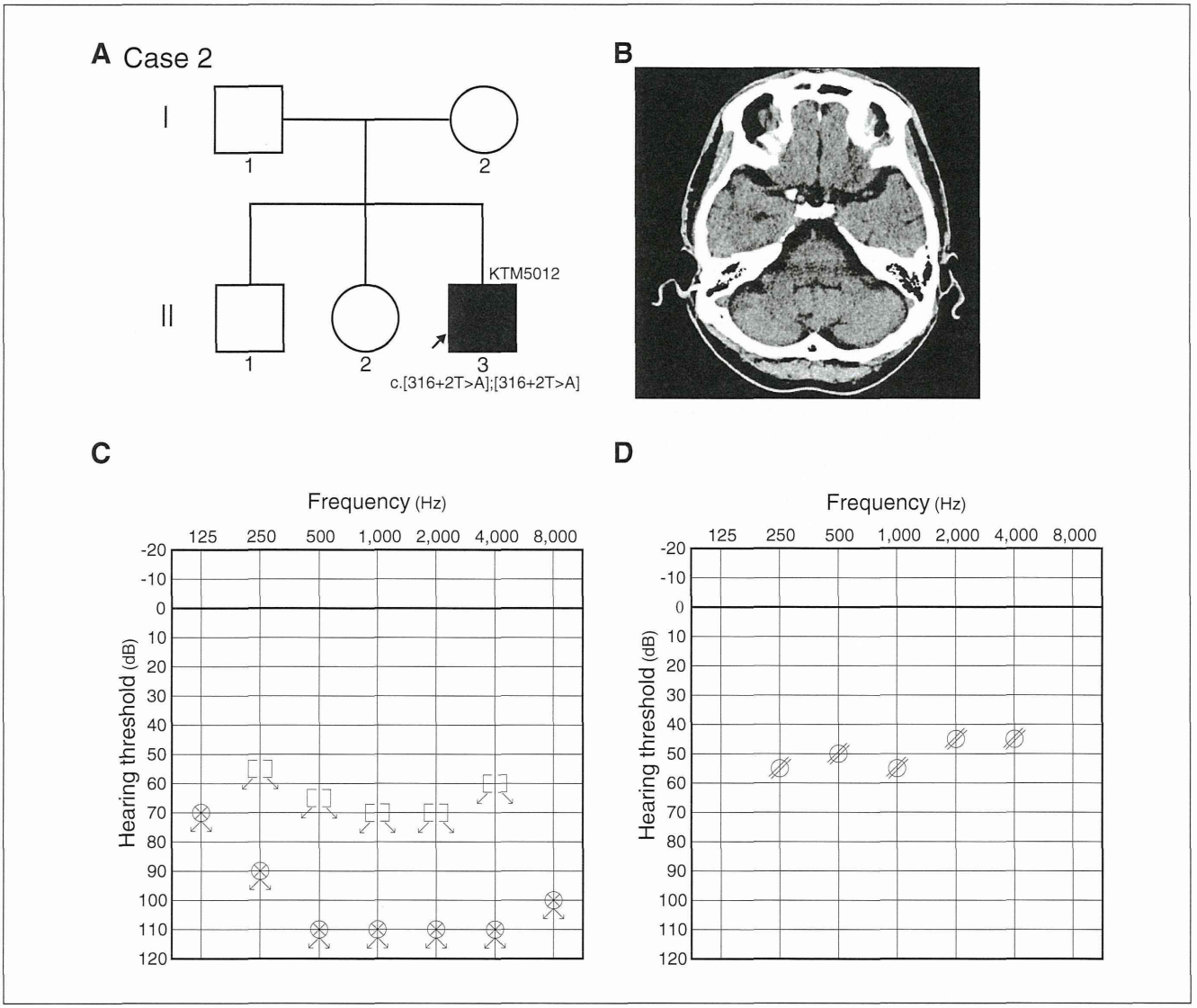

Figure 2. Pedigree of the patient (KTM 5012), brain computed tomography (CT) and audiogram (preoperative and postoperative with a cochlear implant [CI]). (A) Pedigree and sequence results for the proband. (B) The brain CT was normal. (C) The preoperative audiogram (left) revealed profound hearing loss. (D) The preoperative audiogram with a CI demonstrated that the CI was effective for the proband.

result of combined deafness and blindness, he uses tactile communication (writing letters on the hand by finger).

From age 63, he began to experience some episodes of fainting without convulsions, and these were followed by confusion. His electroencephalogram showed spikes over the occipital regions and he was diagnosed as having occipital lobe epilepsy (Figure 1D). The use of antiepileptic drugs (Phenytoin 300 mg daily) resulted in a normal electroencephalogram and no recurrence. Computed tomography (CT) of his brain revealed diffuse cerebral and cerebellar atrophy without any focal lesions (Figure 1E).

After that, he visited a department of otolaryngology at age 63 because it was suspected that he might suffer from USH. However, his symptoms were inconsistent with USH because he had epilepsy. His 2 brothers (II-4, II-6; SNS 5548) exhibited similar symptoms (Figure 1A). One (II-6; SNS 5548) had deafness, RP, and epilepsy, whereas the other (II-4) had only deafness and RP. The brother (II-4) without epilepsy died from prostate cancer at age 60.

Based on these findings, we considered that his diagnosis was an autosomal recessive disorder, not USH as had been initially suspected. It is now suspected that this patient has a variation of PHARC.

Case 2 (Figure 2)

The patient (II-3; patient ID: KTM 5012), a 56-year-old Japanese male, was the third child of a healthy father and

mother. He has 2 other healthy brothers (Figure 2A). He was diagnosed with severe HL at age 15. Since then, his HL has gradually deteriorated. By age 19, he could not hear, even when he wore hearing aids, because his HL was profound for all frequencies (Figure 2C). After that, he used sign language. He received a cochlear implant (CI) in the right ear at age 41. This CI made it possible for him to engage in improved verbal communication (Figure 2D).

He was diagnosed with RP at age 22. After that, his visual fields were seriously constricted. In addition, he lost vision in the right eye and had 20/500 vision at that time. He also had cataracts and received cataract surgery in both eyes at age 18 and 20.

At age 27, he visited the department of neurology, and reduced sensory perception in the distal portion of the extremities was identified. Afterward, his ability to walk gradually also decreased. His peripheral nerve conduction velocity decreased. His motor nerve conduction velocities in the median nerve and tibial nerve were 31.4 m/s (normal: 58.4 ± 4.0 m/s) and 25.1 m/s (normal: 48.4 ± 3.2 m/s), respectively. He demonstrated steppage gait and was diagnosed with peripheral neuropathy. He could not walk without a cane at age 56. In addition, the CT of his brain was normal (Figure 2B).

His symptoms were polyneuropathy, hearing loss, RP, and cataracts. We suspect that he has a variation of PHARC.

Methods

Mutation Analysis

Genomic DNA was extracted from peripheral blood leukocytes of the patients and their brothers. Polymerase chain reaction (PCR) was used to amplify all 13 exons and flanking intronic sequences of the *ABHD12* gene. Primers were designed to flank all of the exon-intron boundaries through use of the Primer3 web-based server (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Each genomic DNA sample (40 ng) was amplified with a slight modification and KOD DNA polymerase (Toyobo, Osaka, Japan) for 8.5 minutes at 95°C, followed by 30 three-step cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes, ending with a holding period at 4°C in PCR thermal cycler (Takara, Shiga, Japan). PCR products were treated with ExoSAP-IT (Affymetrix, Santa Clara, California, USA) by incubation at 37°C for 60 minutes, and inactivation at 80°C for 15 minutes. After the products were purified, we performed standard cycle-sequencing reactions with Big Dye terminators in an ABI PRISM 3100 Genetic Analyzer autosequencer (Applied Biosystems, Foster City, California, USA). All subjects gave prior informed consent for participation in the project, and the Ethical Committee of Shinshu University approved the study (No. 387).

Gene accession number. NM_001042472 Homo sapiens abhydrolase domain containing 12 (ABHD12).

Haplotype Analysis

Haplotype pattern within the 1Mbp region surrounding position c.316+2, where the Japanese mutation c.316+2T>A was found in this study, was analyzed using a set of 28 single nucleotide polymorphisms (SNPs) (18 sites upstream and 10 sites downstream). Haplotype analysis was performed by the direct sequencing with the primers for each SNPs method described above (Figure 3).

Results

ABHD12 gene mutation screening revealed that both cases carried a novel splice site mutation (c.316+2T>A) in intron 2 (Figures 1A, 2A).

Haplotype pattern within the 1 Mbp region surrounding position c.316+2 was characterized using a set of 28 SNPs (18 sites upstream and 10 sites downstream). All 3 patients (I-2, I-3, II-1) from both families with c.316+2T>A showed an identical pattern in the allele with c.316+2T>A, although the other allele showed a variety of haplotype patterns.

Discussion

The present study reported 1 novel homozygous *ABHD12* mutation in 2 suspected PHARC families. This splice site mutation was identified as pathogenic because it was a truncating mutation. To date, little has been reported about PHARC. Only 10 mutations in the *ABHD12* gene have been identified in the world (Table 1). Identification of the present novel homozygous mutation is the first report of splice site mutation causing PHARC. It is interesting, based on the haplotype analysis, that c.316+2T>A is likely not a hot spot, but rather could be attributable to a common ancestor (Figure 3). The present study is the first to demonstrate such a recurrent mutation for PHARC.

Nishiguchi et al[5] reported that there was no obvious correlation between genotype and phenotype. In addition, PHARC was shown to have phenotypic variability, even within the same family, in previous reports.[2] In this study, despite the same variant in both cases, 1 (case 1) had epilepsy and the other (case 2) had peripheral neuropathy. Previous studies have reported that all PHARC patients exhibit HL, RP, and cataracts[1,2,6] and experience progressive HL (from severe to profound),[4] which is consistent with the results of this present study.

Distance from the c.316+2T>A mutation (bp)	Case 1 II-3 (+)	Case 1 II-6 (+)	Case 1 II-1 (-)	Case 2 II-3 (+)	Allele frequency				Marker
479966	T	T	T	T	T	0.10	C	0.90	rs6050118
429441	C	C	C	C	C	0.24	T	0.76	rs36090194
364772	A	A	A	A	A	0.10	G	0.90	rs6050255
344783	T	T	T	T	T	0.26	C	0.74	rs910527
344530	G	G	G	G	G	0.22	A	0.78	rs6132792
317484	A	A	A/G	A	A	0.39	G	0.62	rs4813547
312419	T	T	T/C	T	T	0.44	C	0.56	rs6138482
290571	T	T	T/C	T	T	0.36	C	0.64	rs6138487
274069	A	A	A/G	A	A	0.35	G	0.65	rs6138492
265703	G	G	G/C	G	G	0.37	C	0.63	rs6050342
237895	G	G	G/T	G	G	0.40	T	0.60	rs6050374
193742	A	A	A	A	A	0.09	G	0.92	rs6050425
184648	A	A	A/G	A	A	0.39	G	0.61	rs2076559
176843	C	C	C/T	C	C	0.42	T	0.58	rs3787076
171869	A	A	A/G	A	G	0.46	A	0.54	rs11699316
150314	C	C	C	C	C	0.07	T	0.93	rs6050477
139455	A	A	A/G	A	A	0.34	G	0.66	rs3787078
115263	G	G	G/A	G	G	0.23	A	0.77	rs11697384
0	-	-	-	-	-	-	-	-	c.316+2T>A
53947	A	A	A/G	A	A	0.36	G	0.64	rs6138569
295893	G	G	G/A	G	G	0.23	A	0.77	rs6138638
332273	G	G	G/A	G	G	0.23	A	0.77	rs11698047
343646	G	G	G/T	G	G	0.36	T	0.64	rs6138650
346620	T	T	T	T	T	0.06	G	0.94	rs8116474
350601	C	C	C/T	C	C	0.44	T	0.56	rs7260957
351771	A	A	A	A	A	0.06	G	0.94	rs12106173
359107	T	T	T	T	T	0.07	C	0.93	rs6050867
477207	C	C	C/G	C	C	0.40	G	0.60	rs3949256
490417	T	T	T	T	T	0.39	G	0.61	rs6132882

Figure 3. The haplotypes around the c.316+2T>A mutation in the 2 families constructed using SNPs. Each column shows an affected allele. Each base is defined by pure segregation analysis in the family. Allele frequencies are derived from HapMap JPT+CHB samples. Both families shared a large common region in their haplotypes of approximately 1 Mb (in blue).

