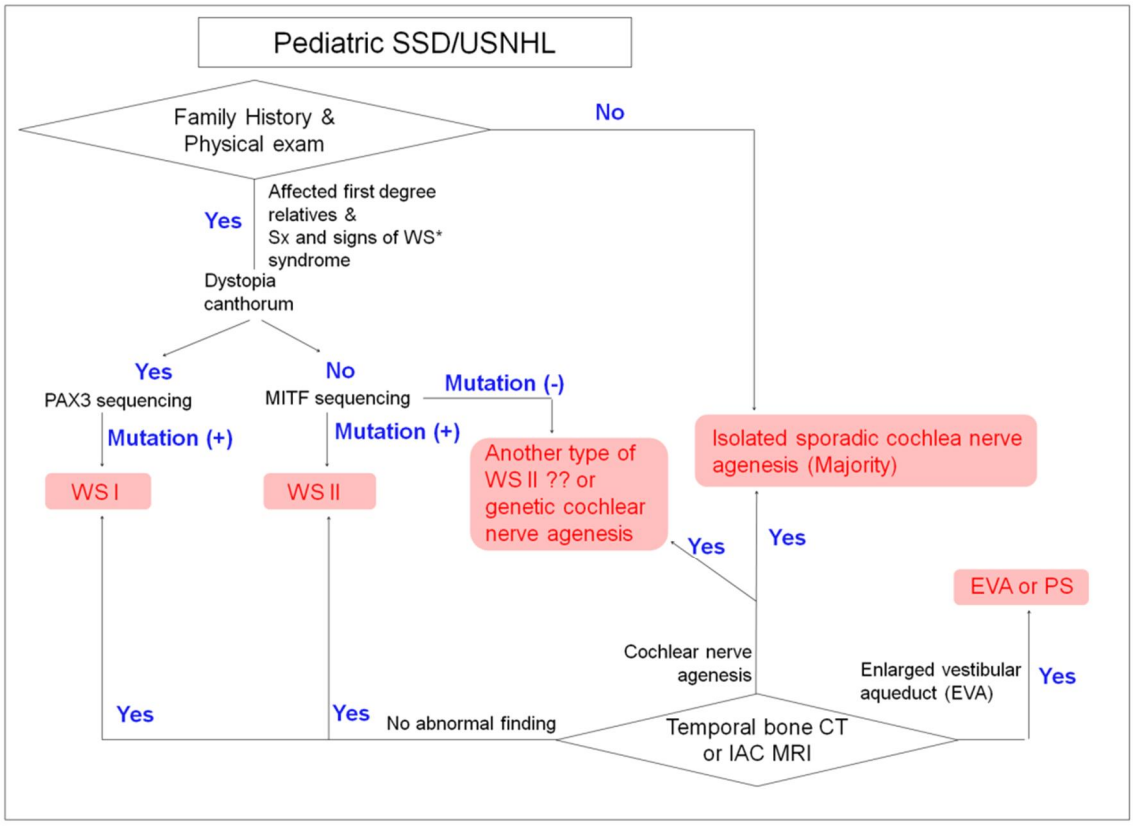


Fig.7. Proposed pipeline designed for diagnostic work up of pediatric single side deafness or unilateral sensorineural hearing loss: A rigorous physical examination, imaging studies and molecular genetic studies are mandatory to reach a correct diagnosis. WS: Waardenburg syndrome. PS: Pendred syndrome, EVA: enlarged vestibular aqueduct.



국문 초록

서론: 일측성 감각신경성난청 (unilateral sensorineural hearing loss, USNHL)/일측성 농 (single side deafness, SSD)은 유소아에서 흔하게 발생하는 장애이다. USNHL/SSD의 원인의 대부분은 아직 밝혀지지 않았으며, 유전적 경향이 있는지에 대해서도 밝혀지지 않았다. 본 연구에서 저자들은 USNHL/SSD의 유전성에 대해 분석하고자 한다.

대상 및 방법: USNHL/SSD를 보이는 50명의 유소아 환자를 모집하였다. 일측성 난청의 원인 진단을 위하여 50명의 환자와 그 가족들에서 난청 과 색소 이상 증상이 있는 지 확인하고, 청력검사와 신체검진을 시행하였다. 일측성 난청의 가족력이 확인된 유소아 환자에서는 혈액 채취 및 분자 진단을 시행하였다.

결과: 50명의 USNHL/SSD 유소아 환자들 중, 4명(SH136, SB173, SB177, SB199) 에서 일측성 난청의 가족력을 확인하였다. 상기 네 가계의 가족 구성원은 일측성 난청 외에도 새치, 주근깨와 같은 색소이상 소견을 보였다. 이로부터 색소이상과 관련된 어떤 유전자의 이상으로 가계 내에서 가족 간에 일측성 난청과 색소이상 소견이 동시 발생 (co-segregation)하는 것으로 사료되었다. 분자 진단 결과 SH136와 SB199의 두 가계에서 각각 *MITF*와 *PAX3* 유전자의 돌연변이가 발견되어, SH136은 와덴버그 증후군 (Waardenburg syndrome) 2형, SB199는 와덴버그 증후군 1형으로 밝혀졌다. 반면 나머지 SB173와 SB177의 두 가계에서는 전장 유전체 시퀀싱 (whole exome sequencing)에서도 유전 변이가 발견되지 않았다.

결론: 본 연구는 처음으로 USNHL/SSD의 유전성을 밝힌 연구이다. 유전되는 일측성 난청과 색소이상 소견이 밀접한 관련이 있음을 시사하였고, 이로부터 유전되는 일측성 난청의 분자 기초에 대한 이해에 새로운 시각을 제시하였다. 따라서 유소아가 일측성 난청을 보이면, 그 가족력을 확인하고 색소이상 소견이

있는 지 면밀한 신체검진을 해야 한다. 특히 일측성 난청의 가족력이 확인되면, 와덴버그 증후군을 의심해야 한다.

주요어: 일측성 감각신경성 난청, 일측성 농, 유전성, 색소이상 질환, 와덴버그 증후군

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