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Original Article

Next-Generation Sequencing to Detect Mutations for the Molecular Diagnosis of Auditory Neuropathy Spectrum Disorder in a Chinese Series

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BACKGROUND: Auditory neuropathy spectrum disorder (ANSD) encompasses a range of hearing impairments caused by disrupted sound transmission from the cochlea to the brain. The atypical symptoms or signs of ANSD often complicate both diagnosis and treatment. To improve the identification of lesion sites and gain insights into the disease mechanisms, we employed next-generation sequencing (NGS) to detect mutations in ANSD-related genes.

METHODS: We studied 23 patients with ANSD from non-consanguineous Chinese families. Clinical data were collected and analyzed from medical records. Genomic DNA was extracted from blood samples, followed by whole-exome capture, NGS, and confirmation through bidirectional Sanger sequencing.

RESULTS: Based on ANSD classification, 10 patients had non-syndromic (NS) ANSD, 7 had syndromic peripheral neuropathy, and 6 had syndromic central neuropathy. Thirteen novel variants (8 missense variants and 1 deletion variant) and 21 previously reported variants were identified in 23 patients. Several cases exhibited mild-to-profound hearing loss.

CONCLUSION: Multiple genes have been identified to cause ANSD. Next-generation sequencing plays a role in differentiating ANSD from other clinical conditions and identifying it as a symptom of syndromic ANSD. Molecular diagnosis offers valuable insights into prognosis and helps guide treatment strategies.

KEYWORDS: ANSD, auditory neuropathy spectrum disorder, hearing loss, neuropathy, next-generation sequencing, NGS

INTRODUCTION

Auditory neuropathy spectrum disorder (ANSD) is a form of hearing loss (HL) characterized by disrupted sound transmission from the cochlea to higher auditory centers, as initially described by Starr et al in 1996.[1] This impairment can occur at various sites, such as inner hair cells (IHC), spiral ganglion cells, synapses between these structures, and auditory nerve fibers within the cochlea. Lesions may affect one or more of these locations simultaneously.[2] Individuals with ANSD typically experience neural HL, marked by normal outer hair cell activity but a disproportionate decline in speech recognition compared to the pure tone threshold. This is evidenced by normal otoacoustic emissions (OAE) and/or cochlear microphonics (CM) alongside absent or severely abnormal auditory brainstem responses (ABR).[3] Auditory neuropathy spectrum disorder can manifest as either non-syndromic (NS) HL or as part of a broader syndromic presentation involving peripheral or central neuropathies. Such syndromic forms of ANSD are linked to conditions like autosomal dominant optic atrophy (ADOA), Brown–Vialetto–Van Laere syndrome (BVVL), Pelizaeus–Merzbacher disease (PMD), Leber's hereditary optic neuropathy (LHON), and Wolfram syndrome.[4-8] The atypical nature of the neuropathic symptoms complicates both diagnosis and treatment.

Accurate etiologic diagnosis of ANSD clarifies the underlying cause and helps predict treatment outcomes. Early interventions, such as the use of hearing aids (HA), cochlear implants (CI), and speech therapy, are crucial for improving speech development in patients with ANSD. Delayed diagnosis poses a challenge for individuals with hearing impairments. In addition, the presence

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of OAE response complicates clinical decision-making regarding the optimal timing of CI surgery. Numerous studies have shown that genetic factors play a significant role in congenital deafness, contributing to more than 50% of cases.[9] Furthermore, substantial evidence suggests that Mendelian inheritance significantly contributes to the development of ANSD.[10] The *OTOF* gene mutation is the most common in isolated ANSD.[11,12] An increasing number of ANSD cases have been associated with inherited syndromic neuropathies. Therefore, genetic testing for ANSD is valuable. Recent advances in next-generation sequencing (NGS) technology have facilitated molecular genetic testing to identify variants associated with sensorineural HL.[13,14] The integration of genetic testing with clinical audiology has enabled researchers to pinpoint the precise locations of lesions and show the specific pathogenesis underlying ANSD.

In this study, we utilized NGS technology to analyze a cohort of Chinese patients, aiming to identify potential disease-causing genetic variations. Our findings emphasize the importance of genetic testing in identifying specific ANSD subtypes, which may yield positive outcomes in individuals undergoing cochlear implantation. This study contributes to the growing body of research on the genetic factors influencing ANSD and highlights the value of genetic testing in guiding treatment decisions for this condition.

