Novel Variant of FDXR as a Molecular Etiology of Postlingual Post-Synaptic Auditory Neuropathy Spectrum Disorder via Mitochondrial Dysfunction: Reiteration of the Correlation between Genotype and Cochlear Implantation Outcomes

Running title: FDXR and Adult-Onset Post-Synaptic ANSD

Bong Jik Kim1,2, Yujin Kim3, Ju Ang Kim4, Jin Hee Han4, Min Young Kim4, Hee Kyung Yang5, Chae-Seo Rhee4, Young Cheol Kang3, Chun-Hyung Kim3, Byung Yoon Choi4

1Department of Otorhinolaryngology, Chungnam National University School of Medicine, Chungnam National University Sejong Hospital, Sejong 30099, South Korea
2Brain Research Institute, Chungnam National University College of Medicine, Daejeon 35015, South Korea
3Paean Biotechnology Inc., Seoul, South Korea
4Department of Otorhinolaryngology – Head and Neck Surgery, Seoul National University Bundang Hospital, Seoul National University College of Medicine, Seongnam 13620, South Korea
5Department of Ophthalmology, Seoul National University Bundang Hospital, Seoul National University College of Medicine, Seongnam 13620, South Korea

Address for correspondence

Byung Yoon Choi, MD, PhD, Department of Otorhinolaryngology – Head and Neck Surgery, Seoul National University Bundang Hospital, Seoul National University College of Medicine, 300 Gumi-dong, Bundang-gu, Seongnam 13620, Republic of Korea. Tel.: +82-31-787-7406, Fax:+82-31-787-4057, Email: choiby@snubh.org
Abstract

Objectives: FDXR encodes the mitochondrial ferredoxin reductase, which is associated with auditory neuropathy spectrum disorder (ANSD) and optic atrophy. Only two studies have described FDXR-related hearing loss. The auditory rehabilitation outcomes of this disease entity have not been investigated, and the pathophysiologic mechanism is not well elucidated. Here we report a hearing-impaired subject with co-segregation of the FDXR variant and post-synaptic type ANSD, who underwent cochlear implantation (CI) with favorable outcomes. We suggest a possible pathophysiologic mechanism of adult-onset ANSD via mitochondrial dysfunction.

Methods: A 35-year-old woman was ascertained to have ANSD. Exome sequencing identified the genetic cause of hearing loss, and functional study measuring mitochondrial activity was performed to provide molecular evidence of pathophysiology. Expression of FDXR in the mouse cochlea was evaluated by immunohistochemistry. Intraoperatively, electrically-evoked compound action potential (ECAP) responses were measured, and mapping parameters were adjusted accordingly. Audiological outcomes were monitored for over 1 year.

Results: In lymphoblastoid cell lines (LCLs) carrying a novel FDXR variant, decreased ATP and MtMP levels and increased ROS levels were observed compared to control LCLs. These dysfunctions were restored by administering mitochondria isolated from umbilical cord mesenchymal stem cells, confirming the pathogenic potential of this variant via mitochondrial dysfunction. Partial ECAP responses during CI and FDXR expression in the mouse cochlea indicate that FDXR-related ANSD is postsynaptic. By increasing the pulse width during mapping, the patient’s CI outcomes showed significant improvement over 1-year post-CI.

Conclusion: Post-synaptic ANSD due to a novel FDXR variant linked to mitochondrial dysfunction was identified first in a Korean, and 1-year post CI outcomes were reported for the first time in the literature. Excellent audiologic results were obtained, and our results reiterate the correlation between genotype and CI outcomes in ANSD.

Keywords: FDXR, postlingual, post-synaptic, auditory neuropathy, optic atrophy, mitochondria, cochlear implantation, auditory rehabilitation
Highlights

- A novel missense variant of the *FDXR* gene (p.Val314Leu) was identified as a causative variant for adult-onset ANSD with optic atrophy in a Korean pedigree for the first time.

- Mitochondrial dysfunction was suggested as a possible pathogenesis of *FDXR*-related hearing loss, based on decreased mitochondrial function in patient-derived cell lines and restoration of function after administration of normal mitochondria to the cell lines.