In general, cochlear implantation has been acknowledged to be effective for patients whose auditory nerve and spiral ganglions are preserved. Until now, the localization of the *ABHD12* gene in the inner ear has been unclear. However, the present patient (case 2) enjoyed the benefits of CI. It is therefore conceivable that etiology is mainly localized within the cochlea. We think that if PHARC patients suffer from profound HL, they should be given the opportunity for cochlear implantation.

All PHARC patients had auditory and visual symptoms. Therefore, a differential diagnosis of deaf-blindness diseases is crucial. Among them, we listed 5 diseases with HL and RP (USH,[3] Cockayne syndrome,[7] Kearns-Sayre syndrome,[8] Flynn Aird's syndrome,[9] and Refsum disease[10]) (Table 2).

If a patient has only auditory and visual symptoms, we may make a diagnosis of Usher syndrome because it is the most common cause of deaf-blindness. Usher syndrome is divided into 3 clinical subtypes.[3] Of them, USH type 1 (USH1) and type 2 (USH2) are characterized by congenital hearing loss (USH1: profound HL; USH2: moderate to severe HL, with a high-frequency sloping configuration). Patients who have other symptoms associated with HL and RP, including PHARC, have late-onset hearing loss.

However, USH3 is characterized by progressive HL and RP. Consequently, we tend to diagnose late-onset HL and

Table 1. *ABHD12* Mutations Found in This Study Together With Previously Reported Mutations.

Mutation Type	Nucleotide Change	Amino Acid Change	Exon/Intron	Domain	Country	Reference
Long deletion	14Kb deletion including exon 1	—	Exon 1	—	UAE	[2]
	59Kb deletion including exon 1	—	Exon 1	—	USA	[6]
Frameshift	c.337_338delGAinsTTT	p.Asp113PhefsX15	Exon 3	—	Norway	[2]
	c.846_852dupTAAGAGC	p.His285fsX1	Exon 9	—	Algeria	[2]
Splicing	c.316+2T>A	—	Intron 2	—		This study
Nonsense	c.193C>T	p.Arg65X	Exon 2	—	Lebanon	[4]
	c.447G>A	p.Trp159X	Exon 4	—	Netherlands	[5]
	c.1054C>T	p.Arg352X	Exon 12	—	USA	[2]
	c.1129A>T	p.Lys377X	Exon 12	—	USA	[6]
Missense	c.557G>A	p.Arg186Pro	Exon 5	EC	Netherlands	[5]
	c.1116C>G	p.His372Gln	Exon 12	EC	Spain	[5]

Abbreviations: EC, extracellular domain; UAE, United Arab Emirates.

Table 2. Genetic Hearing Loss Associated With Retinitis Pigmentosa.

Syndrome or Disease	HL	RP	Associated Symptoms	Hereditary Pattern	Causative Gene	Reference
PHARC	Progressive HL	Present	Polyneuropathy, ataxia, cataracts	AR	*ABHD12*	[2]
Usher syndrome type 1	Congenital profound	Prepubertal onset of RP	Vestibular dysfunction	AR	*MYO7A, USH1C CDH23, PCDH15 USH1G, CIB2*	[3]
Usher syndrome type 2	Congenital moderate to severe HL, with a high-frequency sloping configuration	Present (first or second decade onset)	None	AR	*USH2A GPR98 WHRN*	[3]
Usher syndrome type 3	Progressive hearing loss (variable oneset)	Present (variable oneset)	Impairment of vestibular function (normal to absent)	AR	*CLRN1*	[3]
Cockayne syndrome	Progressive HL	Present	Cachectic dwarfism Progressive microcephaly	AR	*ERCC6 ERCC8*	[7]
Kearns-Sayre syndrome	Present	Present	Progressive external ophthalmoplegia Cardiac conduction defects Encephalomyopathy Muscle weakness Short stature, proximal myopathy	Sporadic Maternal	N/D	[8]
Flynn Aird's syndrome	Progressive	Present	Ataxia, peripheral neuritis, epilepsy Dementia, skin atrophy Chronic ulceration Baldness, striking dental caries	AD	N/D	[9]
Refsum disease	Bilateral mild-to-profound	Present	Anosmia, peripheral neuropathy Cerebellar ataxia	AR	*PHYH* *PEX7*	[10]

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; HL, hearing loss; N/D, not detected; RP, retinitis pigmentosa.

RP patients with USH3. This is because patients with PHARC might initially exhibit no dysfunction of the central and peripheral nervous systems. Subsequently, we should determine what, if any, other associated symptoms patients may suffer from (Table 2). In cases where patients suffer from 1 or more associated symptoms, mutation analysis of the causative genes should be performed, as in this study.

If patients do not exhibit any associated symptoms, we should assume that they suffer from USH, and we ought to conduct genetic analysis using massively parallel DNA sequencing (MPS) to find mutations in the causative USH genes. Massively parallel DNA sequencing analysis is useful because the USH genes themselves are too large for conventional analysis.[11] When we cannot detect pathogenic mutations in the causative USH genes, we should consider 2 possibilities. One is that patients have unknown causative USH gene mutations, and the other is that associated symptoms have not yet been exhibited.

In conclusion, we reported 2 PHARC cases. As evidenced in the present study, PHARC has phenotypic variability, even when caused by the same mutation in the *ABHD12* gene. 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.

Acknowledgments

The authors thank Mr Sean Mehmet for his help in preparing the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by a Health and Labour Sciences Research Grant for Research on Rare and Intractable Diseases and Comprehensive Research on Disability Health and Welfare from the Ministry of Health, Labour and Welfare of Japan (S.U.), and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (S.U.).

References

1. Fiskerstrand T, Knappskog P, Majewski J, Wanders RJ, Boman H, Bindoff LA. A novel Refsum-like disorder that maps to chromosome 20. *Neurology*. 2009;72(1):20-27.
2. Fiskerstrand T, H'Mida-Ben Brahim D, Johansson S, et al. Mutations in ABHD12 cause the neurodegenerative disease PHARC: an inborn error of endocannabinoid metabolism. *Am J Hum Genet*. 2010;87(3):410-417.
3. Yan D, Liu XZ. Genetics and pathological mechanisms of Usher syndrome. *J Hum Genet*. 2010;55(6):327-335.
4. Eisenberger T, Slim R, Mansour A, et al. Targeted next-generation sequencing identifies a homozygous nonsense mutation in ABHD12, the gene underlying PHARC, in a family clinically diagnosed with Usher syndrome type 3. *Orphanet J Rare Dis*. 2012;7:59.
5. Nishiguchi KM, Avila-Fernandez A, van Huet RA, et al. Exome sequencing extends the phenotypic spectrum for ABHD12 mutations: from syndromic to nonsyndromic retinal degeneration. *Ophthalmology*. 2014;121(8):1620-1627.
6. Chen DH, Naydenov A, Blankman JL, et al. Two novel mutations in ABHD12: expansion of the mutation spectrum in PHARC and assessment of their functional effects. *Hum Mutat*. 2013;34(12):1672-1678.
7. Laugel V, Dalloz C, Durand M, et al. Mutation update for the CSB/ERCC6 and CSA/ERCC8 genes involved in Cockayne syndrome. *Hum Mutat*. 2010;31(2):113-126.
8. Maceluch JA, Niedziela M. The clinical diagnosis and molecular genetics of Kearns-Sayre syndrome: a complex mitochondrial encephalomyopathy. *Pediatr Endocrinol Rev*. 2006;4(2):117-137.
9. Flynn P, Aird RB. A neuroectodermal syndrome of dominant inheritance. *J Neurol Sci*. 1965;2(2):161-182.
10. van den Brink DM, Brites P, Haasjes J, et al. Identification of PEX7 as the second gene involved in Refsum disease. *Am J Hum Genet*. 2003;72(2):471-477.
11. Yoshimura H, Iwasaki S, Nishio SY, et al. Massively parallel DNA sequencing facilitates diagnosis of patients with Usher syndrome type 1. *PLoS One*. 2014;9(3):e90688.

OPEN ACCESS Freely available online

PLOS | ONE

Massively Parallel DNA Sequencing Facilitates Diagnosis of Patients with Usher Syndrome Type 1

Hidekane Yoshimura[1], Satoshi Iwasaki[2,3], Shin-ya Nishio[1], Kozo Kumakawa[4], Tetsuya Tono[5], Yumiko Kobayashi[6], Hiroaki Sato[6], Kyoko Nagai[7], Kotaro Ishikawa[8], Tetsuo Ikezono[9], Yasushi Naito[10], Kunihiro Fukushima[11], Chie Oshikawa[12], Takashi Kimitsuki[13], Hiroshi Nakanishi[14], Shin-ichi Usami[1]*

1 Department of Otorhinolaryngology, Shinshu University School of Medicine, Matsumoto, Nagano, Japan, **2** Department of Hearing Implant Sciences, Shinshu University School of Medicine, Matsumoto, Nagano, Japan, **3** Department of Otorhinolaryngology, International University of Health and Welfare, Mita Hospital, Minatoku, Tokyo, Japan, **4** Department of Otolaryngology, Okinaka Memorial Institute for Medical Research, Toranomon Hospital, Minatoku, Tokyo, Japan, **5** Department of Otorhinolaryngology-Head and Neck Surgery, Miyazaki University School of Medicine, Miyazaki, Miyazaki, Japan, **6** Department of Otolaryngology-Head and Neck Surgery, Iwate Medical University, Morioka, Iwate, Japan, **7** Department of Otolaryngology-Head and Neck Surgery, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan, **8** Department of Otolaryngology, National Rehabilitation Center for Persons with Disabilities, Tokorozawa, Saitama, Japan, **9** Department of Otolaryngology, Saitama Medical University, Moroyama, Saitama, Japan, **10** Department of Otolaryngology, Kobe City Medical Center General Hospital, Kobe, Hyougo, Japan, **11** Department of Otorhinolaryngology, Head and Neck Surgery, Okayama University Postgraduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama, Okayama, Japan, **12** Department of Otorhinolaryngology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Fukuoka, Japan, **13** Department of Otorhinolaryngology, Kyushu Central Hospital, Fukuoka, Fukuoka, Japan, **14** Department of Otorhinolaryngology, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, Japan