METHODS

Subject and Clinical Evaluations

Twenty-three patients with ANSD from non-consanguineous Chinese families were identified, including 4 patients with a family history of HL (pedigrees depicted in Figure 1). A detailed medical history was obtained, followed by comprehensive audiological evaluations. These included pure tone audiometry, speech audiometry, tympanometry, distortion product otoacoustic emissions (DPOAEs), ABR, auditory steady-state response (ASSR), CM, stapedial reflex testing, and a physical examination. Auditory neuropathy spectrum disorder diagnoses were made in accordance with clinical guidelines and conducted in a specialized soundproof room. In addition to diagnosing ANSD, all patients underwent clinical consultations and physical examinations to identify signs of syndromic ANSD. High-resolution CT scans of the temporal bone and brain MRI were performed to rule out other potential neuropathic or anatomical abnormalities.

This study, including all protocols involving Chinese families with ANSD, adhered to ethical guidelines and received approval from the Medical Ethics Committee of Peking University First Hospital (approval number: (2020)24, date: October 11, 2020). Before participation, written informed consent was obtained from all participants, and for minors, from their parents or guardians. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Genomic DNA Sample Collection

Blood samples were collected from the patients and their guardians at the Department of Otolaryngology and Head-neck Surgery, Peking University First Hospital. Genomic DNA was extracted using the Qiagen Blood DNA Extraction Kit (Qiagen, Hilden, Germany).

Whole Exome Capture and Library Construction

The human exome sequencing followed Illumina's TruSeq Exome Enrichment Guide (Illumina, San Diego, CA, USA) using the TruSeq Exome Enrichment kit, which targets 62 Mb of probe sets. Initially, approximately 5 μg of genomic DNA in Buffer EB (Qiagen) was sheared into 100-500 bp fragments using a Bioruptor UCD-200 (Diagenode, Belgium). The subsequent polymerase chain reaction (PCR) product was purified by quantitative real-time PCR analysis, and the DNA concentration was determined by measuring the absorbance at 260 nm. Captured DNA libraries were sequenced on an Illumina HiSeq 2000 analyzer (Illumina) with 200 (2 × 100) bp paired-end reads using the V2 reagent. Whole-exome sequencing analysis commenced with the conversion of raw sequencing data into FASTQ format. Bioinformatics analysis was conducted using the Genome analysis toolkit (https://gatk.broadinstitute.org/) for variant calling, the Burrows–Wheeler Aligner (http://bio-bwa.sourceforge.net/) to align reads to the reference human genome (hg19, NCBI Build 38), and Picard for duplicate removal. Filtering criteria for variants excluded synonymous variants, non-coding intronic variants, and UTR variants with a minor allele frequency <1% in databases such as dbSNP Human Build 147, 1000 Genomes (https://www.internationalgenome.org/), the NHLBI GO Exome Sequencing Project (https://evs.gs.washington.edu/), and the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org).

After filtration, all candidate variants were confirmed using bidirectional Sanger sequencing. Polymerase chain reaction amplification and sequencing of the variants were performed using appropriate primers. Sequences containing chromatograms were then aligned to the reference sequence for each gene region using SeqMan software version 5.00© (DNASTAR, Madison, WI, USA). Additionally, variants present in the Human Gene Mutation Database professional database (http://www.hgmd.cf.ac.uk/) and the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) were reviewed. Further investigation of relevant literature was conducted to explore the novelty of the variant and its association with HL. The variant nomenclature followed the recommended Human Genome Variation Society naming convention.[15]

Prediction of Pathogenicity of Mutation

We annotated the mutations using SIFT, PolyPhen2, and MutationTaster to evaluate the impact of amino acid substitutions

MAIN POINTS

- This study reveals the importance of genetic testing in the identification of a specific subtype of auditory neuropathy spectrum disorder (ANSD). According to the classification of ANSD, there were a total of 10 patients with non-syndromic, 7 with syndromic peripheral neuropathy and 6 with syndromic central neuropathy.
- Next-generation sequencing (NGS) technology has identified potential disease-causing genetic variations in individuals with ANSD, as well as emerging variants that have not been previously reported. In total, 13 novel variants were identified, including 8 missense variants and 1 deletion variant, alongside 21 previously reported variants, across 23 patients.
- Mild to profound hearing loss is observed in many cases, consistent with the clinical presentation of ANSD.
- This study underscores the potential benefits of NGS in yielding positive outcomes for individuals undergoing cochlear implantation.