- Immunohistochemistry and intraoperative electrophysiological findings suggest that alteration of *FDXR* manifests as post-synaptic ANSD, posing concerns about the outcomes of CI.

- For the first time in the literature, 1-year post-CI outcomes for *FDXR*-related ANSD were reported, with favorable results after increasing the pulse width during mapping.

- Our results could provide a scientific basis for advancing CI timing with clinical confidence for post-synaptic ANSD caused by alterations in genes with similar expression to *FDXR*.
Introduction

Hearing and visual impairment rarely co-occur due to common genetic etiologies (1-3). The ferredoxin reductase (FDXR) gene is a common etiology of these conditions, and alterations in FDXR have been associated with auditory neuropathy spectrum disorder (ANSD) and optic atrophy (OMIM #617717)(4). Other common etiologies include alterations of the OPA1 and TMEM126A genes (1, 2). Among them, FDXR was most recently reported in 2017; since then, only one other article regarding FDXR-related hearing loss has been published (5). Thus, the phenotypic manifestations of this condition, including auditory rehabilitation outcomes, have not been well described. Moreover, there has been no report on the outcomes of cochlear implantation (CI) in this disease entity.

FDXR encodes a mitochondrial ferredoxin reductase involved in the biosynthesis of iron-sulfur (Fe-S) clusters and heme formation (6). A previous study reporting the first case of FDXR-related hearing loss confirmed the critical role of Fe-S biogenesis in the function of auditory and optic neurons. It also suggested indirect evidence of mitochondrial iron overload in ARH1-null mutation (arh1Δ) yeast cells expressing the human mutant protein, where Arh1 is the ortholog of human ferredoxin reductase (4). Furthermore, increased mitochondrial ROS production was observed in fibroblasts from individuals affected by FDXR-related hearing loss. However, direct assessments of mitochondrial functions other than ROS production have not been performed in this disease entity.

ANSD, which is associated with FDXR mutation, is a distinct type of hearing loss characterized by absent auditory brainstem response (ABR) and normal otoacoustic emissions (OAEs), with poor speech discrimination given the pure tone audiometry threshold (7). There exist several etiologies for ANSD, and alterations in OTOF are the most common (8). CI has been a mainstay treatment for successful auditory rehabilitation in several, if not all, types of ANSD (9, 10). Recently, OTOF-related AN (DFNB9) has become the first target for successful inner ear gene therapy (11). However, given the paucity of literature regarding FDXR-related ANSD, auditory rehabilitation data are limited for this disease entity, and CI outcomes have never been reported.

In the study, we report the first Korean pedigree with FDXR-related ANSD and show the audiologic outcome of CI in this disease entity for the first time in the literature. We also provide evidence for mitochondria-related pathogenesis by directly assessing the mitochondrial functions in
control- and mutant-derived lymphoblastoid cell lines and further suggest mitochondrial administration as a potential treatment for this disease.

Materials and Methods

Subject and Clinical features

A 35-year-old woman (SB1113-1825) presented with progressive hearing loss on the left side since childhood and on the right side, which had started 2 years ago. She particularly complained of difficulty hearing in noisy environments. History-taking revealed that visual impairment, more severe on the left side, had been present since childhood. Audiologic tests showed no response on both sides in the auditory brainstem response (ABR) and normal responses on both sides in the distortion product otoacoustic emissions (DPOAE) and transient evoked otoacoustic emissions (TEOAE) tests. These findings were consistent with a diagnosis of Auditory Neuropathy Spectrum Disorder (ANSD) (Figure 1). Intraoperative ECAP thresholds were measured in every channel for the subject under an automated measurement mode of neural response telemetry (NRT), as previously described (9, 10, 12, 13). Preoperative and postoperative evaluations of auditory rehabilitation were performed using the speech recognition test and CAP score, as previously described (14, 15). An ophthalmologic consultation was conducted to investigate the involvement of the eye. The human subjects research in this study was approved by the Institutional Review Board of Seoul National University Bundang Hospital (IRB-B-1007-105-402), and written informed consent was obtained from all subjects.

