Table 2. NOG mutations reported in SYM1, SYNS1, TCC, BDB2 and TCS families

Nucleotide change	Amino acid	Family information	Phenotype	Evolutionary conservation +	Domain/structure/motif ++	Authors
c. 58delC	Frameshift	Japanese, AD	SYNS1	—	—	Takahashi (8)
c. 103C>G	p. P35A	German, AD	BDB2	Conserved	Finger/clip region Interface of NOG and BMP7	Lehmann (4)
c. 103C>T	p. P35S	Turkish, AD	BDB2	Conserved	Finger/clip region Interface of NOG and BMP7	Lehmann (4)
c. 103C>T	p. P35S	Israeli, AD	SABTT	Conserved	Finger/clip region Interface of NOG and BMP7	Hirshoren (9)
c. 103C>T	p. P35S	Italian, AD	SYM1	Conserved	Finger/clip region Interface of NOG and BMP7	Mangino (10)
c. 104C>G	p. P35R	NI, sporadic	SYM1	Conserved	Finger/clip region Interface of NOG and BMP7	Gong (1)
c. 104C>G	p. P35R	NI, AD	TCC	Conserved	Finger/clip region Interface of NOG and BMP7	Dixon (2)
c. 106G>C	p. A36P	Danish, AD	BDB2	Almost conserved[a]	Finger/clip region Interface of NOG and BMP7	Lehmann (4)
c. 110C>G	p. P37R	Belgian, AD	TCC	Conserved	Finger/clip region Interface of NOG and BMP7	Debeer (11)
c. 124C>G	p. P42A	Belgian, *de novo*	TCC	Conserved	Finger/clip region Interface of NOG and BMP7	Debeer (12)
c. 125C>G	p. P42R	NI, AD	SYNS1	Conserved	Finger/clip region Interface of NOG and BMP7	Oxley (13)
c. 129-130dup	Frameshift	Dutch, AD	SABTT	—	—	Weekamp (14)
c. 142G>A	p. E48K	Japanese, sporadic	SYM1	Conserved	Finger/clip region Interface of NOG and BMP7	Kosaki (15)
c. 142G>A	p. E48K	Iranian, AD	BDB2	Conserved	Finger/clip region Interface of NOG and BMP7	Lehmann (4)
c. 149C>G	p. P50R	Belgian, *de novo*	TCC	Conserved	Finger/clip region Interface of NOG and BMP7	Debeer (12)
c. 252-253 insC	Frameshift	NI, AD	SABTT	—	—	Brown (3)
c. 304delG	Frameshift	Dutch	SYM1	—	—	Thomeer (16)
c. 328C>T	p. Q110X	Italian, AD	SABTT	Conserved	—	Brown (3)
c. 386T>A	p. L129X	Japanese, AD	SYM1	Almost conserved[b]	—	Takahashi (8)
c. C391C>T	p. Q131X	Dutch	SYM1	Almost conserved[b]	—	Thomeer (16)
c. 463T>A	p. C155S	Japanese, AD	SYM1	Conserved	Conserved cysteine of cysteine knot I	Present study
c. 499C>G	p. R167G	North American, sporadic	BDB2	Conserved	—	Lehmann (4)
c. 551G>A	p. C184Y	Japanese, sporadic	SYM1	Conserved	Conserved cysteine of cysteine knot III	Takahashi (8)
c. 551G>T	p. C184F	Japanese, sporadic	SYM1	Conserved	Conserved cysteine of cysteine knot III	Present study
c. 559C>T	p. P187S	British, AD	BDB2	Conserved	—	Lehmann (4)
c. 561del	Frameshift	Dutch, AD	SABTT	—	—	Weekamp (14)
c. 565G>T	p. G189C	Dutch, AD	SYM1	Conserved	—	Gong (1)
c. 568A>G	p. M190V	NI, AD	SYNS1	Conserved	—	Oxley (13)
c. 608T>C	p. L203P	Dutch, AD	SABTT	Conserved	β-Sheet 3 of NOG structure	Weekamp (14)
c. 611G>T	p. R204L	NI, AD	TCC	Conserved	β-Sheet 3 of NOG structure	Dixon (2)
c. 614G>A	p. W205X	sporadic	SYNS1	Conserved	—	Dawson (17)
c. 615G>C	p. W205C	Belgian, AD	SYNS1	Conserved	β-Sheet 4 of NOG structure	Declau (18)
c. 615G>C	p. W205C	American, sporadic	SABTT	Conserved	β-Sheet 4 of NOG structure	Emery (19)
c. 645C>A	p. C215X	Japanese, AD	SABTT	Conserved	Disulphide bounds in cysteine knot motif to stabilize finger 2 structure	Present study
c. 649T>G	p. W217G	Hawaiian, AD	SYNS1	Conserved	β sheet 4 of NOG structure	Gong (1)
c. 659-660TC>AT	p. I220N	Belgian, AD	SYM1	Almost conserved[a]	Interaction region to BMP-type binding epitope	Gong (1)
c. 659T>A	p. I220N	NI, AD	SYM1	Almost conserved[a]	Interaction region to BMP-type binding epitope	Gong (1)
c. 664T>G	p. Y222D	Belgian, AD	SYM1	Conserved	Interaction region to BMP-type binding epitope	Gong (1)
c. 665A>G	p. Y222C	American, AD	SYM1	Conserved	Interaction region to BMP-type binding epitope	Gong (1)
c. 665A>G	p. Y222C	NI, AD	TCC	Conserved	Interaction region to BMP-type binding epitope	Dixon (2)
c. 668C>T	p. P223L	NI, AD	SYM1	Conserved	Interaction region to BMP-type binding epitope	Gong (1)
c. 696C>G	p. C232W	Germany, AD	SYM1	Conserved	Intermolecular disulphide bounds to stabilize NOG dimmer structure	Rudnik-Schöneborn (20)
17q22 long deletion		Japanese, sporadic	SYNS1	—	—	Shimizu (21)

