Table 1. Reported *CLRN1* Mutations.

Mutation Type	Nucleotide Change	Amino Acid Change	Exon	Domain	Reference
Frame shift	c.149_152delinsTGTCCAAT	p.S50Lfs	0	—	Fields 2002
	c.165delC	p.D55fs	0	—	Fields 2002
	c.187_209del	p.Y63fs	0	—	Adato 2002
	c.459_461del	p.I153_L154delinsM	3	—	Joensuu 2001
	c.502_503insA	p.I168fs	3	—	Ebermann 2007
Nonsense	c.189C>A	p.Y63X	0	—	Adato 2002
	c.528T>G	p.Y176X	3	—	Joensuu 2001
Missense	c.118T>G	p.C40G	0	EC	Aller 2004
	c.144T>G	p.N48K	0	EC	Adato 2002
	c.313T>C	p.S105P	2	TM	Sadeghi 2005
	c.359T>A	p.M120K	2	TM	Joensuu 2001
	c.368C>A	p.A123D	2	TM	Ebermann 2007
	c.449T>C	p.L150P	3	TM	Fields 2002
	c.503T>A	p.I168N	3	EC	Garcia 2012
	c.606T>G	p.N202K	3	TM	This study
	c.619C>T	p.R207K	3	TM	Garcia 2012

Abbreviations: EC, extracellular domain; TM, transmembrane domain.

in the plasma membranes surrounding the ribbon synapses of the inner ear and retina transport.[8] The mutation that we identified was located in the transmembrane domain in the clarin-1 protein. Isosomppi et al[12] reported that 2 *CLRN1* missense mutations, located in the transmembrane domain, led to the missorting and mistrafficking of mutated clarin-1 proteins, and resulted in cell degeneration. These findings support our speculation that the *CLRN1* mutation in this study is pathogenic.

USH3 is characterized by variable onset of progressive HL and of RP, as well as the normal to absent impairment of vestibular function.[1] HL in USH3 exhibits phenotypic variability, including variations in the age of onset, degree and type, rate of progression, and clinical outcomes.[13] The age at which USH3 patients were diagnosed as having HL was reported to range from infancy to greater than 35 to 40 years.[13,14] The deterioration of hearing occurred over only a few years in some patients, while in others it occurred over several decades.[13] Sadeghi et al[13] reported that HL in USH3 first affected the high frequencies, and progressed to involve the mid frequencies later in life. Subsequently, USH3 patients had profound HL with some residual hearing at the lower frequencies at the age of 40 years or older. In this study, no obvious HL deterioration in either proband was detected during the observation period (case 1: 8 years, case 2: 5 years). However, the follow-up might have been too short for the detection of whether they had progressive HL or not. Their HL was late-onset (at age 40), which is different from USH1 and USH2 which present as congenital HL, and, therefore, the clinical diagnosis was probably thought to be USH3.

Vestibular function, as determined by electronystagmography and caloric tests, is either normal or hypofunctional in patients with USH3.[15] Of USH3 patients, 45% had vestibular hypofunction or dysfunction, despite a normal walking age (<16 months), which indicated a late-onset vestibular function disturbance suggestive of progressive vestibular dysfunction.[13] Both patients in the present study had normal vestibular function. However, the results of vestibular testing were not the definitive key parameter for distinguishing USH3 from other subtypes in our patients.

With regard to the onset of RP, Pakarinen et al[16] reported that the average age of RP diagnosis in USH3 ranged from 3 to 51 years (mean: 17 years), which was consistent with the findings of our patients. The onset of visual symptoms may precede that of auditory symptoms in USH3, thus differing USH1 and USH2 which show congenital HL and later-onset RP.[14] We considered that the features of HL and RP were clinically important for differentiating USH subtypes, and our cases were thought to be USH3.

Based on the above findings, we considered that the 2 patients were correctly diagnosed as having USH3 caused by the *CLRN1* gene mutation. In Finnish USH3 patients, the most common mutation is the nonsense mutation in the *CLRN1* gene, c.[528T>G] (p.[Y176X]), which is responsible for 94% of USH3 patients.[17] Among Ashkenazi Jews, the missense mutation in the *CLRN1* gene, c.[144T>C] (p.[N48K]), is responsible for all USH3 patients.[14] Accordingly, it has been well accepted that the *CLRN1* gene is responsible for the majority of USH3 patients. In contrast, there have been some controversial reports. Some studies have shown that mutations in the *CLRN1* gene were identified in USH1 and USH2.[18-20] Conversely, Bonnet et al[21] found that biallelic mutations in *USH2A* were identified in 3 USH3 patients. Comprehensive studies, including both detailed clinical findings as well as genetic analysis, are necessary to clarify the relationship between clinical and molecular diagnoses.