Abstract

Usher syndrome is an autosomal recessive disorder manifesting hearing loss, retinitis pigmentosa and vestibular dysfunction, and having three clinical subtypes. Usher syndrome type 1 is the most severe subtype due to its profound hearing loss, lack of vestibular responses, and retinitis pigmentosa that appears in prepuberty. Six of the corresponding genes have been identified, making early diagnosis through DNA testing possible, with many immediate and several long-term advantages for patients and their families. However, the conventional genetic techniques, such as direct sequence analysis, are both time-consuming and expensive. Targeted exon sequencing of selected genes using the massively parallel DNA sequencing technology will potentially enable us to systematically tackle previously intractable monogenic disorders *and improve molecular diagnosis. Using this technique combined with direct sequence analysis, we screened* 17 unrelated Usher syndrome type 1 patients and detected probable pathogenic variants in the 16 of them (94.1%) who carried at least one mutation. Seven patients had the *MYO7A* mutation (41.2%), which is the most common type in Japanese. Most of the mutations were detected by only the massively parallel DNA sequencing. We report here four patients, who had probable pathogenic mutations in two different Usher syndrome type 1 genes, and one case of *MYO7A/PCDH15* digenic inheritance. This is the first report of Usher syndrome mutation analysis using massively parallel DNA sequencing and the frequency of Usher syndrome type 1 genes in Japanese. Mutation screening using this technique has the power to quickly *identify mutations of many causative genes while maintaining cost-benefit performance. In addition, the simultaneous* mutation analysis of large numbers of genes is useful for detecting mutations in different genes that are possibly disease modifiers or of digenic inheritance.

Citation: Yoshimura H, Iwasaki S, Nishio S-y, Kumakawa K, Tono T, et al. (2014) Massively Parallel DNA Sequencing Facilitates Diagnosis of Patients with Usher Syndrome Type 1. PLoS ONE 9(3): e90688. doi:10.1371/journal.pone.0090688

Editor: Klaus Brusgaard, Odense University hospital, Denmark

Received September 6, 2013; **Accepted** February 3, 2014; **Published** March 11, 2014

Funding: This study was supported by a Health and Labour Sciences Research Grant for Comprehensive Research on Disability Health and Welfare from the Ministry of Health, Labour and Welfare of Japan (S.U.), and by a Grant-in-Aid for Scientific Research from the (then) Ministry of Education, Science and Culture of Japan (http://www.mext.go.jp/english/) (S.U.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: usami@shinshu-u.ac.jp

Introduction

Usher syndrome (USH) is an autosomal recessive disorder characterized by hearing loss (HL), retinitis pigmentosa (RP) and vestibular dysfunction. Three clinical subtypes can be distinguished. USH type 1 (USH1) is the most severe among them because of profound HL, absent vestibular responses, and prepubertal onset RP. USH type 2 (USH2) is characterized by congenital moderate to severe HL, with a high-frequency sloping configuration. The vestibular function is normal and onset of RP is in the first or second decade. The onset of the visual symptoms such as night blindness in USH usually occurs several years later than in USH1. USH type 3 (USH3) is characterized by variable onset of progressive HL, variable onset of RP, and variable impairment of vestibular function (normal to absent) [1,2].

To date, nine genetic loci for USH1(*USH1B-H*, *J*, and *K*) have been mapped to chromosomes 11q13.5, 11p15.1, 10q22.1, 21q21, 10q21-q22, 17q24-q25, 15q22-q23 (*USH1H* and *J*), and 10p11.21–q21.1 [2,3,4]. Six of the corresponding genes have been identified: the actin-based motor protein myosin VIIa

(*MYO7A*, USH1B) [5]; two cadherin-related proteins, cadherin 23 (*CDH23*, USH1D) [6] and protocadherin 15 (*PCDH15*, USH1F) [7]; and two scaffold proteins, harmonin (*USH1C*) [8] and sans (*USH1G*) [9]; the Ca^{2+}- and integrin-binding protein (*CIB2*, USH1J) [4]. In Caucasian USH1 patients, previous studies showed that mutations in *MYO7A*, *USH1C*, *CDH23*, *PCDH15*, and *USH1G*, were found in 39–55%, 7–14%, 7–35%, 7–11%, and 0–7%, respectively (the frequency of *CIB2* is still unknown) [10,11,12]. In Japanese, Nakanishi et al. showed that *MYO7A* and *CDH23* mutations are present in USH1 patients [13], however, the frequency is not yet known. In addition, mutations in three corresponding genes (usherin *USH2A* [14], G protein-coupled receptor 98; *GPR98* [15], and deafness, autosomal recessive 31; *DFNB31* [16]) have been reported so far in USH2, and USH3 is caused by mutations in the clarin 1 (*CLRN1*) [17] gene.

Comprehensive molecular diagnosis of USH has been hampered both by genetic heterogeneity and the large number of exons for most of the USH genes. The six USH1 genes collectively contain 180 coding exons [4,9,10] the three USH2 genes comprise 175 coding exons [15,16,18], and the USH3 gene has five coding exons [17]. In addition some of these genes are alternatively spliced ([4,7,8,16,17] and NCBI database: http://www.ncbi.nlm.nih.gov/nuccore/). Thus far, large-scale mutation screening has been performed using direct sequence analysis, but that is both time-consuming and expensive. We thought that targeted exon sequencing of selected genes using the Massively Parallel DNA Sequencing (MPS) technology would enable us to systematically tackle previously intractable monogenic disorders and improve molecular diagnosis.

Therefore, in this study, we have conducted genetic analysis using MPS-based genetic screening to find mutations in nine causative USH genes (except *CIB2*) in Japanese USH1 patients.

Results

Mutation analysis of the nine USH genes in 17 unrelated USH1 patients revealed 19 different probable pathogenic variants, of which 14 were novel (Table 1).

All mutations were detected in only one patient each and sixteen of the 17 patients (94.1%) carried at least one mutation, while one patient had no mutations. Thirteen of the 16 mutation carriers each had two pathogenic mutations (Table 2).

Nonsense, frame shift, and splice site mutations are all classified as pathogenic, whereas missense mutations are presumed to be probable pathogenic variants based on results of prediction software for evaluation of the pathogenicity of missense variants (Table 1).

Of the 19 probable pathogenic mutations that we found, 17 were detected by MPS. The remaining two (p.Lys542GlnfsX5 in *MYO7A* and c.5821-2A>G in *CDH23*) were sequenced by direct sequence analysis.

Of our 17 USH patients, seven had *MYO7A* mutations (41.2%), three had *CDH23* mutations (17.6%), and two had *PCDH15* mutations (11.8%). We did not find any probable pathogenic mutations in *USH1C*, *USH1G*, and USH2/3 genes.

Four USH1 patients (Cases #3, 5, 8, 15) had probable pathogenic mutations in two different USH genes, with one being a biallelic mutation (Table 3). The other heterozygous/homozygous mutations were missense variants. Three of these patients (Cases #3, 5, 8) presented with earlier RP onset (night blindness) than in the other patients with two pathogenic mutations (Cases #1, 6, 7, 9, 11, 16) ($p = 0.007$) (Fig. 1).

One patient (Case #4) had heterozygote mutations in two USH1 genes (p.Ala771Ser in *MYO7A* and c.158-1G>A in *PCDH15*). His parents and one brother were found to also be carriers for these mutations. Another brother had no variants (Fig. 2).

Discussion

For USH1, early diagnosis has many immediate and several long-term advantages for patients and their families [1]. However, diagnosis in childhood, based on a clinical phenotype, has been difficult because patients appear to have only non-syndromic HL in childhood and RP develops in later years. Although early diagnosis is now possible through DNA testing, performing large-scale mutation screening for USH genes in all non-syndromic HL children has been both time-consuming and expensive. Therefore, the availability of MPS, which facilitates comprehensive large-scale mutation screening [19] is a very welcome advance.

MPS technology enabled us to detect pathogenic mutations in USH1 patients efficiently, identifying one or two pathogenic/likely pathogenic mutations in 16 of 17 (94.1%) cases. This was comparable to previous direct sequence analysis results such as Bonnet et al. who detected one or two mutations in 24 out of 27 (89%) USH1 patients [11] and Le Quesne Stabej et al. who detected one or two mutations in 41 out of 47 (87.2%) USH1 patients [12].

In addition, MPS assists in the analysis of disease modifiers and digenic inheritance because it simultaneously investigates many causative genes for a specific disease, such as in our case, USH. Previous reports have described several USH cases with pathogenic mutations in two or three different USH genes [11,12,20]. In our study, four patients had two pathogenic mutations in one gene and missense variants in a different gene (Table 3). We considered the latter to possibly be a disease modifier. For example, *USH1C*:p.Tyr813Asp, which occurred in 0/384 control chromosomes and was predicted to be "probably damaging" by the Polyphen program, was found with a homozygous *CDH23* nonsense mutation (p.Arg2107X) (Case #15). As for what the variant "modifies", we speculate that for USH1 patients with a disease modifier, RP symptoms such as night blindness show an earlier onset. However, we think that profound HL and the absence of vestibular function in USH1 patients are not affected by modifiers as they are congenital and therefore not progressive.

Ebermann et al. described a USH2 patient with "digenic inheritance." a heterozygous truncating mutation in *GPR98*, and a truncating heterozygous mutation in PDZ domain-containing 7 (*PDZD7*), which is reported to be a cause of USH [20]. Our USH1 patient (Case #4) had segregated *MYO7A*:p.Ala771Ser and *PCDH15*:c.158-1G>A. Molecular analyses in mouse models have shown many interactions among the USH1 proteins [2]. In particular, *MYO7A* directly binds to *PCDH15* and both proteins are expressed in an overlapping pattern in hair bundles in a mouse model [21]. *PCDH15*:c.158-1G>A, predicted to alter the splice donor site of intron 3, has been classified as pathogenic. *MYO7A*:p.Ala771Ser is a non-truncating mutation, but was previously reported as disease-causing [13]. So, we consider the patient to be the first reported case of *MYO7A*/*PCDH15* digenic inheritance.

However, we should be aware of two limitations of MPS technology. First, the target region of MPS cannot cover all coding exons of USH genes. Actually, the coverage of the target exons was 97.0% in our study. So, it is impossible to detect a mutation in a region which is not covered using this system (Case #9: c.5821-2A>G). Secondarily, the MPS system used in this study, is not effective for detecting homo-polymer regions, for example poly C stretch [22] (Case #8: p.Lys542GlnfsX5). In addition, concerning

Table 1. Possible pathogenic variants found in this study.