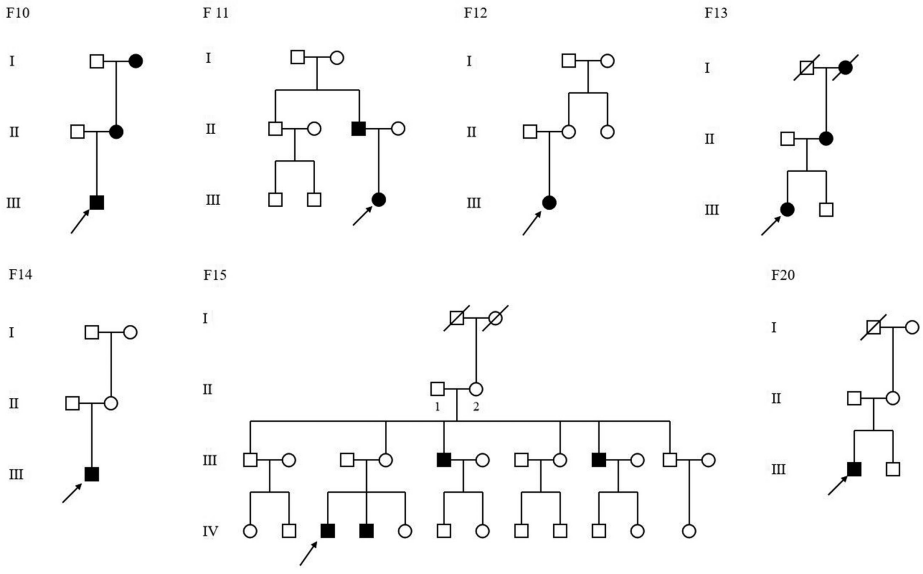

Figure 1. Pedigrees of families.

on protein function.[16] Several in silico tools, including CADD (https://cadd.gs.washington.edu/), FSPLICE (http://www.softberry.com/), MutationTaster (http://www.mutationtaster.org/), NetGene2 Server (www.cbs.dtu.dk/), PANTHER (http://www.pantherdb.org/), and SIFT (https://sift.bii.a-star.edu.sg/), were employed to analyze the deleterious effects of splice variants on protein function. Genetic variants were classified according to the guidelines provided by the American College of Medical Genetics and Genomics (ACMG).[17]

RESULTS

Clinical Characteristics

Table 1 provides an overview of the demographic and clinical characteristics of 23 patients with ANSD. The mean age was 4.3 years (SD = 3.8), with 13 male patients (56.5%) and 10 female patients (43.5%). According to the ANSD classification, 10 patients had NS ANSD, 7 had syndromic peripheral neuropathy (SP), and 6 had syndromic central neuropathy (SC). Eight of the 10 patients with NS were carriers of the autosomal recessive (AR) *OTOF* gene, and all presented with isolated ANSD. Two other patients with isolated ANSD had AD mutations in the *SLC17A8* and *DIAPH1* genes. In the SP group, 2 patients had ADOA, 2 had X-linked inherited peripheral neuropathy (XIPN), 1 had LHON, and 1 had mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes syndrome (MELAS). Most patients in this group exhibited AD genetic characteristics, except for those with LHON and MELAS, which are mitochondrial diseases. Patients with SC have related diseases such as BVVL syndrome, epilepsy with *DFNB86* deafness, Pelizaeus–Merzbacher disease, microcephalic primordial dwarfism, hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC), and Rahman syndrome.

Analysis of Hearing Performance

To elucidate the degree of HL more precisely, we collected hearing threshold data from the patients (Table 2). Hearing loss ranged from mild to profound, with 1 patient showing severe hearing impairment. However, we observed normal hearing in 2 individuals (numbers 20 and 23). Audiometric patterns varied, with 6 ears displaying low-frequency loss, flat type in 9 ears, and normal in 2 ears. All patients with NS ANSD presented with early-onset HL. Most patients with SP had late-onset HL, except for 1 patient with isolated ANSD and 1 with LHON. In contrast, in SC, only 1 patient presented with the late-onset type. Psychophysical evaluations revealed poor speech discrimination ability (SDS) in many cases. Additionally, we collected objective assessments, such as OAEs, CM, and ABRs, to further understand the auditory patterns. Most patients exhibited typical results, including preserved OAEs and CM with absent or severely abnormal ABR waveforms. However, there are exceptional cases and 3 types of abnormal ABRs. Two patients with LNON and MELAS showed abnormal OAEs and Type 1 ABR, characterized by poor bilateral waveform repeatability, a bilateral wave V threshold at 95 dBnHL, and a latent period of wave V exceeding 10 ms. The bilateral waveform repeatability of type 2 was good; however, only wave I differentiation was observed on both sides (Figure 2). The bilateral wave I threshold was 30 dB nHL, whereas waves III and V were absent. ABR waveforms of patients treated with Pelizaeus–Merzbacher and H-ABC were also analyzed. A patient with Rahman syndrome presented with type 3 ABR (with good bilateral waveform repeatability, differentiation of waves I and III on both sides, and a bilateral wave V threshold at 80 dB HL) (Figure 3). None of the other patients underwent ABRs. In contrast, abnormal or absent ASSRs were observed in patients with ANSD.