Genetic diagnosis and generation of lymphoblastoid cell lines

Genomic DNA samples from the blood of the proband and her siblings were collected and analyzed for genetic diagnosis. Initially, 11 recurrent variants of five deafness genes were screened as described elsewhere (16-19). However, these did not identify potential candidate variants to explain the hearing loss in the pedigree. Subsequently, we proceeded with exome sequencing (ES) and
narrowed down the candidate variants using bioinformatics filtering steps (20). The potential causative variant identified through the above steps was confirmed by Sanger sequencing and was checked for co-segregation with the phenotype in the pedigree. Whole blood in the EDTA bottle from the proband was sent to Seoul Clinical Laboratories in Gyeonggi-do, South Korea, for the generation of lymphoblastoid cell lines (LCLs).

**Cell culture**

Human umbilical cord-derived mesenchymal stem cells (UC-MSCs) were obtained through the primary culture of the umbilical cord from a healthy pregnant woman who provided informed consent. The study was approved by the Public Institutional Review Board (IRB) designated by the Ministry of Health and Welfare, Korea (IRB No. P01-202002-31-008). UC-MSCs were cultured in Minimum Essential Medium Eagle Alpha Modification (α-MEM; Hyclone, USA) supplemented with 10% fetal bovine serum and 10 ng/mL basic fibroblast growth factor (FGF-2; CHA Meditech Co., Korea).

**Isolation of mitochondria from UC-MSC**

UC-MSCs were used at passage 7 for mitochondria preparation. Cells were harvested from culture flasks, depressurized in SHE buffer [0.25 M sucrose, 20 mM HEPES (pH 7.4), 2 mM EGTA, 0.1% defatted bovine serum albumin (BSA)] using nitrogen cavitation (Parr Instrument, USA) (21), and then centrifuged at 2,000 x g for 10 minutes at 4°C to remove cellular debris and nuclei. The supernatant was subsequently centrifuged at 12,000 x g for 15 minutes at 4°C to pellet the mitochondria. The pellet was washed twice by resuspension in 500 μL SHE buffer followed by centrifugation at 20,000 x g for 10 minutes at 4°C. The final pellet was resuspended in 100 μL of suspending buffer and kept on ice until use. Isolated mitochondria were quantified by determining protein concentrations using a bicinchoninic acid assay. The treatment of mitochondria was as follows: 1.0 x 10^5 LCLs were seeded in 24-well plates and cultured overnight, followed by the administration of ten mg of the isolated mitochondria.
Measurement of intracellular ATP

Intracellular ATP concentration was measured using a luciferin-luciferase reaction with CellTiter-Glo Luminescent reagent (Promega, USA), following the manufacturer's recommendations. A total of $5 \times 10^3$ LCLs were seeded into a 96-well white tissue culture plate and incubated with PN-101 for 24 hours. After washing with DPBS, 100 µL of CellTiter-Glo® reagent (Promega, USA) was added to each well. The LCLs were then incubated for 10 minutes at room temperature on an orbital shaker to induce cell lysis. The luminescence signal was measured using a SYNERGY HTX multi-mode reader (BioTek, USA), and the data were analyzed using Gen5 3.09 software. The study was performed in triplicate to ensure consistent results. All data are presented as the percentage of control.

Intracellular ROS measurements

ROS production in cell lysates was evaluated using CM-H2DCFDA (Invitrogen, USA) according to the supplier's instructions. Briefly, 50 µL of cell lysate was treated with 1 µM CM-H2DCFDA at 37°C for 60 minutes. After staining with CM-H2DCFDA, ROS production was measured using a SYNERGY HTX multi-mode reader (BioTek, USA), and the data were analyzed using Gen5 3.09 software. Relative CM-H2DCFDA fluorescence intensities were normalized to Hoechst 33342. The study was performed in triplicate. All data are presented as the percentage of control.

Measurement of mitochondrial membrane potential

The mitochondrial membrane potential was measured by the fluorescence of the potential-dependent TMRE. Briefly, cells were incubated with 500 nM TMRE at 37°C for 30 minutes, protected from light. After staining with TMRE, fluorescence was measured using flow cytometry. Cells were collected, suspended in PBS, and analyzed with a CytoFLEX LX flow cytometer. Data analysis was performed using CytExpert software.