Gene	Mutation type	Nucleotide change	Amino acid change	exon/intron number	Domain	control (in 384 alleles)	SIFT Score	PolyPhen Score	Reference
MYO7A	Frameshift	c.1623dup	p.Lys542GlnfsX5	Exon 14	-	N/A	-	-	Le Quesne Stabej et al. (2012)
		c.4482_4483insTG	p.Trp1495CysfsX55	Exon 34	-	N/A	-	-	This study
		c.6205_6206delAT	p.Ile2069ProfsX6	Exon 45	-	N/A	-	-	This study
	Nonsense	c.1477C>T	p.Gln493X	Exon 13	-	N/A	-	-	This study
		c.1708C>T	p.Arg570X	Exon 15	-	N/A	-	-	This study
		c.2115C>A	p.Cys705X	Exon 18	-	N/A	-	-	This study
		c.6321G>A	p.Trp2107X	Exon 46	-	N/A	-	-	This study
	Missense	c.2074G>A	p.Val692Met	Exon 17	Motor domain	0	0.09	0.982	This study
		c.2311G>T	p.Ala771Ser	Exon 20	IQ 2	0.0026	0.01	0.825	Nakanishi et al. (2010)
		c.6028G>A	p.Asp2010Asn	Exon 44	FERM 2	0	0	0.925	Jacobson et al. (2009)
CDH23	Frameshift	c.3567delG	p.Arg1189ArgfsX5	Exon 30	-	N/A	-	-	This study
		c.5780_5781delCT	p.Ser1927Cysfs16	Exon 44	-	N/A	-	-	This study
	Splicing	c.5821-2A>G	?	Intron 44	-	N/A	-	-	This study
	Nonsense	c.6319C>T	p.Arg2107X	Exon 48	-	N/A	-	-	Nakanishi et al. (2010)
PCDH15	Splicing	c.158-1G>A	?	Intron 3	-	N/A	-	-	This study
	Nonsense	c.1006C>T	p.Arg336X	Exon 10	-	N/A	-	-	This study
		c.2971C>T	p.Arg991X	Exon 22	-	N/A	-	-	Roux et al. (2006)
		c.3337G>T	p.Glu1113X	Exon 25	-	N/A	-	-	This study
	Missense	c.3724G>A	p.Val1242Met	Exon 28	Cadherin 11	0	0	1	This study

Computer analysis to predict the effect of missense variants on MYO7A protein function was performed with sorting intolerant from tolerant (SIFT; http://sift.jcvi.org/), and polymorphism phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/).

N/A: not applicable.

doi:10.1371/journal.pone.0090688.t001

Table 2. Details of phenotype and genotype of 17 USH1 patients.

Sample No.	Age	Sex	Allele1	Allele2	Hereditary form	Onset of night blindness	Cataract	Hearing Aid	Cochlear Implant
MYO7A									
1	37	M	p.Gln493X	p.Trp1495CysfsX55	sporadic	13	no	unilateral	unilateral
2	41	W	p.I2069fsX6	p.I2069fsX6	AR	unknown	both eyes	bilateral	no
5	54	M	p.Val692Met	p.Val692Met	AR	5	both eyes	no	no
6	54	W	p.Arg570X	p.Arg570X	sporadic	6	no	no	no
8	14	M	p.Lys542GlnfsX5	p.Lys542GlnfsX5	sporadic	6	no	unilateral	unilateral
11	54	M	p.Asp2010Asn	p.Trp2107X	sporadic	13	no	no	no
17	56	W	p.Cys705X	p.Cys705X	sporadic	unknown	no	no	no
CDH23									
7	12	W	p.Arg1189ArglfsX5	p.Arg1189ArglfsX5	sporadic	12	both eyes	no	bilateral
9	9	M	p.Ser1927Cysfs16	c.5821-2A>G	sporadic	8	no	unilateral	unilateral
15	16	W	p.Arg2107X	p.Arg2107X	sporadic	unknown	no	no	no
PCDH15									
3	47	W	p.Glu1113X	p.Glu1113X	sporadic	5	both eyes	no	no
16	28	W	p.Arg991X	p.Arg991X	AR	10	no	no	no
10	62	M	p.Arg962Cys	unknown	sporadic	9	both eyes	no	no
12	52	M	p.Arg336X	unknown	sporadic	3	no	no	no
13	51	M	p.Val1242Met	unknown	sporadic	10	no	no	no
MYO7A[*1]/*PCDH15*[*2]									
4	21	M	p.Ala771Ser[*1]	c.158-1G>A[*2]	sporadic	10	no	unilateral	unilateral
unknown									
14	64	W	unknown	unknown	sporadic	15	both eyes	unilateral	no

*All subjects have congenital deafness and RP.

doi:10.1371/journal.pone.0090688.t002

pathogenecity of mutations identified, functional analysis will be necessary to draw the final conclusion in the future.

In UK and US Caucasian USH1 patients, USH1B (*MYO7A*) has been reported as the most common USH1 genetic subtype [11,12], while USH1F (*PCDH15*) has been reported as the most common USH1 genetic subtype in North American Ashkenazi Jews [23]. In Japanese, our study revealed that the most common type was *MYO7A* (41.7%), which was similar to the frequency in the above Caucasian patients (46.8~55%) [11,12]. However, the small number of USH1 patients in our study might have biased the frequency and further large cohort study will be needed in the future.

In addition, most of our detected mutations were novel. We have previously reported genes responsible for deafness in Japanese patients and observed differences in mutation spectrum between Japanese (who are probably representative of other Asian populations) and populations with European ancestry [24].

In conclusion, our study was the first report of USH mutation analysis using MPS and the frequency of USH1 genes in Japanese. Mutation screening using MPS has the potential power to quickly identify mutations of many causative genes such as USH while maintaining cost-benefit performance. In addition, the simultaneous mutation analysis of large numbers of genes was useful for detecting mutations in different genes that are possibly disease modifiers or of digenic inheritance.

Materials and Methods

Subjects

We screened 17 Japanese USH1 patients (aged 9 to 64 years): three from autosomal recessive families (non-affected parents and two or more affected siblings), and 14 from sporadic families. There were 9 males and 8 females. None of the subjects had any other noteworthy symptoms. All subjects or next of kin on the behalf of the minors/children gave prior written informed consent for participation in the project, and the Ethical Committee of Shinshu University approved the study and the consent procedure.

Amplicon Library Preparation

An Amplicon library of the target exons was prepared with an Ion AmpliSeq Custom Panel (Applied Biosystems, Life Technologies, Carlsbad, CA) designed with Ion AmpliSeq Designer (https://www.ampliseq.com/browse.action) for nine USH genes by using Ion AmpliSeq Library Kit 2.0 (Applied Biosystems, Life Technologies) and Ion Xpress Barcode Adapter 1–16 Kit (Applied Biosystems, Life Technologies) according to the manufacturers' procedures.

In brief, DNA concentration was measured with Quant-iT dsDNA HS Assay (Invitrogen, Life Technologies) and Qubit Fluorometer (Invitrogen, Life Technologies) and DNA quality was confirmed by agarose gel electrophoresis. 10 ng of each genomic DNA sample was amplified, using Ion AmpliSeq HiFi Master Mix (Applied Biosystems, Life Technologies) and AmpliSeq Custom primer pools, for 2 min at 99°C, followed by 15 two-step cycles of

Table 3. The patients with mutations in two different genes.

Sample	Genes with two pathogenic mutations	Gene with one heterozygous mutation	Nucleotide change	Amino acid change	control	SIFT score	PolyPhen score	Referense
5	*MYO7A*	*CDH23*	c.C719T	p.P240L*	0.26	0.06	0.999	Wagatsuma et al. (2007)
8	*MYO7A*	*CDH23*	c.2568C>G	p.Ile856Met	0	0.08	1	This study
15	*CDH15*	*USH1C*	c.2437T>G	p.Tyr813Asp	0	0.19	0.932	This study
3	*PCDH15*	*USH1G*	c.28C>T	p.Arg10Trp	0	0.19	1	This study

*homozygotes.

doi:10.1371/journal.pone.0090688.t003

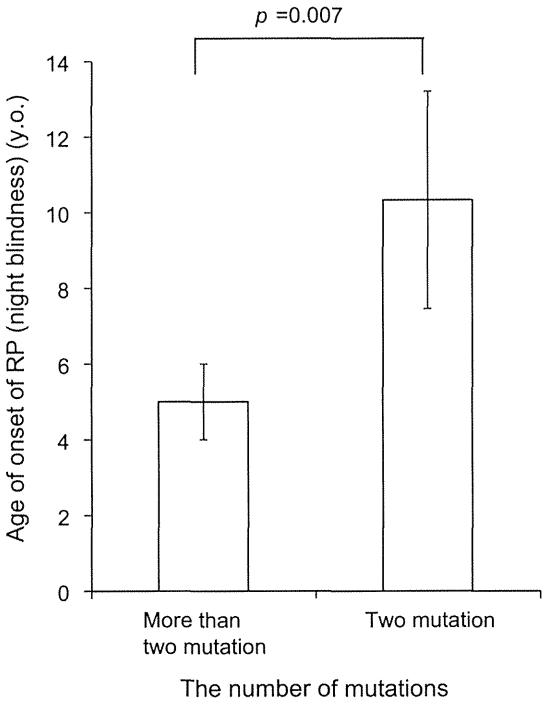

Figure 1. The number of mutations and the age of RP onset in Usher syndrome type 1 patients. The age of RP onset is earlier in the patients with more than two pathogenic mutations. RP: retinitis pigmentosa.

doi:10.1371/journal.pone.0090688.g001

99°C for 15 sec and 60°C for 4 min, ending with a holding period at 10°C in a PCR thermal cycler (Takara, Shiga, Japan). After the Multiplex PCR amplification, amplified DNA samples were digested with FuPa enzyme at 50°C for 10 min and 55°C for 10 min and the enzyme was successively inactivated for 60°C for 20 min incubation. After digestion, diluted barcode adapter mix including Ion Xpress Barcode Adapter and Ion P1 adaptor were ligated to the end of the digested amplicons with ligase in the kit for 30 min at 22°C and the ligase was successively inactivated at 60°C for 20 min incubation. Adaptor ligated amplicon libraries were purified with the Agencourt AMPure XP system (Beckman Coulter Genomics, Danvers, MA). The amplicon libraries were quantified by using Ion Library Quantitation Kit (Applied Biosystems, Life Technologies) and the StepOne plus realtime PCR system (Applied Biosystems, Life Technologies) according to the manufacturers' procedures. After quantification, each amplicon library was diluted to 20 pM and the same amount of the 12 libraries for 12 patients were pooled for one sequence reaction.

Emulsion PCR and Sequencing

The emulsion PCR was carried out with the Ion OneTouch System and Ion OneTouch 200 Template Kit v2 (Life Technologies) according to the manufacturer's procedure (Publication Part Number 4478371 Rev. B Revision Date 13 June 2012). After the emulsion PCR, template-positive Ion Sphere Particles were enriched with the Dynabeads MyOne Streptavidin C1 Beads (Life Technologies) and washed with Ion OneTouch Wash Solution in the kit. This process were performed using an Ion OneTouch ES system (Life Technologies).