Identified Variants

Multiple variants were identified in each sample, and we determined the causative gene in the patients following the ACMG guidelines. The most common causative gene was *OTOF*, found in 9 patients, which accounts for nearly half of the total cohort. Pathogenic mutations in *OPA1* were detected in 2 patients, as well as apoptosis inducing factor mitochondria-associated 1 (*AIFM1*) mutations in another 2 patients. Mutations in 10 other genes (*SLC17A8*, *SLC52A2*, *DIAPH1*, *MT-ND6*, *MT-TL1*, *TBC1D24*, *PLP1*, *CEP135*, *TUBB4A*, *H1-4*) were observed in only 1 patient or family each. We examined all variants through ClinVar records and assessed their potential deleterious effects using in silico analysis, supported by relevant literature, ultimately classifying them according to ACMG criteria. Among the 23 patients with

Table 1. Demographic Characteristics and Variants Identified in 23 Patients with ANSD

Number	Gender	Age of Test (Years)	Related Disease	NS or SP or SC	Family History	Gene	Mode of Inheritance	Mutation	Reported	ACMG Criteria	ACMG
1	F	1.5	Isolated ANSD	NS	No	OTOF	AR	c.3277G-A(p.E 1093K)/ c.1535C-T(p.T512I)	Yes/no	PS1 PM1 PM2 PM6/ PS2 PM2	P/LP
2	M	5	Isolated ANSD	NS	No	OTOF	AR	c.4023+1G-A/c.2438G-A(p.R813Q)	Yes/yes	PVS1 PM4 PP3/ PM1	P/VUS
3	M	2.5	Isolated ANSD	NS	No	OTOF	AR	c.5197G-A(p.E1733K)/ c.1493A-C(p.Q498P)	Yes/no	PM1 PM2 PP2 PP3/ PS2 PM2 PP2	LP/LP
4	M	0.5	Isolated ANSD	NS	No	OTOF	AR	c.5098G-C(p.E1700Q)/ c.157G-A(p.A53T)	Yes/yes	PS1 PS3 PM1 PP3/ PM2	P/VUS
5	F	1	Isolated ANSD	NS	No	OTOF	AR	c.5567G-A(p.R1856Q)/ c.2712G-A(p.W904X)	Yes/no	PS1 PS3 PM1/ PS2 PM1 PM2 PP2 PP5	P/P
6	M	1	Isolated ANSD	NS	No	OTOF	AR	c.3514C-T(p.R1172W)/ c.1697G-C(p.R566P)	No/yes	PS2 PM2/ PS1 PS3 PM2 PP3	VUS/P
7	M	2	Isolated ANSD	NS	No	OTOF	AR	c.4023+1G-A/c.1194T-A(p.D398E)	Yes/yes	PVS1 PM4 PP3/ PM2 PP2	P/VUS
8	F	1.7	Isolated ANSD	NS	No	OTOF	AR	c.3683T-C(p.L1228P)/c.*20G-A	Yes/no	PM2 PM3 PP2 PP5/ PVS1 PS2 PM2 PP3	LP/P
9	M	5	Isolated ANSD	NS	No	OTOF	AR	c.4023+1G-A/c.1194T-A(p.D398E)	Yes/yes	PVS1 PM4 PP3/ PM2 PP2	P/VUS
10	M	4	Isolated ANSD	NS	Yes	SLC17A8	AD	c.223C-T(p.R75C)	Yes	PM1 PM2 PP1 PP4	LP
11	F	4	Isolated ANSD	SP	Yes	DIAPH1	AD	c.791C-A(p.S264Y)	No	PM1 PM2 PP1 PP4	LP
12	F	7	ADOA	SP	No	OPA1	AD	c.1555G-A(p.E519K) (de novo)	Yes	PM1 PM2 PP2 PP5	LP
13	F	12	ADOA	SP	Yes	OPA1	AD	c.1466A-G(p.K489R)	Yes	PM1 PM2 PP1 PP4	LP
14	M	13	XIPN	SP	No	AIFM1	AD	c.697G-A(p.V233I)	No	PS1 PS2 PM1 PM5 PP2 PP3	P
15	M	9	XIPN	SP	Yes	AIFM1	AD	c.1463C>T(p.P488L)	No	PM2 PP1 PP4	VUS
16	F	1.8	LHON	SP	No	MT-ND6	M	m.14502T-C	Yes	PP3	VUS
17	F	8	MELAS	SP	No	MT-TL1	M	m.3243A-G	Yes	PS3 PM1 PM2 PP2 PP3	P
18	M	9	BVVL	SC	No	SLC52A2	AR	c.124C-G(p.P42A)/ c.1024dup(p.L342Pfs*103)	Yes/no	PM2 PM3 PP2 PP3/ PVS1 PS2	LP/P
19	F	2	Epilepsy with DFNB86 deafness	SC	No	TBC1D24	AR	c.241_252del(p.I81_K84del)/c.1525+1del (p.G510Efs*13)	No/no	PVS1 PS2 PP3/ PVS1 PS2 PP3	P/P
20	M	7	Pelizaeus–Merzbacher disease	SC	No	PLP1	XR	c.623G>T(p.G208V)	No	PS1 PS2 PM1 PM5 PP2	P
21	M	0.5	Microcephalic primordial dwarfism	SC	No	CEP135	AR	c.1364A-T(p.D455V) (Homozygous)	Yes	PM1 PM3 PP2 PP3	LP
22	F	1	H-ABC	SC	No	TUBB4A	AD	c.785G-A(p.R262H) (de novo)	No	PS2 PM1 PM2 PM5 PP2 PP5	P
23	M	0.6	Rahman syndrome	SC	No	H1-4	AD	c.441dupC(p.K148Qfs*48) (de novo)	Yes	PVS1 PM1 PP3	P