In conclusion, we identified 2 USH3 patients possessing the *CLRN1* gene mutation. This is the first report of its kind in Asian populations. As evidenced in the present study, the validation of the presence of clinical findings is imperative for proper differentiation among USH subtypes. In addition, mutation screening using MPS has the ability to identify causative mutations in USH for confirming the clinical diagnosis of this phenotypically variable disease.

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Declaration of Conflicting Interests

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USH2 Caused by *GPR98* Mutation Diagnosed by Massively Parallel Sequencing in Advance of the Occurrence of Visual Symptoms

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Abstract

Objective: We present 2 patients who were identified with mutations in the *GPR98* gene that causes Usher syndrome type 2 (USH2).

Methods: One hundred ninety-four (194) Japanese subjects from unrelated families were enrolled in the study. Targeted genomic enrichment and massively parallel sequencing of all known nonsyndromic hearing loss genes were used to identify the genetic causes of hearing loss.

Results: We identified causative mutations in the *GPR98* gene in 1 family (2 siblings). The patients had moderate sloping hearing loss, and no progression was observed over a period of 10 years. Fundus examinations were normal. However, electroretinograms revealed impaired responses in both patients.

Conclusion: Early diagnosis of Usher syndrome has many advantages for patients and their families. This study supports the use of comprehensive genetic diagnosis for Usher syndrome, especially prior to the onset of visual symptoms, to provide the highest chance of diagnostic success in early life stages.

Keywords

hearing loss, genetics, *GPR98*, Usher syndrome, massively parallel sequencing

Introduction

Usher syndrome (USH) is an autosomal recessive disorder characterized by hearing loss (HL), retinitis pigmentosa (RP), and vestibular dysfunction. There are 3 clinical subtypes classified by the severity and onset of HL, onset of RP, and vestibular symptoms. However, it can be difficult to recognize these clinical manifestations due to its extremely heterogeneous nature. USH type 1 (USH1) has the most severe forms that are characterized by congenital profound HL, prepubertal onset RP, and absent vestibular responses. USH type 2 (USH2) shows congenital moderate to severe with a high-frequency sloping HL and normal vestibular functions. The onset of RP is later than in USH1, and visual symptoms such as night blindness in USH2 usually occur in the second decade. USH type 3 (USH3) has variable onset and severity in these 3 manifestations.[1,2] Due to its complexity, diagnosis of USH in childhood, based on clinical phenotype, can be difficult because patients appear to have only nonsyndromic HL in childhood and RP develops in later years. Based solely on frequency, if a patient has only HL, it is hard to diagnose accurately syndromic HL.

Early diagnosis has many immediate and several long-term advantages for patients and their families and is now possible through genetic testing.[1] The advantage of genetic testing for the diagnostic approach to USH has been fully established. We also previously reported a case in which *MYO7A* mutation analysis allowed the diagnosis of USH1

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in advance of the appearance of the visual symptoms, and genetic testing allowed us to give appropriate genetic counseling.[3] To date, 10 corresponding genes have been identified as a cause of USH: *MYO7A* (USH1B), *USH1C* (USH1C), *CDH23* (USH1D), *PCDH15* (USH1F), *USH1G* (USH1G), and *CIB2* (USH1J) for USH1, *USH2A* (USH2A), *GPR98* (USH2C), and *DFNB31* (USH2D) for USH2, and *CLRN1* (USH3A) for USH3.[4] However, these targeted genes are significant in size and number of exons, and much labor and expense are necessary for analyzing whole genes corresponding to USH. Recent advances in targeted genomic enrichment with massively parallel sequencing (TGE+MPS) have made possible the sequencing of all known causative genes simultaneously.[5,6]

In this study, we performed genetic testing on 194 Japanese hearing loss patients. Here, we describe 2 patients with hearing loss in whom we identified novel mutations in the *GPR98* gene. Based on the result of genetic testing, we performed ophthalmological tests for the patients and diagnosed USH2 even before they suffered from any visual symptoms. This is the first report of a diagnosis of USH2 caused by *GPR98* mutations in advance of visual defects in the cohort of nonsyndromic HL patients and highlights the importance of comprehensive genetic testing for use in the clinical diagnosis of hearing loss patients.

Subjects and Methods

Subjects

One hundred ninety-four (194) Japanese subjects (114 females) from unrelated and non-consanguineous families were ascertained through 33 otolaryngology clinics in 28 prefectures across Japan. All subjects had presumed nonsyndromic HL. For each proband, informed consent was obtained to participate in this study, which was approved by the human subjects ethical committee associated with each clinic.