After the Ion Sphere Particle preparation, MPS was performed with an Ion Torrent Personal Genome Machine (PGM) system

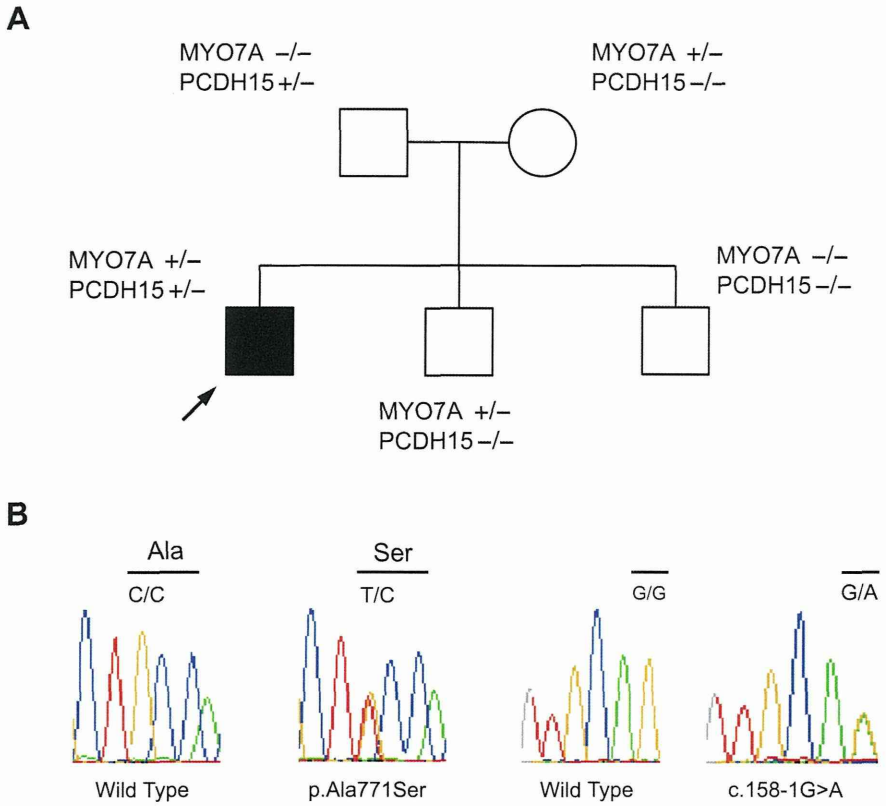

Figure 2. Pedigree and sequence chromatograms of the patient with the p.Ala771Ser in *MYO7A* and c.158-1G>A in *PCDH15* mutations. (A) The pedigree and sequence results of the proband and family. (B) Sequence chromatograms from wild-type and mutations. The proband, his mothor and one brother carried a heterozygous 2311G>T transition in exon 20, which results in an alanine to a serine (Ala771Ser) in *MYO7A*. Another variation, 158-1G>A in intron 3 of *PCDH15*, was derived from the proband and his father. Another brother had no variants.
doi:10.1371/journal.pone.0090688.g002

using the Ion PGM 200 Sequencing Kit and Ion 318 Chip (Life Technologies) according to the manufacturer's procedures.

Base Call and Data Analysis

The sequence data were processed with standard Ion Torrent Suite Software and Torrent Server successively mapped to human genome sequence (build GRCh37/hg19) with Torrent Mapping Alignment Program optimized to Ion Torrent data. The average of 562.33 Mb sequences with about 4,300,000 reads was obtained by one Ion 318 chip. The 98.0% sequences were mapped to the human genome and 94% of them were on the target region. Average coverage of depth in the target region was 314.2 and 93.8% of them were over 20 coverage.

After the sequence mapping, the DNA variant regions were piled up with Torrent Variant Caller plug-in software. Selected variant candidates were filtered with the average base QV (minimum average base quality 25), variant frequency (40–60% for heterozygous mutations and 80–100% for homozygous mutations) and coverage of depth (minimum coverage of depth 10). After the filtrations, variant effects were analyzed with the wANNOVAR web site [25,26] (http://wannovar.usc.edu) including the functional prediction software for missense variants: Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), and Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/). The sequencing data was available in the DNA databank of Japan (Accession number: DRA001273).

Algorithm

Missense, nonsense, and splicing variants were selected among the identified variants. Variants were further selected as less than 1% of: 1) the 1000 genome database (http://www.1000genomes.org/), 2) the 5400 exome variants (http://evs.gs.washington.edu/EVS/), and 3) the in-house control. Candidate mutations were confirmed by Sanger sequencing and the responsible mutations were identified by segregation analysis using samples from family members of the patients. In addition, the cases with heterozygous or no causative mutation were fully sequenced by Sanger sequencing for USH1 genes in order to verify the MPS results.

Direct Sequence Analysis

Primers were designed with the Primer 3 plus web server (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Each genomic DNA sample (40 ng) was amplified using Ampli Taq Gold (Life Technologies) for 5 min at 94°C, followed by 30 three-step cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 5 min, ending with a holding period at 4°C in a PCR thermal cycler (Takara, Shiga, Japan). The PCR products were treated with ExoSAP-IT (GE Healthcare Bio, Buckinghamshire, UK) and by incubation at 37°C for 60 min, and inactivation at 80°C for 15 min. After the products were purified, we performed standard cycle sequencing reaction with ABI Big Dye terminators in an ABI 3130xl sequencer (Life Technologies).

Accession numbers

MYO7A, [NM_000260.3]; *USH1C*, [NM_ 153676.3]; *CDH23*, [NM_ 022124.5]; *PCDH15*, [NM_ 033056.3]; *USH1G*, [NM_ 173477.2]; *USH2A*, [NM_206933.2]; *GPR98*, [NM_ 032119.3]; *DFNB31*, [NM_ 015404.3]; *CLRN1*, [NM_ 174878.2]; *PDZD7*, [NM_ 001195263.1].

Acknowledgments

We thank A. C. Apple-Mathews for help in preparing the manuscript.

Author Contributions

Conceived and designed the experiments: HY SI SN SU. Performed the experiments: HY SN. Analyzed the data: HY SN SU. Contributed reagents/materials/analysis tools: HY SI SN KK TT YK HS KN KI TI YN KF CO TK HN SU. Wrote the paper: HY SN SU.

References

1. Kimberling WJ, Hildebrand MS, Shearer AE, Jensen ML, Halder JA, et al. (2010) Frequency of Usher syndrome in two pediatric populations: Implications for genetic screening of deaf and hard of hearing children. Genet Med 12: 512–516.
2. Yan D, Liu XZ (2010) Genetics and pathological mechanisms of Usher syndrome. J Hum Genet 55: 327–335.
3. Jaworek TJ, Bhatti R, Latief N, Khan SN, Riazuddin S, et al. (2012) USH1K, a novel locus for type I Usher syndrome, maps to chromosome 10p11.21-q21.1. J Hum Genet 57: 633–637.
4. Riazuddin S, Belyantseva IA, Giese AP, Lee K, Indzhykulian AA, et al. (2012) Alterations of the CIB2 calcium- and integrin-binding protein cause Usher syndrome type 1J and nonsyndromic deafness DFNB48. Nat Genet 44: 1265–1271.
5. Weil D, Blanchard S, Kaplan J, Guilford P, Gibson F, et al. (1995) Defective myosin VIIA gene responsible for Usher syndrome type 1B. Nature 374: 60–61.
6. Bork JM, Peters LM, Riazuddin S, Bernstein SL, Ahmed ZM, et al. (2001) Usher syndrome 1D and nonsyndromic autosomal recessive deafness DFNB12 are caused by allelic mutations of the novel cadherin-like gene CDH23. Am J Hum Genet 68: 26–37.
7. Ahmed ZM, Riazuddin S, Bernstein SL, Ahmed Z, Khan S, et al. (2001) Mutations of the protocadherin gene PCDH15 cause Usher syndrome type 1F. Am J Hum Genet 69: 25–34.
8. Verpy E, Leibovici M, Zwaenepoel I, Liu XZ, Gal A, et al. (2000) A defect in harmonin, a PDZ domain-containing protein expressed in the inner ear sensory hair cells, underlies Usher syndrome type 1C. Nat Genet 26: 51–55.
9. Mustapha M, Chouery E, Torchard-Pagnez D, Nouaille S, Khrais A, et al. (2002) A novel locus for Usher syndrome type I, USH1G, maps to chromosome 17q24-25. Hum Genet 110: 348–350.
10. Ouyang XM, Yan D, Du LL, Hejtmancik JF, Jacobson SG, et al. (2005) Characterization of Usher syndrome type I gene mutations in an Usher syndrome patient population. Hum Genet 116: 292–299.
11. Bonnet C, Grati M, Marlin S, Levilliers J, Hardelin JP, et al. (2011) Complete exon sequencing of all known Usher syndrome genes greatly improves molecular diagnosis. Orphanet J Rare Dis 6: 21.
12. Le Quesne Stabej P, Saihan Z, Rangesh N, Steele-Stallard HB, Ambrose J, et al. (2012) Comprehensive sequence analysis of nine Usher syndrome genes in the UK National Collaborative Usher Study. J Med Genet 49: 27–36.
13. Nakanishi H, Ohtsubo M, Iwasaki S, Hotta Y, Takizawa Y, et al. (2010) Mutation analysis of the MYO7A and CDH23 genes in Japanese patients with Usher syndrome type 1. J Hum Genet 55: 796–800.
14. Eudy JD, Yao S, Weston MD, Ma-Edmonds M, Talmadge CB, et al. (1998) Isolation of a gene encoding a novel member of the nuclear receptor superfamily from the critical region of Usher syndrome type IIa at 1q41. Genomics 50: 382–384.
15. Weston MD, Luijendijk MW, Humphrey KD, Moller C, Kimberling WJ (2004) Mutations in the VLGR1 gene implicate G-protein signaling in the pathogenesis of Usher syndrome type II. Am J Hum Genet 74: 357–366.
16. Aller E, Jaijo T, van Wijk E, Ebermann I, Kersten F, et al. (2010) Sequence variants of the DFNB31 gene among Usher syndrome patients of diverse origin. Mol Vis 16: 495–500.
17. Joensuu T, Hamalainen R, Yuan B, Johnson C, Tegelberg S, et al. (2001) Mutations in a novel gene with transmembrane domains underlie Usher syndrome type 3. Am J Hum Genet 69: 673–684.
18. Nakanishi H, Ohtsubo M, Iwasaki S, Hotta Y, Usami S, et al. (2011) Novel USH2A mutations in Japanese Usher syndrome type 2 patients: marked differences in the mutation spectrum between the Japanese and other populations. J Hum Genet 56: 484–490.
19. Miyagawa M, Nishio SY, Ikeda T, Fukushima K, Usami S (2013) Massively parallel DNA sequencing successfully identifies new causative mutations in deafness genes in patients with cochlear implantation and EAS. PLoS One.
20. Ebermann I, Phillips JB, Liebau MC, Koenekoop RK, Schermer B, et al. (2010) PDZD7 is a modifier of retinal disease and a contributor to digenic Usher syndrome. J Clin Invest 120: 1812–1823.
21. Senften M, Schwander M, Kazmierczak P, Lillo C, Shin JB, et al. (2006) Physical and functional interaction between protocadherin 15 and myosin VIIa in mechanosensory hair cells. J Neurosci 26: 2060–2071.
22. Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, et al. (2012) Performance comparison of benchtop high-throughput sequencing platforms. Nat Biotechnol 30: 434–439.
23. Ben-Yosef T, Ness SL, Madeo AC, Bar-Lev A, Wolfman JH, et al. (2003) A mutation of PCDH15 among Ashkenazi Jews with the type 1 Usher syndrome. N Engl J Med 348: 1664–1670.
24. Usami S, Wagatsuma M, Fukuoka H, Suzuki H, Tsukada K, et al. (2008) The responsible genes in Japanese deafness patients and clinical application using Invader assay. Acta Otolaryngol 128: 446–454.
25. Wang K, Li M, Hakonarson H (2010) ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 38: e164.
26. Chang X, Wang K (2012) wANNOVAR: annotating genetic variants for personal genomes via the web. J Med Genet 49: 433–436.