AD, autosomal dominant; ADOA, autosomal dominant optic atrophy; AIFM1, apoptosis inducing factor mitochondria-associated 1; AMCG, American College of Medical Genetics and Genomics; ANSD, auditory neuropathy spectrum disorder; AR, autosomal recessive; BVVL, Brown–Vialetto–Van Laere syndrome; CEP135, 135-kD centrosomal protein; DIAPH1, diaphanous 1; H1-4, histone protein 1 informs E; H-ABC, hypomyelination with atrophy of the basal ganglia and cerebellum; LHON, Leber's hereditary optic neuropathy; M, mitochondrial disease; MELAS, mitochondrial encephalomyopathy lactic acidosis and stroke like episodes syndrome; MT-ND6, NADH dehydrogenase 6; MT-TL1, mitochondrial tRNA gene for leucine 1; NS, non-syndromic; OPA1, optic atrophy 1; OTOF, otoferlin; PLP1, proteolipid protein 1; SC, syndromic central neuropathy; SLC52A2, solute carrier family 52, member 2; SLC17A8, solute carrier family 17, member 8; SP, syndromic peripheral neuropathy; TBC1D24, Tre2-Bub2-Cdc1 domain 1 and domain 24; TUBB4A, human tubulin beta class IVa; XIPN, X-linked inherited peripheral neuropathy; XR, X-linked recessive; Criteria of Pathogenicity Very Strong (PVS), Strong (PS), Moderate (PM), Supporting (PP); VUS, variant of unknown significance; P, pathogenic; LP, likely pathogenic.

Table 2. Hearing Performance in 23 Patients with ANSD

Number	Hearing Impairment Phenotype Hearing Degree (PTA)	Type of Audiometry	Early-Onset or Late-Onset	SDS	OAE	CM	ABR	ASSR
1	Profound	Upsloping	Early-onset	/	N	/	Absent	90 dBnHL
2	Profound	/	Early-onset	0%	N	/	Absent	Absent
3	Profound	Upsloping	Early-onset	/	N	/	Absent	80 dBnHL
4	Profound	Flat	Early-onset	0%	N	/	Absent	85 dBnHL
5	Profound	Flat	Early-onset	/	N	/	Absent	80 dBnHL
6	Profound	Flat	Early-onset	/	N	/	Absent	76 dBnHL
7	Profound	Upsloping	Early-onset	/	N	/	Absent	85 dBnHL
8	Profound	Flat	Early-onset	/	N	/	Absent	95 dBnHL
9	Profound	/	Early-onset	/	N	/	Absent	Absent
10	Profound	/	Early-onset	/	N	/	Absent	Absent
11	Moderate	Flat	Early-onset	72%	A	Present	Absent	Absent
12	Moderate	Flat	Late-onset	32%	N	/	Absent	/
13	Moderate	Flat	Late-onset	25%	N	/	Absent	/
14	Profound	Upsloping	Late-onset	0%	N	/	Absent	/
15	Severe	Upsloping	Late-onset	0%	N	/	Absent	Absent
16	/	/	Early-onset	/	A	Present	Bilateral waveform is not good, bilateral wave V threshold is 95 dBnHL, latent period of wave V is more than 10 ms.	Absent
17	Moderate	Upsloping	Late-onset	60%	A	Present	Bilateral waveform is not good, bilateral wave V threshold is 95 dBnHL, latent period of wave V is more than 10 ms.	60 dBnHL
18	Mild	Flat	Late-onset	25%	N	/	Absent	/
19	Mild	Flat	Early-onset	/	N	/	Absent	50 dBnHL
20	<25 dB	/	Early-onset	85%	N	/	Bilateral waveform repeatability is good, only wave I differentiation can be seen on both sides. Bilateral wave I threshold is 30 dBnHL; wave III and wave V are absent.	55 dBnHL
21	/	/	Early-onset	/	N	/	Absent	80 dBnHL
22	/	/	Early-onset	/	N	/	Bilateral waveform repeatability is good, only wave I differentiation can be seen on both sides. Bilateral wave I threshold is 30 dBnHL; wave III and wave V are absent.	40 dBnHL
23	<25 dB	/	Early-onset	/	N	/	Bilateral waveform repeatability is good, wave I and wave III differentiation can be seen on both sides. Bilateral wave V threshold is 80 dBnHL.	<30 dBnHL