Clinical information and blood samples were obtained for each proband and for all consenting affected and unaffected relatives.

Targeted Genomic Enrichment and Massively Parallel Sequencing

Genomic DNA was assessed for quality by gel electrophoresis and spectrophotometry (Nanodrop 1000; Thermo Fisher Scientific, Waltham, Massachusetts, USA; 260/280 ratio of 1.8-2.2) and for quantity by fluorometry (Qubit 2.0 Fluorometer; Life Technologies, Carlsbad, California, USA). TGE of all exons of all genes implicated in nonsyndromic HL, including nonsyndromic HL mimics, was completed as described, targeting 89 genes as part of the OtoSCOPE v5 platform. Libraries were prepared using a modification of the solution-based Agilent SureSelect target enrichment system (Agilent Technologies, Santa Clara, California, USA).[7] Of the 194 samples, 58 samples were processed manually; the remainder was prepared robotically using the Sciclone NGS Workstation.

In brief, 3 μg gDNA was randomly fragmented to an average size of 250 bp (Covaris Acoustic Solubilizer; Covaris Inc, Woburn, Massachusetts, USA), fragment ends were repaired, A-tails were added, and sequencing adaptors were ligated before the first amplification. Solid phase reverse immobilization purifications were performed between each enzymatic reaction. Hybridization and capture with RNA baits was followed by a second amplification before pooling for sequencing. Minimal amplification was used—typically 8 cycles for the pre-hybridization polyremase chain reaction (PCR; range, 8-10 cycles) using NEB Phusion HF Master Mix (New England BioLabs Inc, Ipswich, Massachusetts, USA) and 14 cycles for the post-hybridization PCR (range, 12-16 cycles) using Agilent Herculase II Fusion DNA Polymerase. All samples were barcoded and multiplexed before sequencing on either an Illumina MiSeq or HiSeq (Illumina Inc, San Diego, California, USA) in pools of 4 to 6 or 48, respectively, using 100-bp paired-end reads.

Bioinformatics Analysis

Data were analyzed as described using a local installation of the open-source Galaxy software and the following open-source tools: BWA[8] for read mapping, Picard for duplicate removal, GATK[9] for local re-alignment and variant calling and NGSRich[10] for enrichment statistics.[6] We reported and annotated variants with custom software.

Variant Confirmation

All pathogenic variants were confirmed by Sanger sequencing and segregation analysis using exon-specific custom primers.

Results

Mutation Analysis

We indentified novel causative mutations including 1 frame-shift mutation and 1 missense mutation in *GPR98* in the cohort of this study (194 hearing loss patients). The former mutation corresponded to c.16604_16611delGTACCCAG (NM_032119) and led to a frame-shift mutation and truncation (p.Ser5535ArgfsX6). The second mutation was c.9464C>A (p.Ala3155Asp). We also performed Sanger sequencing for the family segregation study and confirmation of the variant MPS outputted result. As shown in Figure 1, Sanger sequencing results revealed that the parents had 1 of either mutation in the heterozygote, and the proband's brother had biallelic mutations.