+, evolutionary conservation was evaluated by the NCBI data base; ++, the domain/structure/motif are based on a hypothesized protein structure; AD, autosomal dominant; BDB2, brachydactyly type B2; BMP, bone morphogenetic protein; FOP, fibrodysplasia ossificans progressiva; NI, no information; NOG, noggin; SABTT, stapes ankylosis with broad thumbs and toes; SYNS1, multiple synostosis syndrome; SYM1, proximal symphalangism; TCC, trasal–carpal coalition syndrome.

[a]Residue is conserved across mammalians except for zebrafish.

[b]Residue is conserved across mammalians except for zebrafish and chicken.

broad thumb and toes/Teunissen and Cremer syndrome. One mutation was a nonsense mutation (C215X), leading to a truncated protein, and was likely to be a pathologic mutation. The other two mutations are also likely to be pathologic rather than functionally neutral polymorphic changes because: (i) none were found in any of the controls, (ii) the alignment of NOG sequences from human, mouse, chicken, *Xenopus laevis* and zebrafish showed that C155 and C184 are well-conserved amino acids in all species (data not shown), and (iii) all affected subjects showed similar phenotypes.

To date, 36 *NOG* mutations have been reported in SYM1, SYNS1, TCC, BDB2 and TCS families (Table 2). Although the *NOG* mutations have been reported mainly in dominant families (Table 2), *de novo NOG* mutations have also been reported in sporadic SYM1 (8) and sporadic SYNS1 (1). Therefore, genetic investigation may be needed for determining pathogenesis of congenital stapes ankylosis with stiffness of the PIP joints, even in sporadic cases. A milder phenotype (3) as well as the present case with minor joint anomalies in family 2 indicated that it may be clinically important to check such skeletal abnormalities when diagnosing and treating patients with stapes ankylosis, because it may be difficult to differentiate congenital stapes ankylosis from otosclerosis when conductive hearing loss is delayed to adulthood.

Whether *NOG* mutations can be found more frequently in sporadic conductive hearing loss patients is an interesting question. In this study, mutations were not found in any otosclerosis patients who did not have any associated abnormality. Therefore, typical otosclerosis is not a continuum of the category of diseases associated with *NOG* mutations. These results, together with the previous literature, indicate that the *NOG* mutations are restrictively found within patients with various skeletal abnormalities regardless of severity. It is noted that the reported *NOG* mutation in mild cases (patients with stapes ankylosis without symphalangism) have minor skeletal abnormalities such as broad thumbs and great toes (3), but these cases had symphalangism in the little fingers only.