A, significantly decreased amplitude or no response; ABR, auditory brainstem response; ANSD, auditory neuropathy spectrum disorder; ASSR, auditory steady state response; CM, cochlear microphonic; N: 100% detection rate at each frequency; OAE, observable otoacoustic emission; PTA, pure tone audiometry; SDS, speech discrimination abilities.

ANSD, all were found to carry pathogenic, likely pathogenic variants, or variants of unknown significance (VUS) (Table 1), with some patients harboring more than 1 variant. Of the 34 candidate variants, 13 were newly discovered, while 21 had been reported previously. The majority 26 were missense variants, followed by 4 splicing variants, 3 frameshift insertion/deletion variants, and 1 promoter variant. Ten candidate variants were classified as likely pathogenic, while 17 were categorized as pathogenic according to the ACMG guidelines. Evaluated by at least 1 interpreter, 7 of the 34 candidate variants were clearly classified as VUS. Additionally, 1 homozygous variant and 3 de novo variants were identified.

DISCUSSION

Inherited ANSD is a group of syndromic and NS diseases caused by gene mutations, most leading to syndromic peripheral or central neuropathy. The relationship between the genotype and phenotype of the primary disease with ANSD is crucial for pinpointing the precise site of a lesion through molecular diagnosis. Molecular diagnosis also helps to formulate different treatment strategies based on the other disease characteristics. Over the past 2 decades, discovering numerous genes involved in ANSD has significantly enhanced our understanding of the condition's underlying mechanisms. Auditory neuropathy spectrum disorder can present as

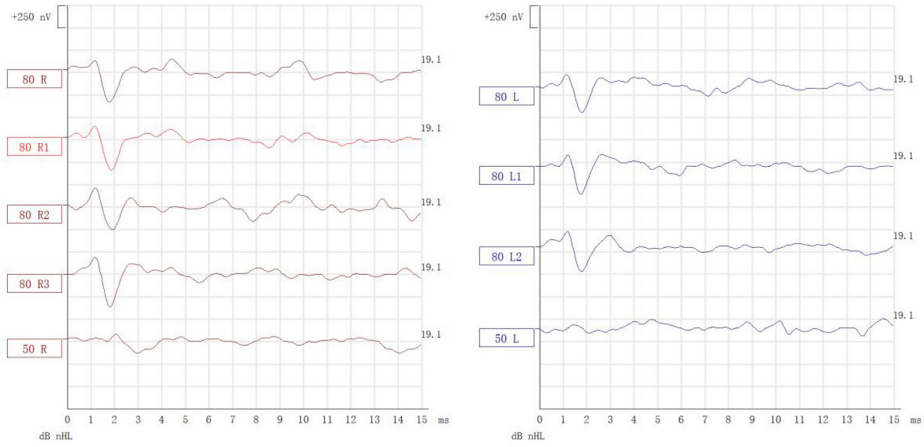

Figure 2. Bilateral ABR waveform with only wave I present.