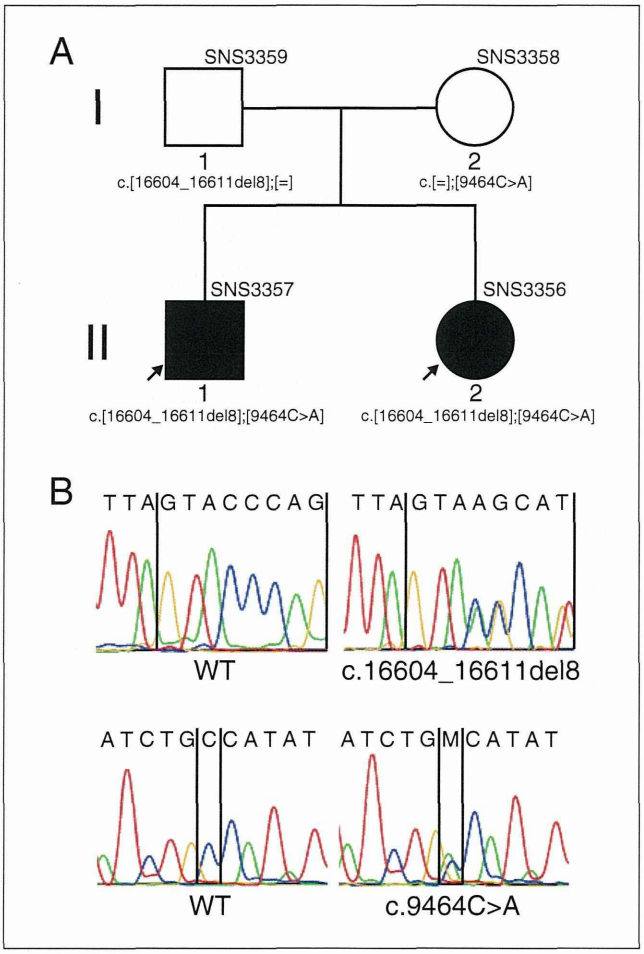

Figure 1. Pedigree of the patients ID: SNS 3356 and 3357, and Sanger sequence results. The affected patients carried c.[16604-16611delGTACCCAG] (p.Ser5535ArgfsX6) and c.[9464C>A] (p.Ala3155Asp) mutations. These mutations were segregated in this family.

Case Details

The proband is a 16-year-old female (II-2; SNS 3356). She had no complications in the perinatal period. She had not undergone newborn hearing screening. At the age of 6, HL was suspected at an elementary school wellness check-up, and she was referred to Shinshu University Hospital, Department of Otolaryngology for audiological examinations. An older brother (II-1; SNS 3357) (8 years old) visited the hospital at the time she received her consultation as he had not undergone newborn hearing screening. They were diagnosed with moderate HL, with a high-frequency sloping configuration (Figures 2A, 3A). Subsequently, they began to wear hearing aids. No subsequent deterioration in their HL was observed over a period of 10 years (Figures 2A, 3A).

They participated in this study in 2004; however, we could not find the responsible genes within the common genes, such as *GJB2* and mitochondrial 1555AG mutations. Genetic testing using MPS was carried out in 2013. Mutations in the *GPR98* gene as mentioned previously were detected, thus we considered that they might have USH2C rather than nonsyndromic HL. At the time of testing, the brother (II-1) was aware of night blindness at the age of 18. However, the proband (II-2) had no apparent nyctalopia or dark adaptation problems at the age of 16. Ophthalmological testing showed that the fundus examinations of both patients were normal (Figures 2C, 3C). However, his electroretinogram (ERG) revealed a complete bilateral absence (Figure 3D), and her ERG revealed a weaker response than that of normal levels (Figure 2D).

In addition, their vestibular functions (determined by means of caloric tests and cervical vestibular evoked myogenic potential [cVEMP]) were both normal (Figures 2B, 3B). With these findings, they were diagnosed with USH2C caused by *GPR98* mutations.

Discussion

In this report, we identified novel heterogygous mutations in the *GPR98* gene among autosomal recessive inherited and presumably nonsyndromic HL and finally diagnosed USH2C. One (p.Ser5535ArgfsX6) was considered pathogenic due to a truncating mutation. The second (p.Ala3155Asp) was strongly suspected as pathogenic, and this mutation was not described in any mutation databases. PhyloP and GERP showed this residue is well conserved among various species. We also employed functional prediction software (Polyphen2, SIFT, Mutation Taster, and LRT) that indicated the second mutation to be damaging (1.00, 0.00, 0.99, and 1.00, respectively).

The *GPR98* gene (also previously known as the *VLGR1* gene) is localized on chromosome 5q13 and contains 90 exons and has a range of 600 kb. *GPR98* was first implicated in USH2 in 2004.[11] Weston et al[11] identified mutations in *GPR98* among patients who had deaf-blindness, and also showed that the expression of *GPR98* was observed in human fetal retina and cochlea by RT-PCR. Subsequently, there have been several cases identified, thus, it was considered to be a USH2 causative gene.[12-14] Mutations of some USH causative genes (*MYO7A, USH1C, CDH23, PCDH15, CIB2, DFNB31*) can result in both nonsyndromic hearing loss and USH,[4] while mutations of the *GPR98* gene are only responsible for USH. The *GPR98* protein (VLGR1) is one of the major components of the ankle link of the hair bundle in the cochlea[15] and plays an important role in the normal development of cochlea hair bundles.[16] McGee et al[16] reported that *Vlgr1* mutated mice exhibited early hair bundle defects resulting in hearing loss at high frequencies, whereas normal vestibular function was observed. Normal transduction currents were measured in vestibular hair cells. These findings are also consistent with our present cases that have normal vestibular functions.