Antibody validation
The GFP-tagged FDXR (NM_001258012), Human Tagged ORF Clone (RG23436, Origene, USA), was transfected into HEK293 cells using Lipofectamine 3000 (Invitrogen). For controls, HEK293 cells were transfected with either distilled water (negative control) or pCMV6-AC plasmid DNA (empty vector; mock). Twenty-four hours post-transfection, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature, followed by three additional washes with PBS. They were then treated with blocking buffer (2% bovine serum albumin, 0.1% Triton X-100, 5% normal goat serum) for 1 hour at room temperature. For the primary antibody reaction, the cells were incubated with rabbit anti-FDXR antibody (ab204310, Abcam, England) diluted 1:200 for 2 hours at room temperature and washed three times with PBS. The secondary antibody, Alexa Fluor 555 goat anti-rabbit (A21428, Invitrogen), diluted 1:500, was used, and phalloidin-647 (A22287, Invitrogen) was added for 1 hour at room temperature. After three washes with PBS, the cells were counterstained with mounting medium containing DAPI (H-1200, Vector Laboratories, USA) to prepare slides for imaging with the LSM800 (Zeiss, Germany) confocal microscope.

**Immunohistochemistry**

C57BL/6 mice, aged 4 weeks, were sacrificed, and their whole mount cochleae were fixed in 4% paraformaldehyde at room temperature for 1 hour, followed by three washes with PBS. The organ of Corti was then isolated from each fixed cochlea. The samples were treated with a blocking buffer containing 2% bovine serum albumin, 0.1% Triton X-100, and 5% normal goat serum for 1 hour at room temperature. The samples were incubated with anti-FDXR antibody diluted 1:100 at 4°C for 2 days and subsequently washed three times with PBS. The secondary antibody, Alexa Fluor 488 goat anti-rabbit (ab150077, Abcam), was diluted 1:400 and mixed with Rhodamine phalloidin (Invitrogen, R415), then incubated overnight at 4°C. After three washes with PBS, the samples were mounted with a mounting medium containing DAPI to prepare slides for imaging with the THUNDER Imager microscope (Leica, Germany).
Results

Audiologic characteristics of hearing loss from SNUBH1113

Sporadic asymmetric hearing loss was observed in a 35-year-old patient (SNUBH1113-1825) from family SNUBH1113. The diagnosis of ANSD was confirmed by absent ABR responses and normal otoacoustic emissions in DPOAE/TEOAE. This was further supported by discrepancies between pure tone audiometry thresholds and word recognition scores, characterizing the audiologic phenotype of SNUBH1113-1825 as postlingual ANSD (Figure 1A-C). During the ophthalmologic examination, her best-corrected visual acuities were 20/30 in the right eye and 20/40 in the left eye. Fundus photography revealed temporal pallor of the optic disc in both eyes (OU) and mild thinning of the retinal nerve fiber layer. More severe involvement of the papillomacular bundle was observed on optical coherence tomography OU, which is characteristic of mitochondrial optic neuropathy (Supplementary Figure 1).

Genetic diagnosis of postlingual ANSD from SNUBH1113

ES identified a potentially causative, novel variant of FDXR (c.940G>T: p.Val314Leu) in a homozygous state, and the variant status was confirmed with Sanger sequencing. The patient's phenotypes of ANSD and optic atrophy (ANOA) matched perfectly with previous reports in the literature related to FDXR (4). Additionally, the variant co-segregated well with the hearing loss phenotype in the pedigree, as expected for autosomal recessive inheritance (Figures 1A and 1D). Detailed genetic information related to the pathogenic potential of the variant is summarized in Table 1.

Mitochondrial dysfunction of LCLs derived from patients harboring FDXR variants

To investigate whether the FDXR variant (c.940G>T: p.Val314Leu) is indeed pathogenic and thus
affects mitochondrial functions, intracellular ATP, reactive oxygen species (ROS), and mitochondrial membrane potential (MtMP) were evaluated in LCLs derived from both a control and a patient with the FDXR variant (SNUBH1113-1825). As shown in Figures 2A and 2B, intracellular ATP and MtMP were significantly reduced in FDXR-mutant LCLs compared to the control, whereas intracellular ROS was remarkably increased (Figure 2C).