either an isolated hearing issue (NS ANSD) or multiple systemic complications (syndromic ANSD). Typically, isolated ANSD involves presynaptic impairments, whereas ANSD with multisystem complications is often attributed to postsynaptic damage affecting the auditory nerve.[18] The *OTOF* gene encodes otoferlin, a protein that plays a crucial role in hearing by facilitating the transmission of sound signals from sensory hair cells in the inner ear to auditory nerve fibers. It is expressed at the presynaptic site and is the most common mutant gene causing isolated congenital ANSD.[11] In this study, 9 of 23 patients with *OTOF* gene compound mutations had early-onset ANSD. Our findings further confirm that the *OTOF* gene is the most common gene responsible for congenital isolated ANSD and show the value of genetic diagnosis. Conversely, the etiology differs in individuals with late-onset ANSD and is linked to optic atrophy, sensorimotor neuropathy, and other peripheral neuropathies. Apoptosis inducing factor mitochondria-associated 1 is a key genetic factor in late-onset cases.[19] It encodes apoptosis-inducing factor (AIF), which anchors to the inner mitochondrial membrane and translocates to the nucleus to induce apoptosis when stimulated by apoptosis. X-linked *AIFM1*, a mutation resulting from inherited peripheral neuropathies, was first identified as being associated with ANSD in 2015.[20] The 2 cases in this study with delayed-onset ANSD were linked to *AIFM1* mutations.

In our study, a *DIAPH1* mutation was also identified in a family with isolated ANSD, with the proband carrying the same mutation of *DIAPH1* as her father. In 1997, *DIAPH1* was initially recognized as the causative gene for NS deafness *DFNA1* in a family from Costa Rica.[21] Six deleterious heterozygous variations in *DIAPH1* were identified in 7 studies. There was no description or consideration of ABR and DPOAE tests. In 2020, an ANSD phenotype was linked to a family with inherited deafness caused by *DIAPH1*.[22] This gene may have a role in the regulation of actin polymerization in hair cells of the inner ear. The family in our study with the *DIAPH1* mutation further validates that mutations in the *DIAPH* gene can lead to ANSD.

Except for NS ANSD, syndromic ANSD includes LHON,[23] MELAS syndrome,[24] ADOA,[25] BVVL syndrome,[26] and Pelizaeus–Merzbacher disease.[7]

Leber's hereditary optic neuropathy, the most common maternally inherited optic neuropathy, is associated with mitochondrial DNA (mtDNA) point mutations affecting Complex I subunits, frequently at nucleotide position 11778.[27] Leber's hereditary optic neuropathy is a rapid, bilateral, often sequential, painless loss of central vision due to retinal ganglion cell death and optic nerve atrophy, resulting in severe, permanent bilateral visual impairment in most cases.

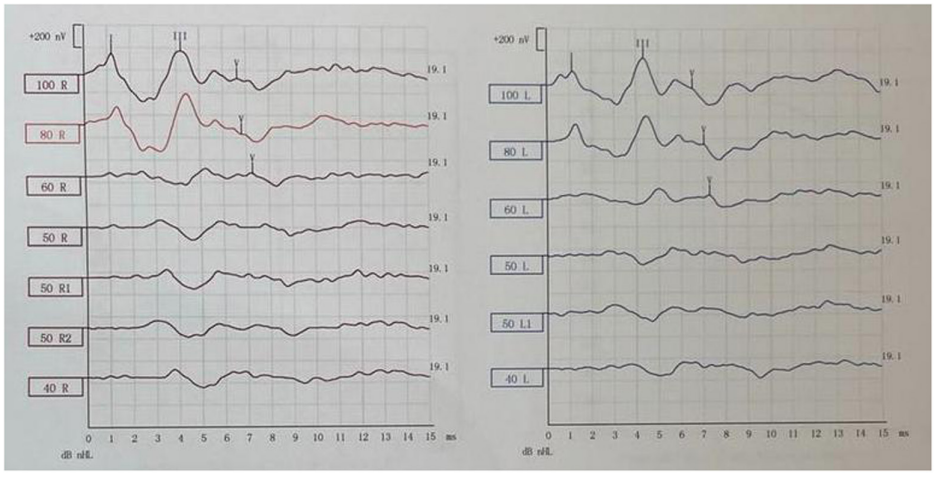

Figure 3. Bilateral ABR waveform with differentiation of wave I and wave III. The wave V threshold at 80 dBnHL.