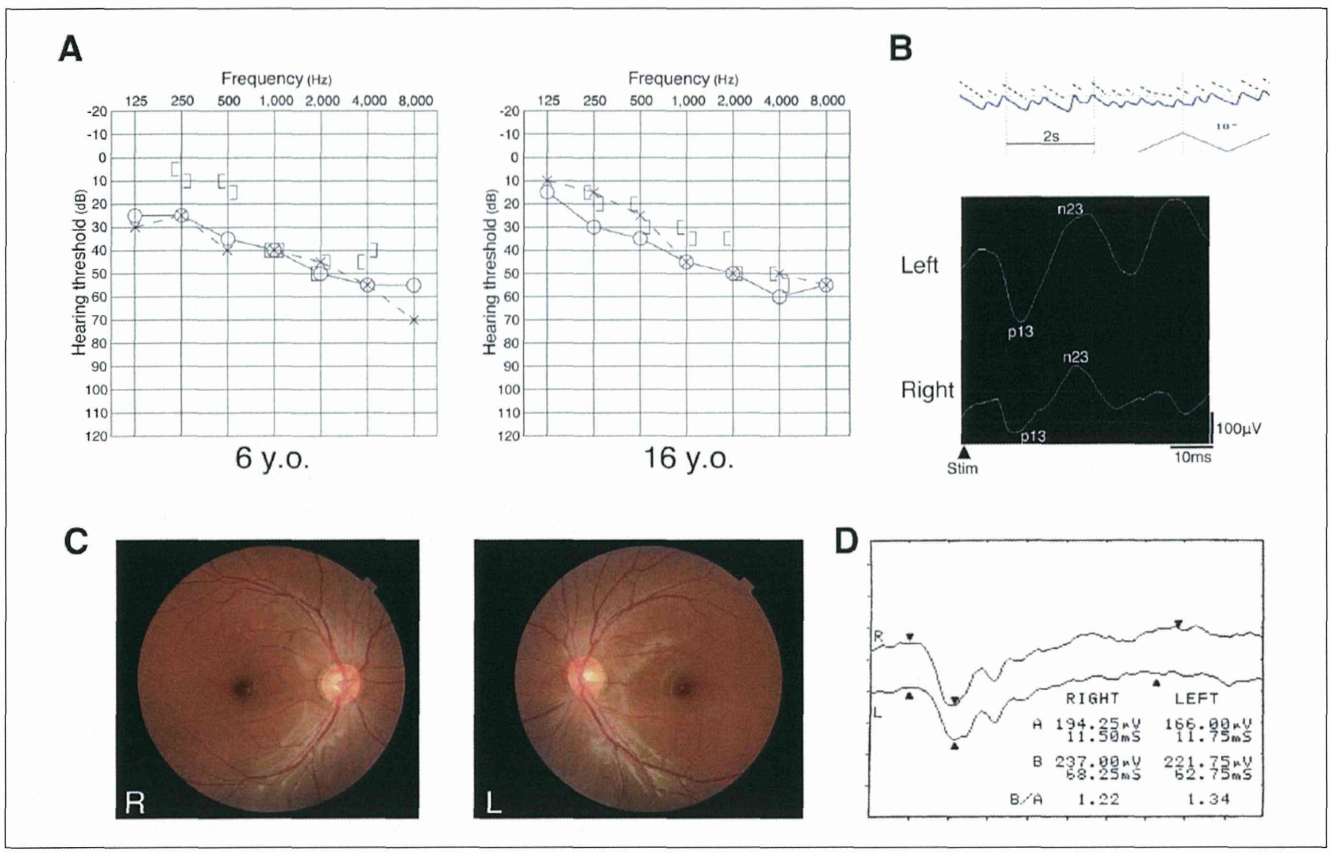

Figure 2. Clinical findings of the patient, II-2; SNS 3356. (A) Left: Audiogram at the age of 6 shows moderate hearing loss. Right: Audiogram at the age of 16 shows no progression of hearing loss. (B) Upper: Caloric testing for the left ear shows a normal response. Lower: There were no obvious differences between both ears in the cervical vestibular evoked myogenic potential (cVEMP). (C) Fundus examination at the age of 18 was normal. (D) Full-field electroretinogram results show a weaker response.

In USH2 patients, USH2A (*USH2A*) has been reported as the most common USH2 genetic subtype (57%~95.8%),[17-19] while USH2C (*GPR98*) and USH2D (*DFNB31*) accounted for approximately 5.2% to 19% and 0% to 9.5%.[14,17-19] All published mutations in USH genes have been recorded in the public database, USHbases.[20] Nakanishi et al[21] reported the frequency of USH2 genetic subtypes in Japanese USH patients and that *USH2A* gene mutations were found in 8 of 10 patients (80%). Our study is the first to identify the *GPR98* gene mutations. However, we recruited a cohort of suspected nonsyndromic HL patients, so that the frequency of USH2C in a Japanese population is still unclear.