Next, we attempted to rule out the possibility that the alterations in intracellular ATP, ROS, and MtMP were secondarily induced by factors other than mitochondrial dysfunction itself. To accomplish this, we investigated whether the impairments in intracellular ATP, ROS, and MtMP observed in FDXR-mutant LCLs could be reversed through direct mitochondrial administration. For this purpose, functionally intact mitochondria were isolated from human UC-MSCs and designated as PN-101. We confirmed that PN-101 could be successfully introduced into FDXR-mutant LCLs by simple coinubcation using fluorescence-labeled PN-101 (Figure 2D). Interestingly, treatment with PN-101 restored the intracellular ATP and MtMP levels to those of the control in FDXR-mutant LCLs. Although not statistically significant, PN-101 tended to decrease the intracellular ROS in the FDXR-mutant LCLs (Figure 2A-C). Collectively, these observations confirmed that this FDXR variant (c.940G>T: p.Val314Leu) is clearly pathogenic and significantly affects mitochondrial functions.

Expression pattern of FDXR in the mouse cochlea

The specificity of the anti-FDXR antibody was validated in HEK293 cells transfected with GFP-tagged FDXR (FDXR-GFP), distilled water (negative control), and an empty vector (mock) (Supplementary Figure 2). Subsequently, the expression of FDXR was examined in the whole mount of the mouse cochlea, which revealed prominent expression in the spiral ganglion neuron area and moderate expression in the inner hair cell area (Figure 3). The localization of FDXR in the cochlea suggests a potential role for FDXR in the synaptic region and the spiral ganglion, thus leading to the hypothesis that ANSD due to alterations in FDXR would be primarily post-synaptic.

Intraoperative electrophysiological findings and CI outcomes
According to the IAC-MRI oblique sagittal view, the cochlear nerve exhibits significant atrophy. Therefore, the prognosis for CI does not appear favorable (Figure 4A). Indeed, during CI, after the electrode insertion, when measuring the ECAP response in the default mode with a pulse width of 25, ECAP responses were only obtained in the nine apical channels (channels 14-22). This type of ECAP pattern, where meaningful responses were obtained but only in some consecutive electrodes, was previously proposed to be associated with post-synaptic pathologies (9). Therefore, the intraoperative electrophysiological findings (Figure 4A) correspond well with expression pattern of FDXR in the mouse cochlea (Figure 3). However, after increasing the pulse width to 50, ECAP responses were obtained in all channels (Figure 4B). Therefore, this parameter was maintained during the switch-on and subsequent mapping sessions.

Given these mapping parameters, post-CI audiologic outcomes demonstrated gradual improvements, almost plateauing at 6 months postoperative follow-up, with a sentence recognition score of 98% and a CAP score of 6 (Figure 4C). The speech discrimination score on the left side also increased from 4% with a hearing aid to 56% with the CI at the 1-year post-CI follow-up.

Discussion

Since the first report of FDXR gene as a deafness gene, only one paper has supported the gene as a cause of ANSD, with five FDXR-related hearing loss pedigrees (4, 5). In this study, FDXR was reconfirmed as a gene related to ANSD, in a Korean pedigree for the first time, suggesting its relevance across various ethnicities, including previous reports from Tunisian, Algerian, French, Azerbaijani, Russian, and Chinese families. Furthermore, we provided evidence that FDXR-related ANSD is postsynaptic, through expression studies using immunohistochemistry and intraoperative ECAP response measurements. Our study also presents the first report in the literature on post-CI auditory rehabilitation outcomes for this type of postsynaptic ANSD due to FDXR mutation, which demonstrated successful outcomes for at least one year. Regarding the underlying pathophysiology of this disease entity, significant mitochondrial dysfunction was identified through functional studies using
a patient-derived cell line, consistent with a previous report (4). An interesting finding is that the alterations in intracellular ATP, ROS, and MtMP observed in the patient-derived cells were significantly ameliorated by the introduction of healthy mitochondria into these cells. This finding not only provides stronger evidence that the FDXR variant causes mitochondrial dysfunction but also suggests mitochondrial administration as a potential therapeutic approach for FDXR alterations in the future.