Annals of Otology, Rhinology & Laryngology
1–7

DOI: 10.1177/0003489415575044
aor.sagepub.com
SAGE

Non-ocular Stickler Syndrome With a Novel Mutation in *COL11A2* Diagnosed by Massively Parallel Sequencing in Japanese Hearing Loss Patients

Yoh-ichiro Iwasa, MD, PhD[1], Hideaki Moteki, MD, PhD[1,2], Mitsuru Hattori, PhD[1], Ririko Sato, MD[1], Shin-ya Nishio, PhD[1,2], Yutaka Takumi, MD, PhD[1,2], and Shin-ichi Usami, MD, PhD[1,2]

Abstract

Objectives: This study aims to document the clinical features of patients with *COL11A2* mutations and to describe the usefulness of massively parallel sequencing.

Methods: One thousand one hundred twenty (1120) Japanese hearing loss patients from 53 ENT departments nationwide participated in this study. Massively parallel sequencing of 63 genes implicated in hearing loss was performed to identify the genetic causes in the Japanese hearing loss patients.

Results: A novel mutation in *COL11A2* (c.3937_3948delCCCCCAGGGCCA) was detected in an affected family, and it was segregated in all hearing loss individuals. The clinical findings of this family were compatible with non-ocular Stickler syndrome. Orofacial features of mid-facial hypoplasia and slowly progressive mild to moderate hearing loss were also presented. Audiological examinations showed favorable auditory performance with hearing aid(s).

Conclusion: This is the first case report of the genetic diagnosis of a non-ocular Stickler syndrome family in the Japanese population. We suggest that it is important to take both genetic analysis data and clinical symptoms into consideration to make an accurate diagnosis.

Keywords

hearing loss, genetics, *COL11A2*, autosomal dominant, Stickler syndrome, massively parallel sequencing

Introduction

Stickler syndrome is a connective tissue disorder characterized by ocular and orofacial features, arthritis, and hearing loss that was first reported by Stickler et al.[1] This syndrome has heterogeneous conditions that are categorized into 3 types; types I and II are associated with ocular symptoms, while type III does not have ocular symptoms. All types are inherited in an autosomal dominant fashion and show varied manifestations. Mutations in different genes, *COL2A1, COL11A1, COL11A2, COL9A1*, and *COL9A2*, have been reported to be associated with Stickler syndrome.[2]

Mutations in *COL11A2* result in several syndromes: non-ocular Stickler syndrome (categorized in type III Stickler syndrome),[3] otospondylomegaepiphyseal dysplasia (OSMED),[4] Weissenbacher-Zweymuller syndrome,[5] and Robin sequence.[6] Other mutations in *COL11A2* lead to autosomal dominant nonsyndromic hearing loss (DFNA13)[7] or autosomal recessive nonsyndromic hearing loss (DFNB53).[8] There have been many manifestations reported in several phenotypes owing to mutations in the single gene, *COL11A2*, although only 3 non-ocular Stickler syndrome families have been found.

Recently, targeted genome sequencing and massively parallel sequencing (MPS) technology have improved genetic diagnosing and have enabled us to diagnose intractable disorders that could have genetic causes. We have recently reported that targeted exon sequencing using MPS is a powerful tool for identifying rare gene mutations in hearing loss patients.[9] In this study, using MPS, we identified a novel mutation in *COL11A2* in an autosomal dominant hearing loss family diagnosed with non-ocular Stickler

[1]Department of Otorhinolaryngology, Shinshu University School of Medicine, Matsumoto, Japan
[2]Department of Hearing Implant Sciences, Shinshu University School of Medicine, Matsumoto, Japan

Corresponding Author:
Shin-ichi Usami, MD, PhD, Department of Otorhinolaryngology, Shinshu University School of Medicine, Department of Hearing Implant Sciences, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan.
Email: usami@shinshu-u.ac.jp

syndrome. We describe the detailed clinical findings, including the efficacy of their hearing aids for their hearing loss.

Subjects and Methods

Subjects. One thousand one hundred twenty Japanese hearing loss patients (autosomal dominat, 266; autosomal recessive, 600; unknown, 254) from 53 ENT departments nationwide participated in this study. Written informed consent was obtained from all subjects (or from their next of kin, caretaker, or guardian on the behalf of minors/children) prior to enrollment in the project. This study was approved by the Shinshu University Ethical Committee as well as the respective Ethical Committees of the other participating institutions.

Amplicon library preparation. Amplicon libraries were prepared using an Ion AmpliSeq Custom Panel (Applied Biosystems, Life Technologies, Carlsbad, California, USA) for 63 genes reported to cause nonsyndromic hearing loss according to the manufacturer's instructions. The detailed protocol was described elsewhere. [9] After preparation, the amplicon libraries were diluted to 20 pM, and equal amounts of 6 libraries for 6 patients were pooled for 1 sequence reaction.

Emulsion polymerase chain reaction and sequencing. Emulsion polymerase chain reaction (PCR) and sequencing were performed according to the manufacturer's instructions. The detailed protocol was described elsewhere. [9] MPS was performed with an Ion Torrent Personal Genome Machine (PGM) system using an Ion PGM 200 Sequencing Kit and an Ion 318 Chip (Life Technologies).

Base call and data analysis. The sequence data were mapped against the human genome sequence (build GRCh37/hg19) with a Torrent Mapping Alignment Program. After sequence mapping, the DNA variant regions were piled up with Torrent Variant Caller plug-in software. After variant detection, their effects were analyzed using ANNOVAR software.[10,11] The missense, nonsense, insertion/deletion, and splicing variants were selected from among the identified variants. Variants were further selected as less than 1% of (1) the 1000 genome database,[12] (2) the 6500 exome variants,[13] (3) the Human Genetic Variation Database (data set for 1208 Japanese exome variants),[14] and (4) the 269 in-house Japanese normal hearing controls.

To predict the pathogenicity of missense variants, the following functional prediction software was used: PhyloP,[15] Sorting Intolerant from Tolerant (SIFT),[16] Polymorphism Phenotyping,[17] LRT,[18] MutationTaster,[19] and GERP++.[20]

Candidate mutations were confirmed by Sanger sequencing, and the responsible mutations were identified by segregation analysis using samples from among the patients' family members.

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

Results

We identified 1 family (Figure 1A) that had a causative mutation in *COL11A2* in the cohort of this study (266 autosomal dominant hearing loss patients).

Case Details

The proband is a 9-year-old female. She had no particular perinatal complications, and she passed a newborn hearing screening. When she was 5 years old, the parents suspected hearing loss, as she seemed unaware of sounds. She visited Shinshu University Hospital, Department of Otolaryngology for a hearing examination. The audiogram showed bilateral sensorineural hearing loss (SNHL) at approximately 36 dBHL in both ears. Computed tomography (CT) of the temporal bone showed no abnormalities in the middle or inner ears. She started to wear a hearing aid on the right ear at the age of 8.

Simultaneously, aged 7, she consulted a plastic surgeon because she had a distinctly depressed nasal bridge and a sharply upturned nose. Three-dimensional CT imaging of the head showed maxilla hypoplasia (Figure 2): Binder syndrome[21] was diagnosed. A submucous cleft palate was also diagnosed.

Her brother (III-3), father (II-2), uncle (II-3), and cousin (III-5) also had hearing loss and similar orofacial appearance. The pedigree was compatible with an autosomal dominant mode of inheritance (Figure 1A). The grandmother of the patient (I-2) suffered from otitis media bilaterally in childhood and underwent middle ear surgery. Thus, her (I-2) hearing loss was not inherited but was due to bilateral otitis media. All affected family members had slowly progressive, mild to moderate hearing loss. They did not have any ocular or joint symptoms. The patient was 120 cm in height at age 8, which is slightly below average.

Audiological Findings

As shown in Figure 1B, all affected patients had mild to moderate hearing loss. No substantial air-bone gaps were observed. Pure-tone audiometry (PTA) shows varied types of hearing loss; patients I-1 and II-2 had sloping hearing loss at high frequencies, II-3 had a flat audiogram, whereas III-1 and III-5 had U-shaped mid-frequency hearing loss. Patient III-1 and her uncle, II-3, wore hearing aids. Figure 3 shows their speech discrimination test (SDT) results with

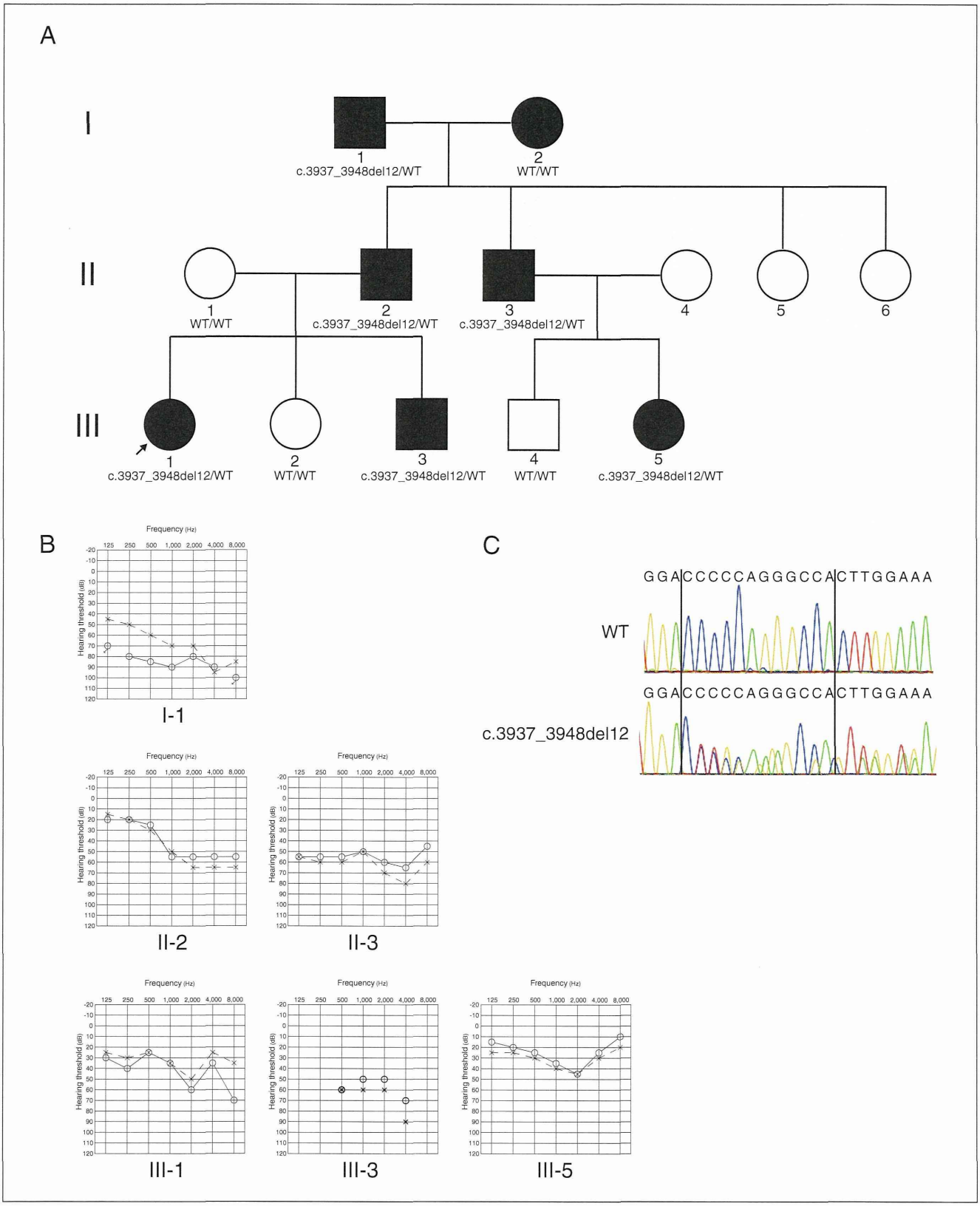

Figure 1. Pedigree of the patient. (A) Pedigree showed an autosomal dominant mode of inheritance. (B) Pure-tone audiometry showed mild to moderate hearing loss in affected patients. The hearing level of III-3 was assessed by Auditory Steady State Response (ASSR). (C) The electropherogram showed the mutation.