Abadie et al[22] reported that in USH2C, moderate HL was predominant (76%), and a gently down-sloping configuration characterized most audiograms (66%). This is consistent with our cases. It has been shown that USH2C patients had more severe HL than USH2A; however, it is impossible to predict the candidate gene based on audiograms due to the heterogeneity of USH.[22]

This is the first report of mutations in *GPR98* identified by genetic testing using MPS in which we were able to diagnose USH2C before the patients suffered from obvious visual symptoms. With regard to USH2C patients, the median age of HL diagnosis was 5 years, although the median age at which USH2 was diagnosed was 34.5 years.[22] That is because visual symptoms with RP appear later in life, mostly in the second decade.[23] In general, diagnosis of RP can be possible before the appearance of visual symptoms by ERG[24]; however, there is usually no indication to carry out an ERG before RP symptoms appear. Based on the present cases, we suggest that if mutations are found in genes concerned with USH, ophthalmological testing should be provided to the patients for differential diagnosis. We previously recommended ophthalmologic tests for young nonsyndromic HL patients in whom candidate mutations in the USH gene had been found.[3]

Further evolution of genetic testing, such as MPS, will make more accurate diagnosis of HL possible, but we should also be more rigorous in confirmation of the phenotypes, including hearing loss and other manifestations. Regarding HL caused by mutations in *GPR98*, all clinicians should provide optimal management of hearing abilities in order to improve patient's quality of life. We should provide genetic