So far in the literature, only nine subjects from five pedigrees have been reported to have FDXR-related hearing loss and visual impairment (4, 5). Previous studies have reported that patients exhibited a diverse onset of hearing loss, ranging from congenital to 20 years old. However, all reported patients shared characteristics of both ANSD and visual impairment, as seen in our patient. Our patient exhibited a relatively late onset of hearing loss and asymmetric hearing loss, which is distinct from other reported cases. To date, eight missense variants, including those identified in our study, and one nonsense variant have been reported to be associated with FDXR-related hearing loss and visual impairment. Although the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) variant interpretation guidelines for genetic hearing loss classified the c.1069G>T variant in our study as of uncertain significance (Table 1) (22, 23), the characteristic phenotype of ANSD and optic atrophy was highly suggestive of a causative variant in the pedigree, and PP4 can be added through the curation of ClinGen. Additionally, a functional study on mitochondrial function could support the pathogenic potential of the variant.

The cochlea is an important organ of the peripheral auditory system with a high energy demand. Because mitochondria are the powerhouses that produce intracellular energy in the form of ATP through oxidative phosphorylation, mitochondrial function in the cochlea is critical for maintaining auditory capacity. In this context, mitochondrial abnormalities have frequently been implicated in hearing loss caused by mutations in either mitochondrial DNA or nuclear DNA-encoding proteins, which are closely associated with mitochondrial function (24-26). For example, the mitochondrial 12SrRNA gene A1555G mutation results in non-syndromic hereditary hearing loss, and mutations in genes coding for nuclear DNA encoding proteins (e.g., OPA1, MFN2, and MSRB3) contribute to mitochondrial dysfunction, leading to sensorineural deafness (2, 27-29). Therefore, the best option for treating hearing loss would be one that directly repairs or replaces damaged mitochondria. Recently, it was reported that mitochondria isolated from different cell sources can be taken up by cells and tissues via local or
systemic injections (30, 31). Moreover, intravenously administered mitochondria preferentially migrate to tissues and cells with damaged mitochondria (10). In this context, administering healthy mitochondria to replace damaged ones has received attention as an attractive therapeutic strategy for treating diseases caused by mitochondrial dysfunction. The current study demonstrated that the FDXR variant induced mitochondrial dysfunction, including decreases in ATP production and mitochondrial membrane potential, and an increase in ROS. Interestingly, UC-MSC-derived mitochondria (PN-101) could restore the mitochondrial damage induced by the FDXR variant, suggesting that mitochondrial replacement can be developed as a novel therapeutic approach for hearing loss.

CI is a definitive solution for many patients with severe-to-profound hearing loss, including ANSD. The postoperative audiologic improvements in this study provide additional evidence for the important role of CI in the auditory rehabilitation of FDXR-related adult-onset ANSD, which is postsynaptic. Generally, a hearing aid, which could play a crucial role in bridging hearing ability to CI, can be beneficial for most types of hearing loss. However, as observed in our pedigree where the word recognition score was very poor (4%) despite relatively preserved pure tone audiometry thresholds (45 dBHL), the role of hearing aids in ANSD is less efficacious in bridging to CI and thus, CI instead of hearing aid should cover a significant portion of the gap between no assistive device and CI. This may support early CI implementation (Figure 5). The clinical decision to advance the timing of CI drastically, while accepting the risk of some damage to residual pure-tone hearing, should be based on an accurate etiological assessment, including molecular genetic diagnosis. Another consideration for the auditory rehabilitation of ANSD, which differs from that of general sensorineural hearing loss (SNHL), is that cochlear nerve dystrophy can progress relatively rapidly. This progression could be supported by our in-house database (unpublished) and a similar tendency in optic nerve dystrophy, sharing a common etiology with FDXR-related ANSD (32). Therefore, in cases of ANSD, a more proactive approach to CI is necessary compared to the standard indications for CI in general SNHL. The proband in our study experienced significantly worse hearing on the left side, and even on the right side, which was better, she had poor speech discrimination scores (50% for 1-syllable words, 75% for 2-syllable words, and 80% for sentences). These scores could not be adequately compensated using a hearing aid. As a working professional, it was difficult for her to maintain a normal daily life, which led to her desire for a CI. Furthermore, magnetic resonance imaging of the temporal bone in the patient revealed severe atrophy of the cochlear nerve on the left side (red arrow).
and progressing atrophy on the right side, despite an almost normal pure tone threshold (Supplementary Figure 3). Overall, genetic diagnosis in post-synaptic, postlingual ANSD might have significance in providing a basis for confidently advancing CI. Previously, Kim et al. (2023) demonstrated that even in post-synaptic ANSD involving the spiral ganglion neuron or cochlear nerve, there is potential for better synchronous stimulation, indicating that the post-synaptic nature of ANSD does not preclude the benefits of CI. In fact, in their study, by performing early CI and adjusting mapping parameters, significant improvement in speech discrimination after CI in these post-synaptic ANSD cases was demonstrated (9). In our present study, we confirmed these findings once again in FDXR-related ANSD.