A review of the reported 41 mutations showed that, interestingly, the majority of *NOG* mutations are located in the evolutionally well conserved and therefore functionally critical region (Table 2), suggesting that this region might be functionally relevant in NOG polypeptides. This study added three novel *NOG* mutations in conserved cysteine residue within the cystine knot motif and confirmed that *NOG* is a causative gene for this category of disease. In addition, there was no particular racial-specific founder mutation within this gene (Table 2). With regard to a genotype–phenotype correlation, phenotypes seem to be independent of the location of the mutation and type of mutations (Table 2). Other genetic factors and/or interacted proteins may also be involved in determining clinical phenotypes.

With regard to surgical outcome, stapes surgery for conductive hearing loss due to *NOG* mutations may be a good therapeutic option for most cases. In fact, two of these seven patients who underwent stapes surgery (#991 and #4106) had hearing deterioration 3–10 years after the initial surgery, in accordance with a previous report (3, 22), hypothesizing that excessive bony overgrowth and refixation of the ossicle chain may occur after initially successful surgery. The other cases in this study maintained good hearing even after long-term follow-up periods (more than 10 years). Therefore, surgical outcome should be carefully evaluated after long-term observation. Careful explanation of possible limitations of surgical treatment and alternative treatment options such as a bone-anchored hearing aid may be appropriate for such patients with this genetic background.

The identification of the causative genes responsible for various middle/inner ear diseases will enable us to classify new congenital deafness groups in the future, and lead to clinical application in the diagnosis of middle ear disorders and better counseling for the selection of ideal intervention.

Supporting Information

The following Supporting information is available for this article:

Fig. S1. (**a**) Pedigree of family 1. Filled symbol represents the affected individual. (**b**) Sequence analysis of noggin (NOG). Arrow indicates a G to T change at nucleotide 551 in patient #991. This substitution causes codon 184 to change from TGC (cysteine: C) to TTC (phenylalanine: F).

Fig. S2. (**a**) Pedigree of family 2. Filled symbols represent the affected individuals. (**b**) Sequence analysis of noggin (NOG). Arrow indicates a T to A change at nucleotide 463 in patients #3925 and #3926. This substitution causes codon 155 to change from TGC (cysteine: C) to AGC (serine: S).

Fig. S3. (**a**) Pedigree of family 3. Filled symbols represent affected symptoms (conductive hearing impairment, hyperopia, and finger malformation. (**b**) Sequence analysis of noggin (NOG). Arrow indicates a C to A change at nucleotide 645 in patients #4106 and #4351. These two patients had a heterozygous C215X mutation. This nonsense mutation (C215X) leads to a truncated protein.

Additional Supporting information may be found in the online version of this article.

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Simultaneous Screening of Multiple Mutations by Invader Assay Improves Molecular Diagnosis of Hereditary Hearing Loss: A Multicenter Study

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Abstract

Although etiological studies have shown genetic disorders to be a common cause of congenital/early-onset sensorineural hearing loss, there have been no detailed multicenter studies based on genetic testing. In the present report, 264 Japanese patients with bilateral sensorineural hearing loss from 33 ENT departments nationwide participated. For these patients, we first applied the Invader assay for screening 47 known mutations of 13 known deafness genes, followed by direct sequencing as necessary. A total of 78 (29.5%) subjects had at least one deafness gene mutation. Mutations were more frequently found in the patients with congenital or early-onset hearing loss, i.e., in those with an awareness age of 0–6 years, mutations were significantly higher (41.8%) than in patients with an older age of awareness (16.0%). Among the 13 genes, mutations in *GJB2* and *SLC26A4* were mainly found in congenital or early-onset patients, in contrast with mitochondrial mutations (12S rRNA m.1555A>G, tRNA(Leu(UUR)) m.3243A>G), which were predominantly found in older-onset patients. The present method of simultaneous screening of multiple deafness mutations by Invader assay followed by direct sequencing will enable us to detect deafness mutations in an efficient and practical manner for clinical use.

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Competing Interests: The authors have read the journal's policy and have the following conflicts. The authors did not receive funding from the Department of Clinical Genomics, Biomedical Laboratories, Inc. They felt that for genetic analysis of patients with hearing impairment in which many gene/gene mutations are involved, Invader Assay is the appropriate choice. However, for patent reasons, the authors cannot develop this method independently. The development of this method was therefore performed in collaboration with Biomedical Laboratories. This relationship had no influence on results and the direct sequencing results were all double checked for accuracy. Although Invader Assay is more efficient, if a method other than Invader Assay had been used, the results would have been identical.