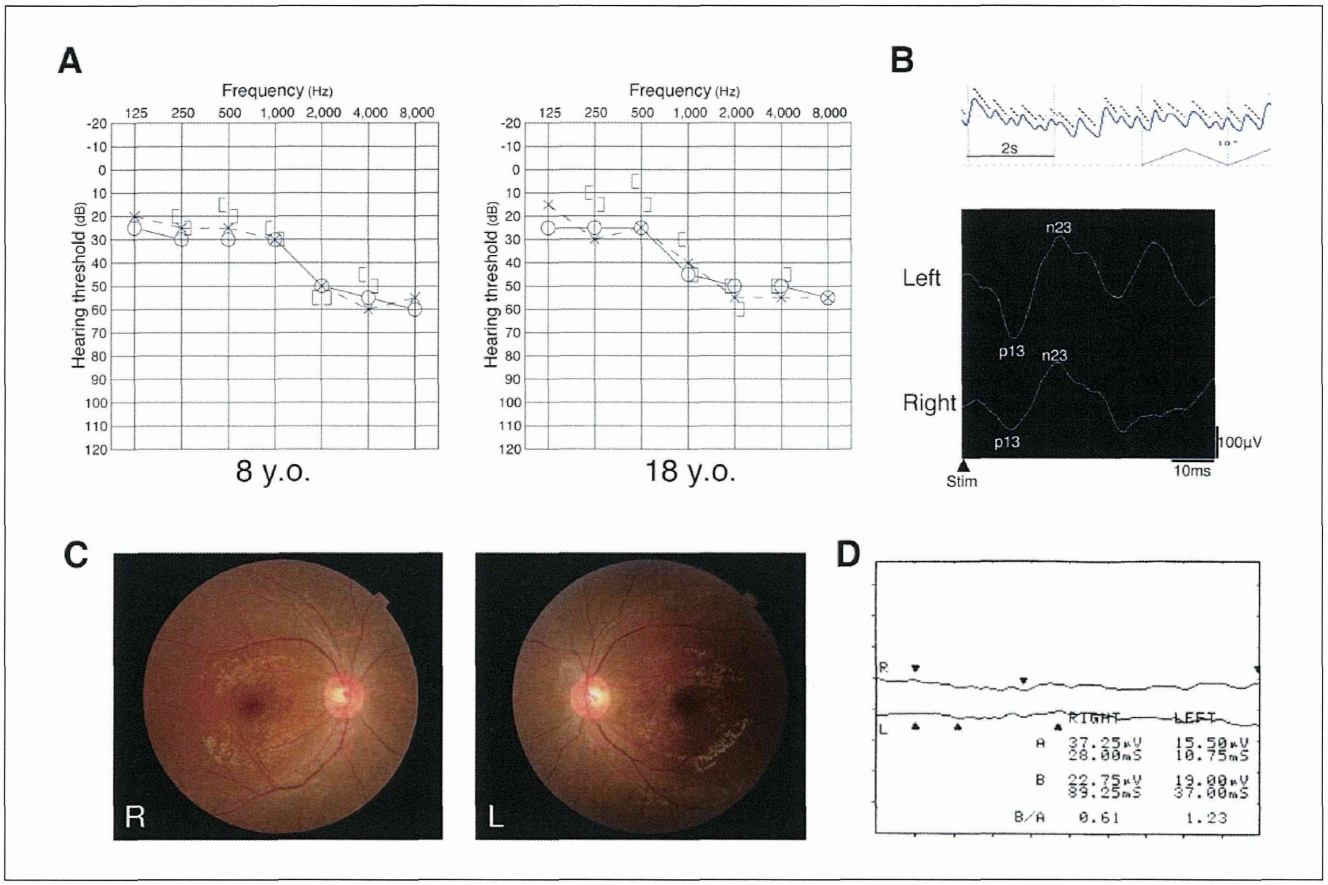

Figure 3. Clinical findings of the patient, II-1; SNS 3357. (A) Left: Audiogram at the age of 8 shows moderate hearing loss. Right: Audiogram at the age of 18 shows no progression of hearing loss. (B) Upper: Caloric testing for the left ear shows normal response. Lower: There were no obvious differences between both ears in the cervical vestibular evoked myogenic potential (cVEMP). (C) Fundus examination at the age of 18 was normal. (D) Full-field electroretinogram results show a complete bilateral absence.

counseling to patients about the risk of future vision loss and also provide applicable educational support.

In conclusion, this study supports the use of comprehensive genetic diagnosis for USH, particularly in advance of visual symptoms, to provide the highest chance of diagnostic success in the early life stages. The benefit of early identification lies in the potential to provide future treatment to prevent RP.[1] Genetic diagnosis using MPS will contribute to early intervention and may provide an opportunity for the development of novel therapeutic possibilities.

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Declaration of Conflicting Interests

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Novel *ABHD12* Mutations in PHARC Patients: The Differential Diagnosis of Deaf-Blindness

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Abstract

Objective: This study examines *ABHD12* mutation analysis in 2 PHARC patients, originally thought to be Usher syndrome.
Methods: The *ABHD12* gene of 2 patients, who suffered from deaf-blindness and dysfunctional central and peripheral nervous systems, were sequenced.
Results: We identified that both cases carried the same novel splice site mutation in the *ABHD12* gene. However, 1 had epilepsy and the other had peripheral neuropathy. Based on haplotype analysis, the mutation is likely not a hot spot, but rather could be attributable to a common ancestor.
Conclusion: This study shows that PHARC has phenotypic variability, even within a family, which is consistent with previous reports. Differential diagnosis of "deaf-blindness" diseases is crucial. Confirming the presence of associated symptoms is necessary for differentiating some deaf-blindness syndromes. In addition, mutation analysis is a useful tool for confirming the diagnosis.

Keywords

PHARC, *ABHD12*, Usher syndrome, deaf-blindness, genetics of hearing loss

Introduction

PHARC is an autosomal recessive disorder that causes polyneuropathy, hearing loss (HL), ataxia, retinitis pigmentosa (RP), and cataracts. Fiskerstrand et al[1] initially reported this syndrome in a Norwegian family. These symptoms are slowly progressive, begin in the childhood or teenage years, and are variable.[2]

PHARC is caused by mutations in the *ABHD12* gene, which encodes serine hydrolase enzyme α/β hydrolase domain-containing 12.[2] The *ABHD12* gene is located on chromosome 20p11.21 and contains 13 exons, encoding a protein of 398 amino acids.

PHARC patients, who have HL, RP, and cataracts, are occasionally diagnosed with "deaf-blindness." In general, the most common cause (50%) of combined deafness and blindness has been Usher syndrome (USH). Usher syndrome is also an autosomal recessive disorder manifesting as HL and RP and having 3 clinical subtypes. In particular, USH type 3 (USH3) is characterized by the variable onset of progressive HL, variable onset of RP, and variable impairment of vestibular function (normal to absent).[3] Eisenberger et al[4] suggested that PHARC should be taken into account as a differential diagnosis for USH, especially in "USH3-like" patients.

In this study, we have conducted a genetic analysis to find mutations in *ABHD12* in PHARC patients with USH3-like symptoms.