In this study, we reconfirmed FDXR as a gene related to ANSD and optic neuropathy, and reported the first Korean pedigree with this disease entity. Post-CI audiological outcomes showed that FDXR-related post-synaptic ANSD could be a good candidate for cochlear implantation, although long-term follow-up is needed. Functional studies also provided a possible link between mitochondrial dysfunction and ANSD, suggesting the potential role of mitochondrial replacement in the treatment of hearing loss.

Conflict of interest

Young Cheol Kang, Yujin Kim, and Chun-Hyung Kim are employees of Paean Biotechnology, Inc. The remaining authors declare no conflicts of interest concerning the research, authorship, and publication of this article.
Acknowledgments

This study is supported by the Basic Science Research Program through the NRF, funded by the Ministry of Education (Grant 2021R1A2C2092038 to Choi, B.Y.), Bio Core Facility center program through the NRF-2022M3A9G1014007 to Choi, B. Y. and also by the Basic Research Laboratory program through the NRF,funded by the Ministry of Education (Grant RS-2023-0021971031482092640001 to Choi, B. Y) and the Technology Innovation Program (K_G012002572001 to Choi, B.Y) funded By the Ministry of Trade, Industry & Energy (MOTIE, Korea). This study is also funded by SNUBH intramural research fund (18-2023-0004, 16-2022-0005, 13-2024-0004, 13-2023-0002, and 16-2023-0002 to Choi, B.Y). This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (2021R1C1C1007980 and RS-2024-00355990 to Kim, B.J.), Chungnam National University Sejong Hospital Research Fund, 2022 and Chungnam National University (Kim, B.J.). This research was supported by Korea Drug Development Fund funded by Ministry of Science and ICT, Ministry of Trade, Industry, and Energy, and Ministry of Health and Welfare (RS-2023-00283926 to Kang, Y.C.).

Author contributions

Conceptualization: BJK, C-HK, BYC.

Data curation: YK, JHH, MYK.

Formal analysis: JHH, YCK.

Funding acquisition: BJK, YCK, BYC.

Methodology: MYK, YK.

Project administration: BJK.

Visualization: BJK, YK, JAK, HKY, BYC.

Writing-original draft: BJK, YK, JAK, C-HK, BYC.

Writing-review and editing: BJK, JAK, HKY, C-SR, C-HK, BYC.
References

    TMEM126A causing autosomal recessive optic atrophy and auditory neuropathy. Mol Vis.

    Mechanisms of Novel OPA1 Mutations Predicted by Molecular Modeling in Patients With Autosomal

    doi: 10.1038/s41598-017-16676-9. PubMed PMID: 29184165; PubMed Central PMCID: PMC5705773.

    Sensorial Neuropathies and Expand the Spectrum of Mitochondrial Fe-S-Synthesis Diseases. Am J
    28965846; PubMed Central PMCID: PMC5630197.

    gene variants in a Chinese boy exhibiting hearing loss, visual impairment, and motor retardation.
    33742450.