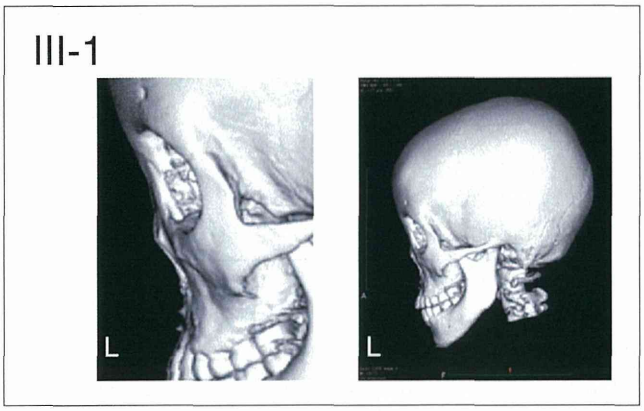

Figure 2. Three-dimensional (3-D) CT imaging of the proband (III-1). (Right side) A whole-head image shows no obvious malformations, except (left side, enlarged) maxilla hypoplasia.

and without hearing aids. Patient III-1 obtained a maximum SDT score of 95% without hearing aids. Patient II-3 obtained maximum SDT scores of 85% (right) and 80% (left) without hearing aids and 90% (right) and 95% (left) with hearing aids, respectively.

Mutation Analysis

We performed comprehensive genetic testing using MPS of all known nonsyndromic hearing loss genes as well as nonsyndromic hearing loss mimic genes. We identified a novel mutation in the *COL11A2* gene, in which 12 nucleotides were deleted. This mutation corresponded to c.3937_3948delCCCCCAGGGCCA (NM_080680.2) and led to a lack of 4 amino acids (p.1312_1315del4). We also performed Sanger sequencing for confirmation of the variant of the proband and for the family segregation study. As shown in Figure 1C, the Sanger sequencing results revealed that this mutation was segregated to all the affected family members (Figure 1A).

Discussion

In this study, we identified a novel mutation in the *COL11A2* gene in an autosomal dominant hearing loss family. The mutations in *COL11A2* caused both syndromic hearing loss (non-ocular Stickler syndrome, otospondylomegaepiphyseal dysplasia [OSMED], and Weissenbacher-Zweymuller syndrome) and nonsyndromic hearing loss (DFNA13, DFNB53). The clinical findings of the present case and affected family members were compatible with non-ocular Stickler syndrome, which was accompanied by orofacial features such as depressed nasal bridge, upturned nose with mid-facial hypoplasia, and slowly progressive mild to moderate hearing loss. Otherwise, no ocular symptoms were diagnosed for this family. This is the first report of a mutation in the *COL11A2* gene causing disease in the Japanese population. This is also the first case of non-ocular Stickler syndrome (type III Stickler syndrome) diagnosed by genetic testing using MPS. Before the genetic testing was performed, we thought that the family including the patient had only nonsyndromic hearing loss with autosomal dominant inheritance, and we did not appreciate that their orofacial features might be related to some other syndrome. Her plastic surgeon independently primarily diagnosed Binder syndrome, based on their typical facial features, and thought that Stickler syndrome would be a secondary diagnosis. However, the plastic surgeon did not appreciate her hearing loss because she communicated easily at the examination. Based on these combined findings, together with the results of genetic testing using MPS, we were able to correctly diagnose non-ocular Stickler syndrome with a mutation in *COL11A2* and discount Binder syndrome.

Rare gene mutations were detected more easily in less time using MPS to sequence all known hearing loss genes simultaneously, suggesting candidate genes in the process. However, since MPS data have been available, it has been difficult to diagnose the actual pathogenic mutations within the data, especially in disease traits attributable to heterozygotic allele mutation. In the present case, we carefully reviewed the patient's clinical findings and noticed her concomitant symptoms. Thus, we suggest that it is important to take both genetic analyzed data and clinical symptoms into consideration for an accurate diagnosis.

Presently, 27 mutations of *COL11A2* have been reported (Table 1). Only 3 mutations were reported in cases of non-ocular Stickler syndrome from Germany, the US, and the UK. Each patient's hearing loss was slowly progressive and did not become severe. It is reported that 94.1% of non-ocular Stickler syndrome patients have hearing loss, which is childhood onset and progresses only slightly, if at all.[2,22] In the present case, affected patients had childhood onset hearing loss, and their hearing loss was slowly progressive. van Beelen et al[2] reported that patients who had hearing loss caused by mutations in *COL11A2* showed better maximum phoneme scores than presbyacusis. Two of the affected patients (III-1 and II-3) were using hearing aids and able to obtain favorable hearing. We suggested that a hearing aid would be a more appropriate option for patients with *COL11A2* mutation and less so for other etiologies of hearing loss, which cause hair cell damage. The *COL11A2* is expressed in the tectorial membrane,[7] not in the hair cells. The cause of the hearing loss in patients who have a mutation in *COL11A2* is thought to be attenuation due to alteration of the tectorial membrane, not due to impairment of the hair cells. Hearing impairment by *COL11A2* mutations has been termed *cochlear conductive hearing loss*.[23] This type of hearing loss was also observed in the hearing loss patients with mutations in the *TECTA* gene (DFNA8/12), which is also expressed in the tectorial membrane.[24] We suspected that amplification by

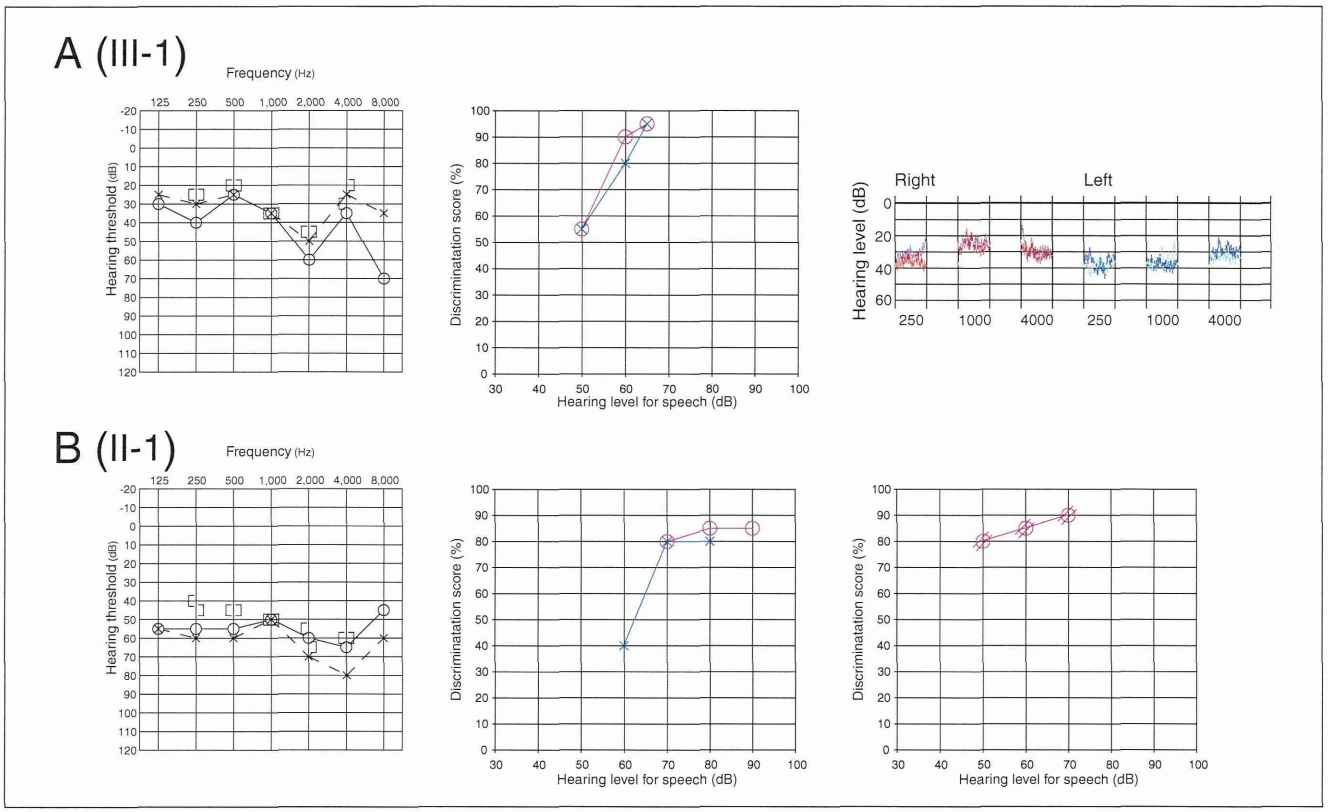

Figure 3. Pure-tone audiograms and speech discrimination test (SDT) scores for (A) patient III-1 and (B) her uncle, II-3. (Upper right) Self-recording audiometry for the patient (III-1) showed Jerger type I in both ears. (Lower right) SDT scores for the patient (II-3) with a hearing aid on both ears were improved for presentation levels 50-70 dB.

Table 1. Known Mutations in the *COL11A2* Gene and Associated Phenotypes.