Clinical Reports

Case 1 (Figure 1)

The patient (II-3; patient ID: SNS 5547), a 64-year-old Japanese male, was the third child of a healthy father and

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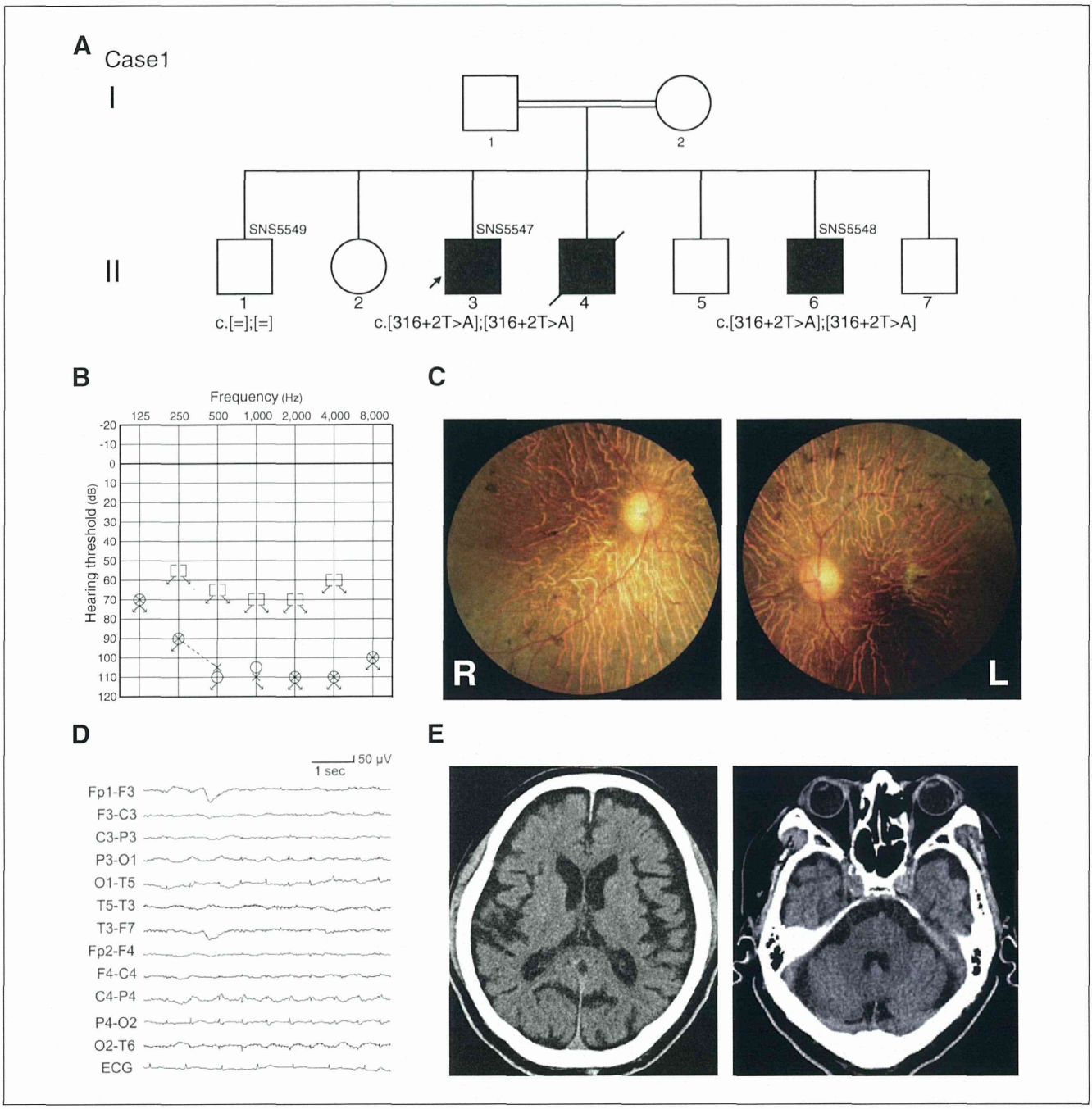

Figure 1. Pedigree of the patient (SNS 5547), audiogram, fundus examination, electroencephalogram (EEG), and brain computed tomography (CT). (A) Pedigree and sequence results for the proband and family. (B) The audiogram of the proband revealed profound hearing loss. (C) The fundus examination revealed a pale optic disc, attenuation of retinal vessels, and bone spicule pigmentation. (D) The EEG in an awake record showed repetitive spikes in the occipital regions. (E) The brain CT displayed diffuse cerebral (left) and cerebellar (right) atrophy.

mother. The patient's parents were consanguineous. He has 6 other brothers (Figure 1A). He became aware of HL in primary school. Since then, HL was gradually progressive. He had profound hearing loss for all frequencies at age 54 (Figure 1B). He has never experienced vertigo. The caloric test was normal.

He also experienced night blindness at about age 30 and was diagnosed with retinitis pigmentosa (RP) at age 45. He suffered from both constricted vision and central scotoma (Figure 1C). In addition, he had cataracts and received cataract surgery in both eyes during the same period. As a