Evidence suggests that mtDNA variations can affect central nervous system function, leading to disorders such as ANSD. In a 2004 study, Luxon et al[23] observed progressive auditory neuropathy in patients with LHON, evidenced by abnormal ABR with prolonged conduction times in early components, despite no observed abnormalities in the IHC or cochlear nucleus. These abnormal ABR findings may indicate a disruption in stimulus-timing-related synchrony, contributing to the auditory dysfunction observed in these patients. In our study, 1 case of an mtDNA *MT-ND6* mutation was diagnosed as LHON with ANSD. Another patient with the mtDNA *MT-TL1* 3243 A-G mutation, diagnosed with MELAS syndrome, also exhibited ANSD. Auditory neuropathy spectrum disorder can occasionally occur in patients with mtDNA disease.[24] In addition to LHON with ANSD, ADOA, which causes prolonged visual impairment that is typically detected in childhood, has recently been observed to exhibit non-ocular neurological features, including ANSD.[25] *OPA1* gene abnormalities are the primary cause of ADOA.[28] The protein encoded by this gene localizes to the inner mitochondrial membrane and helps regulate mitochondrial stability and energy output. In the present study, two patients with *OPA1* missense mutations exhibited the ANSD phenotype.

Brown–Vialetto–Van Laere syndrome, an extremely rare neurological disorder, is also known as riboflavin transporter deficiency, which leads to childhood motor neuron disorders often associated with ANSD.[26] As a progressive neurodegenerative syndrome, BVVL presents with polyneuropathic symptoms and late-onset progressive HL, accompanied by ANSD features, vision loss, and muscular weakness. Reports of unknown-onset ANSD linked to BVVL syndrome have also emerged.[29] Mutations in *SLC52A2* and *SLC52A3* are the origin of the disease, which leads to riboflavin transporter deficiency.[30] This deficiency affects motor neurons and auditory nerve function, leading to ANSD and HL. In this study, a patient with BVVL syndrome and mild HL had a compound heterozygous mutation in *SLC52A2*. After genetic testing, the patient was prescribed vitamin B2 supplements, which helped preserve his hearing. Accurate molecular diagnosis of BVVL syndrome enables timely correction of genetic riboflavin (vitamin B2) deficiency. Cochlear implants are recommended for patients with severe or profound HL and delayed speech development due to BVVL syndrome.[26]

Additionally, this study included 2 cases of hypomyelination leukoencephalopathy: Pelizaeus–Merzbacher disease with an X-linked *PLP1* gene mutation and H-ABC with a *TUBB4A* gene mutation. The encoded protein by *PLP1* may play a role in the compaction, stabilization, and maintenance of myelin sheaths. Pelizaeus–Merzbacher disease is a hereditary condition that affects the development of myelin in the central nervous system and influences various functions, such as auditory and linguistic capabilities.[7] The *TUBB4A* gene encodes a member of the beta tubulin family, which assembles to form microtubules. Hypomyelination with atrophy of the basal ganglia and cerebellum, an inherited hypomyelination leukoencephalopathy caused by a *TUBB4A* mutation, is identified as an allelic variant, with all affected patients displaying de novo mutations,[31] as seen in this study. Both PMD and H-ABC have been reported with ANSD.[32]

In 2014, a gene related to epilepsy, *TBC1D24*, was discovered with a homozygous mutation responsible for *DFNB86* deafness.[33] This gene was predominantly localized to spiral ganglion neurons within the inner ear of mice, suggesting that *DFNB86* deafness could be classified as a disorder within the auditory neuropathy spectrum. The findings in this study, which demonstrate *TBC1D24* gene mutations causing ANSD in a patient with epilepsy, support this classification. Another gene that causes ANSD and central neuropathy is *CEP135*, which is associated with microcephalic primordial dwarfism.[34] These discoveries underscore the importance of genetic testing in children with ANSD, particularly those exhibiting atypical symptoms or signs of underlying conditions.

CONCLUSION

In the present study, we identified several mutations that result in ANSD. Actually, there are still some genes associated with ANSD, especially with syndromic ANSD, such as *PLA2G6*, *WFS1*, and *MPZ*.[4,35] Recognizing these diseases through genetic diagnosis is essential. We confirmed that ANSD can either present as an isolated or a symptom of syndromic ANSD based on clinical diagnosis. Additionally, we found that molecular diagnosis could facilitate clinical assessments. Furthermore, molecular diagnosis allows us to determine the prognosis of the disease, which aids in selecting treatment strategies. Recent advancements in HA and CI technologies have reignited optimism for addressing auditory neurodegeneration. Genetic approaches, including gene replacement and the corrective editing of mutant sequences, show promise for repairing damaged cells, potentially restoring hearing in individuals with ANSD.

Availability of Data and Materials: The datasets generated and/or analyzed during the current study are available in the ClinVar repository, accession number SCV004801097 - SCV004801127.

Ethics Committee Approval: This study was approved by the National Unit of Clinical Trial Ethics Committee of the Peking University First Hospital (approval number: (2020)24; date: October 11, 2020).

Informed Consent: Written informed consent was obtained from the patient as well as children's parents who agreed to take part in the study.

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