Nucleotide Change	Amino acid Change	Type of Mutation	Phenotype	Inheritance	Severity	Reference
c.1105C>T	p.R369W	Missense	Micrognathia	AD	Normal	Melkoniemi et al[25]
c.1357C>T	p.R453W	Missense	Osteoarthritis, early onset	AD	NA	Jakkula et al[26]
c.2165G>A	p.G722E	Missense	DFNA13	AD	Severe	McGuirt et al[6]
c.2652_2662del9		Frameshift	Fibrochondrogenesis	AD	NA	Tompson et al[27]
c.2775_2801del		Frameshift	Non-ocular Stickler syndrome	AD	Mild-moderate	Sirko-Osadsa et al[28]
c.2842C>T	p.R948C	Missense	DFNA13	AD	Severe	McGuirt et al[6]
c.3659G>A	p.G1220D	Missense	Non-ocular Stickler syndrome	AD	Moderate-severe	van Beelen et al[2]
c.3877C>T	p.R1293X	Nonsence	Non-ocular Stickler syndrome	AD	Mild-moderate	Vuoristo et al[29]
c.3937_3948del12		Frameshift	Non-ocular Stickler syndrome	AD	Mild-moderate	This study
c.IVS60-1G>A (c.4134+1G>A)		Frameshift	Non-ocular Stickler syndrome	AD	Moderate	Vikkula et al[3]
c4064G>A	p.G1355E	Missense	Weissenbacher-Zweymuller syndrome	AD	NA	Pihlajamaa et al[30]
c.733delC		Frameshift	OSMED	AR	Moderate-severe	Melkoniemi et al[7]

(continued)

Table 1. (continued)

Nucleotide Change	Amino acid Change	Type of Mutation	Phenotype	Inheritance	Severity	Reference
c.1378C>T	p.R460X	Nonsence	OSMED	AR	Severe	Melkoniemi et al[7]
c.1603C>A	p.P535T	Missense	DFNB53	AR	Profound	Chen et al[8]
c.1660-2A>G		Frameshift	OSMED	AR	Severe	Melkoniemi et al[7]
c.1723G>A	p.G575R	Missense	OSMED	AR	Moderate-severe	Vikkula et al[3]
c.2234C>A	p.S745X	Nonsence	OSMED	AR	Severe-profound	Melkoniemi et al[7]
c.2405_2410ins9		Frameshift	OSMED	AR	Severe	Melkoniemi et al[7]
c.2406_2409del		Frameshift	OSMED	AR	Severe	Melkoniemi et al[7]
c.2505delT		Frameshift	OSMED	AR	Moderate-profound	Kayserili et al[31]
c.3032_3033insC		Frameshift	OSMED	AR	Moderate-severe	Melkoniemi et al[7]
c.3071delC		Frameshift	OSMED	AR	Moderate	Tokgoz-Yilmaz et al[32]
c.3648+5G>A		Frameshift	OSMED	AR	Severe	Melkoniemi et al[7]
c.3704delG		Frameshift	OSMED	AR	Moderate-severe	Temtamy et al[33]
c.3733C>T	p.R1245X	Nonsence	OSMED	AR	Severe	Melkoniemi et al[7]
c.4750G>T	p.G1584X(G1098X)	Nonsence	OSMED	AR	Moderate-severe	Melkoniemi et al[7]
c.4821_4843del		Frameshift	OSMED	AR	Moderate-profound	Melkoniemi et al[7]
c.IVS18+3insG		Frameshift	Fibrochondrogenesis	AR	NA	Tompson et al[27]
c.529C>T	p.R177X	Nonsence	Rovin (nonsyndromic cleft palate)	NA	Normal	Melkoniemi et al[25]

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; NA, not applicable; OSMED, otospondylomegaepiphyseal dysplasia.

hearing aid was properly transmitted without distortion because the hair cells were intact. We suggest that a hearing aid is a suitable amplification method to overcome "cochlear conductive hearing loss" and its use in hearing loss due to purely hair cell defects would be less effective. More precise study of audiological evaluation is necessary for a better understanding of the consequences of mutations in genes encoding proteins in each part of the cochlea and the efficacy of the therapies for hearing loss patients.

Acknowledgments

We thank all the families that participated in the present study. We would also like to thank Ms Matsuda and Ms Suzuki for their technical assistance, Mr Jim George for his help in preparing the manuscript, and Dr Fujita for providing clinical data.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by a Health and Labour Sciences Research Grant for Research on Rare and Intractable Diseases and Comprehensive Research on Disability Health and Welfare from the Ministry of Health, Labour and Welfare of Japan (S.U.) and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (S.U.).

References

1. Stickler GB, Belau PG, Farrell FJ, et al. Hereditary progressive arthro-ophthalmopathy. *Mayo Clin Proc*. 1965;40:433-455.
2. van Beelen E, Leijendeckers JM, Huygen PL, et al. Audiometric characteristics of two Dutch families with non-ocular Stickler syndrome (*COL11A2*). *Hear Res*. 2012;291(1-2):15-23.
3. Vikkula M, Mariman EC, Lui VC, et al. Autosomal dominant and recessive osteochondrodysplasias associated with the *COL11A2* locus. *Cell*. 1995;80(3):431-437.
4. van Steensel MA, Buma P, de Waal Malefijt MC, van den Hoogen FH, Brunner HG. Oto-spondylo-megaepiphyseal dysplasia (OSMED): clinical description of three patients homozygous for a missense mutation in the *COL11A2* gene. *Am J Med Genet*. 1997;70(3):315-323.
5. Harel T, Rabinowitz R, Hendler N, et al. *COL11A2* mutation associated with autosomal recessive Weissenbacher-Zweymuller syndrome: molecular and clinical overlap with otospondylomegaepiphyseal dysplasia (OSMED). *Am J Med Genet A*. 2005;132A(1):33-35.
6. McGuirt WT, Prasad SD, Griffith AJ, et al. Mutations in *COL11A2* cause non-syndromic hearing loss (DFNA13). *Nat Genet*. 1999;23(4):413-419.
7. Melkoniemi M, Brunner HG, Manouvrier S, et al. Autosomal recessive disorder otospondylomegaepiphyseal dysplasia is associated with loss-of-function mutations in the *COL11A2* gene. *Am J Hum Genet*. 2000;66(2):368-377.

8. Chen W, Kahrizi K, Meyer NC, et al. Mutation of *COL11A2* causes autosomal recessive non-syndromic hearing loss at the DFNB53 locus. *J Med Genet*. 2005;42(10):e61.
9. Miyagawa M, Nishio SY, Ikeda T, Fukushima K, Usami S. Massively parallel DNA sequencing successfully identifies new causative mutations in deafness genes in patients with cochlear implantation and EAS. *PLoS One*. 2013;8(10): e75793.
10. Chang X, Wang K. wANNOVAR: annotating genetic variants for personal genomes via the web. *J Med Genet*. 2012;49(7):433-436.
11. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res*. 2010;38(16):e164.
12. Abecasis GR, Auton A, Brooks LD, *et al*. An integrated map of genetic variation from 1,092 human genomes. *Nature*. 2012;491(7422):56-65.
13. NHLBI Exome Sequencing Project (ESP) Exome Variant Server. http://evs.gs.washington.edu/EVS/. Accessed February 10, 2015.
14. Narahara M, Higasa K, Nakamura S, et al. Large-scale East-Asian eQTL mapping reveals novel candidate genes for LD mapping and the genomic landscape of transcriptional effects of sequence variants. *PLoS One*. 2014;9(6):e100924.
15. Pollard KS, Hubisz MJ, Rosenbloom KR, et al. Detection of nonneutral substitution rates on mammalian phylogenies. *Genome Res*. 2010;20(1):110-121.
16. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc*. 2009;4(7):1073-1081.
17. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. *Nat Methods*. 2010;7(4):248-249.
18. Chun S, Fay JC. Identification of deleterious mutations within three human genomes. *Genome Res* 2009;19(9):1553-1561.
19. Schwarz JM, Rodelsperger C, Schuelke M, et al. MutationTaster evaluates disease-causing potential of sequence alterations. *Nat Methods*. 2010;7(8):575-576.
20. Cooper GM, Stone EA, Asimenos G, et al. Distribution and intensity of constraint in mammalian genomic sequence. *Genome Res*. 2005;15(7):901-913.
21. Jain U, Thakur G, Kallury A. Binder's syndrome. *BMJ Case Rep*. 2011;2011:bcr0820114665.
22. Acke FR, Dhooge IJ, Malfait F, De Leenheer EM. Hearing impairment in Stickler syndrome: a systematic review. *Orphanet J Rare Dis*. 2012;7:84.
23. De Leenheer EM, Bosman AJ, Kunst HP, Huygen PL, Cremers CW. Audiological characteristics of some affected members of a Dutch DFNA13/*COL11A2* family. *Ann Otol Rhinol Laryngol*. 2004;113(11):922-929.
24. Plantinga RF, Cremers CW, Huygen PL, Kunst HP, Bosman AJ. Audiological evaluation of affected members from a Dutch DFNA8/12 (*TECTA*) family. *J Assoc Res Otolaryngol*. 2007;8(1):1-7.
25. Melkoniemi M, Koillinen H, Mannikko M, et al. Collagen XI sequence variations in nonsyndromic cleft palate, Robin sequence and micrognathia. *Eur J Hum Genet*. 2003;11(3):265-270.
26. Jakkula E, Melkoniemi M, Kiviranta I, et al. The role of sequence variations within the genes encoding collagen II, IX and XI in non-syndromic, early-onset osteoarthritis. *Osteoarthritis Cartilage*. 2005;13(6):497-507.
27. Tompson SW, Faqeih EA, Ala-Kokko L, et al. Dominant and recessive forms of fibrochondrogenesis resulting from mutations at a second locus, *COL11A2*. *Am J Med Genet A*. 2012;158A(2):309-314.
28. Sirko-Osadsa DA, Murray MA, Scott JA, et al. Stickler syndrome without eye involvement is caused by mutations in *COL11A2*, the gene encoding the alpha2(XI) chain of type XI collagen. *J Pediatr*. 1998;132(2):368-371.
29. Vuoristo MM, Pappas JG, Jansen V, et al. A stop codon mutation in *COL11A2* induces exon skipping and leads to non-ocular Stickler syndrome. *Am J Med Genet A*. 2004;130A(2):160-164.
30. Pihlajamaa T, Prockop DJ, Faber J, et al. Heterozygous glycine substitution in the *COL11A2* gene in the original patient with the Weissenbacher-Zweymuller syndrome demonstrates its identity with heterozygous OSMED (nonocular Stickler syndrome). *Am J Med Genet*. 1998;80(2):115-120.
31. Kayserili H, Wollnik B, Guven G, et al. A novel homozygous *COL11A2* deletion causes a C-terminal protein truncation with incomplete mRNA decay in a Turkish patient. *Am J Med Genet A*. 2011;155A(1):180-185.
32. Tokgoz-Yilmaz S, Sahli S, Fitoz S, et al. Audiological findings in otospondylomegaepiphyseal dysplasia (OSMED) associated with a novel mutation in *COL11A2*. *Int J Pediatr Otorhinolaryngol*. 2011;75(3):433-437.
33. Temtamy SA, Mannikko M, Abdel-Salam GM, et al. Otospondylo-megaepiphyseal dysplasia (OSMED): Clinical and radiological findings in sibs homozygous for premature stop codon mutation in the *COL11A2* gene. *Am J Med Genet A*. 2006;140(11):1189-1195.

資 料 番 号（1）

平成 26 年度 厚生労働科学研究費補助金難治性疾患等政策研究事業
（難治性疾患政策研究事業）

先天性難聴の遺伝子診断
診療指針

※ 日本聴覚医学会・承認

※ 日本耳鼻咽喉科学会・推薦（予定）