6. Shi Y, Ghosh M, Kevtunovych G, Crooks DR, Rouault TA. Both human ferredoxins 1 and 2 and


bmb-55-9-459 [pii]


0094 [pii]


26057

srep26057 [pii]. PubMed PMID: 27184109; PubMed Central PMCID: 4868981.

Figure legends

Figure 1. Pedigree, audiologic tests, and Sanger chromatograms

(A) The pedigree demonstrated a sporadic or autosomal recessive inheritance pattern. (B) The pure-tone audiogram showed asymmetric sensorineural hearing loss, and auditory brainstem response (ABR) testing resulted in no response at 90 dB in both ears. (C) DPOAE and TEOAE showed normal responses in both ears. (D) The Sanger chromatogram identified a homozygous variant only in the proband.

Figure 2. Intracellular mitochondrial function in the FDXR mutant-derived LCLs.

(A) Schematic diagram of the treatment of PN-101 into LCLs. Intracellular ATP, ROS, and mitochondrial membrane potential (MtMP) were measured in LCLs derived from both the control and the FDXR mutant. Compared to the control, the LCLs derived from the FDXR mutant exhibited mitochondrial dysfunction, including decreased intracellular ATP and MtMP (B and C), and increased intracellular ROS (D). Treatment with PN-101 restored the levels of ATP, ROS, and MtMP in the FDXR mutant-derived LCLs to those of normal LCLs (B-D). The introduction of PN-101 into FDXR mutant LCLs was confirmed using fluorescence-labeled PN-101 (E).

The fluorescence of control cells was set to 100%. All data are presented as mean ± standard error of the mean and student’s t-test was used for comparison. *P<0.05 and ***P<0.001 vs. normal and ###P<0.001 vs. non-treatment of PN-101. MT: mitochondria

Figure 3. Expression of FDXR in the mouse cochlea

The organ of Corti was stained with phalloidin (red), FDXR antibody (green), and DAPI (blue). The upper images were captured at 25x magnification, while the lower images are captured at 200X magnification. The white arrow indicates the inner hair cells (IHCs), and the location of the spiral ganglion neuron (SGN) is marked on the image. Scale bar = 100 μm.
Figure 4. Preoperative imaging, intraoperative ECAP response, and postoperative CI outcomes

(A) The atrophic cochlear nerve is shown (red arrow), and a poor initial ECAP response was recorded in only 9 channels.

(B) Increased pulse width (PW) enabled the recording of ECAP responses in all channels.

(C) Both the speech recognition test and CAP score showed gradual improvement over time after CI.

Figure 5. Difference in proposed auditory rehabilitation protocols between ordinary SNHL and ANSD

ANSD needs early CI, unlike ordinary SNHL, which can benefit from hearing aid use bridging to CI.
Supplementary Figure 1. Optic disc atrophy

(A) Fundus photography reveals temporal pallor of the optic discs in both eyes (indicated by white arrows).

(B) Optical coherence tomography reveals mild thinning of the retinal nerve fiber layer and more severe involvement of the papillomacular bundle (indicated by black arrows) in both eyes.

Supplementary Figure 2. Validation of FDXR antibody specificity

To confirm the specificity of the FDXR antibody, HEK293 cells were transfected with GFP-tagged FDXR (FDXR-GFP), distilled water (negative), or an empty vector (mock). The localization of the FDXR-GFP signal in HEK293 cells demonstrates the antibody's specificity (upper panel). Scale bar = 20 μm.

Supplementary Figure 3. Cochlear nerve status on both sides on MRI

Cochlear nerve dystrophy is more prominent on the left side, and even on the right side, which is the better ear with an almost normal pure tone threshold, progressive dystrophy of the cochlear nerve was noted.
Figure 3.

<table>
<thead>
<tr>
<th>Phallloidin</th>
<th>FXDR</th>
<th>DAPI</th>
<th>Merge</th>
</tr>
</thead>
</table>

Figure 4.

Intraoperatively, increase in PW to 50 led to positive responses from all electrodes.
Supplementary Figure 1.