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Introduction

From a series of etiological studies, 60–70% of childhood hearing loss has been estimated to be of genetic etiology, with the rest due to environmental causes, including newborn delivery trouble, acoustic trauma, ototoxic drug use, and prenatal/postnatal infection [1]. However, until now, there has been no multicenter study based on genetic testing. Along with early discovery of hearing loss by newborn hearing screening programs and subsequent intervention programs, much attention has been paid to the determination of the hearing loss etiology. Therefore, genetic testing has become more important for highly accurate diagnosis, prediction of severity of hearing loss, estimation of associated abnormalities, selection of appropriate habilitation options, prevention of hearing loss, and better genetic counseling. Although more than one hundred loci have been mapped and 46 genes reported to be responsible for hereditary hearing loss (Hereditary Hearing Homepage; http://webh01.ua.ac.be/hhh/), many may cause similar phenotypes without any abnormality other than hearing loss. This genetic heterogeneity has made clinical application difficult, in spite of the considerable advances in discovery of deafness genes. We have previously established a screening strategy focusing on recurrent mutations and demonstrated its benefits for clinical application [2]. We carried out the current multicenter study to determine 1) whether the simultaneous screening of the multiple deafness mutations by Invader assay is applicable for clinical use, 2) whether the genetic etiology is truly prevalent among hearing loss patients and 3) whether genetic causes differ by ages.

Materials and Methods

Subjects and clinical status

As summarized in Table 1, two hundred sixty-four Japanese patients with bilateral sensorineural hearing loss from 33 ENT departments nationwide participated in the present study. We first applied the Invader assay for screening forty-seven known mutations of 13 known deafness genes, followed by direct sequencing as necessary.

Table 1. Clinical features of subjects in this study.

	Total (n = 264)	*Early onset (n = 141)*	*Late onset (n = 100)*
Severity of HL			
normal – moderate	148	58	78
severe – profound	95	70	21
unknown	21	13	1
Inheritance			
AD or Mitochondrial	38	9	24
AR or Sporadic	119	69	42
unknown	107	63	34
Other clinical features			
inner ear malformations	52	37	10
EVA	30	22	4
goiter	8	4	3
diabetes mellitus	14	3	11

HL: Hearing loss.
AD: Autosomal dominant.
AR: Autosomal recessive.
EVA: Enlarged vestibular aqueduct.
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Hearing loss was evaluated using pure-tone audiometry (PTA) classified by a pure-tone average over 500, 1000, 2000 and 4000 Hz in the better hearing ears. For children who were unable to be tested by PTA, we used an average over 500, 1000, 2000 Hz in either auditory steady-stem response (ASSR) or conditioned oriented reflex audiometry (COR), or the response threshold (dB) from auditory brainstem response (ABR). Computed tomography (CT) scans were performed to check for congenital inner ear anomalies.

Status of hearing loss in the 264 patients was: mild (21–40 dB) in 39 patients (14.7%), moderate (41–70 dB) in 84 (31.8%), severe (71–94 dB) in 39 (14.8%) and profound (>95 dB) in 56 patients (21.2%). Twenty-four subjects were classified as having normal hearing due to a specific audiogram with hearing loss only in the high or low frequency portions. With regard to onset age (the age of awareness), 141 patients had early onset deafness (below 6 y.o.), 100 had late onset deafness, and the rest had unknown onset ages.

The inheritance composition of the subjects was as follows: 38 subjects from autosomal dominant or mitochondrial inherited families (two or more generations affected); 119 subjects from autosomal recessive families (parents with normal hearing and two or more affected siblings) or subjects with sporadic deafness (also compatible with recessive inheritance or non-genetic hearing loss). None of the patients had an X-linked pattern of inheritance. The numbers of patients with other manifestations were inner ear malformations (52), enlarged vestibular aqueduct (EVA) (30), goiter (8), and diabetes mellitus (14). None of the patients had typical clinical features of Usher syndrome or BOR syndrome.

All subjects gave prior informed consent for participation in the project and the Ethical Committee of Shinshu University as well as the relevant bodies of the participating institutions of the Deafness Gene Study Consortium approved the study.

Invader assay

Invader technology is convenient for mutation genotyping, offering a simple diagnostic platform to detect single nucleotide changes with high specificity and sensitivity from unamplified genomic DNA.

We applied the Invader assay for screening forty-seven known mutations of 13 known deafness genes [*GJB2*(NM_004004.5), *SLC26A4*(NM_000441.1), *COCH*(NM_001135058.1), *KCNQ4*(NM_172163.2), *MYO7A*(NM_000260.3), *TECTA*(NM_005422.2), *CRYM* (NM_001888.3), *POU3F4*(NM_000307.3), *EYA1*(NM_172060.2), mitochondrial 12 s ribosomal RNA, mitochondrial tRNA(Leu), mitochondrial tRNA(Ser), and mitochondrial tRNA(Lys)] (Table 2). Mutations were selected on the basis of a mutation/gene database established in the Japanese deafness population. The detailed methodological protocol was described elsewhere [2]. In brief, 1.2 ul of primary probe/Invader oligonucleotides mixture (containing 0.5 umol/l wild type primary probes, 0.5 umol/l mutant primary probe, 0.05 umol/l Invader oligonucleotide, and 10 mmol/l MOPS) were poured into each well of 384-well plates. Fluorescent resonance energy transfer (FRET)/Cleavase mixture (Third Wave Technologies, Madison, WI) was added to the probe/Invader oligonucleotide-containing plates. Then, 3 ul of 5–100 fmol/l synthetic target oligonucleotides (positive control), 10 ug/ml yeast tRNA (no target control), and denatured genomic DNA samples (>15 ng/ul) were added. Next, 6 ul of mineral oil (Sigma, St. Louis, MO) were overlayed into all reaction wells and incubated at 63°C for 4 hour. After incubation fluorescence was measured by a Cyto Fluor 4000 fluorescent micro plate reader (Applied Biosystems, Foster CA). The heteroplasmy rate for mitochondrial mutations was quantified by detection of fluorescently labeled and digested PCR products through a fluorescence imaging system [2].

Direct sequencing

Dominant mutations and mitochondrial mutations are themselves diagnostic criteria for molecular diagnosis. But a hallmark of recessive mutations, in *GJB2* and *SLC26A4* for example, is the detection of two mutations in the paternal and maternal alleles. In this study, direct sequencing was further carried out as follows: 1) *GJB2* mutation analysis for all subjects, because the authors wanted to clarify whether the number of mutations on the invader panel are enough (saturated) or not. 2) *SLC26A4* mutation analysis for all the subjects with EVA, 3) *SLC26A4* mutation analysis for heterozygous patients for these genes. DNA fragments containing the entire coding region were sequenced as described elsewhere [3,4].

Results

The mutations found by Invader assay and direct sequencing in this study are summarized in Table 2 and 3.

Invader Assay

A total of 74 (28.0%) hearing-impaired subjects (n = 264) were found to have at least one deafness gene mutation. Among the deafness genes situated on the present diagnostic panel, mutations were most frequently found in the *GJB2* gene. Screening of *GJB2* showed mutations of one or both alleles of the gene in 43 (43/264; 16.2%) samples from the subjects, of which 13 cases had only a single mutation, and 30 cases were compound heterozygotes or homozygotes, confirmed by segregation analysis (Table 4). The most common mutation was c.235delC, accounting for nearly 67% (29/43) of all *GJB2* mutated patients. On the other hand, the *GJB2*: c.35delG mutation, which is known to be the most common mutation in Caucasian or other ethnic populations, was not found in this group. The second most common group of *GJB2* mutations consisted of p.[G45E; Y136X], p.V37I, and c.299_300del. These mutations were detected in more than 5 patients each, and their allele frequencies were relatively high. Three mutations (p.T86R, p.R143W, and c.176_191del) were observed in more than one

Table 2. Mutation list of Invader based genetic screening test.

Gene	*Exon*	*Codon location*	*Nucleotide change*	*Frequency of mutant alleles (n = 528)*	*Number of patients with mutations (n = 264)*
GJB2	exon 2	p.L79fs	c.235delC	43 (8.1%)	29 (10.9%)
GJB2	exon 2	p.V37I	c.109G>A	7 (1.3%)	6 (2.3%)
GJB2	exon 2	p.[G45E; Y136X]	c.[134G>A; 408C>A]	10 (1.9%)	10 (3.8%)
GJB2	exon 2	p.G59fs	c.176_191del	3 (0.6%)	3 (1.1%)
GJB2	exon 2	p.R143W	c.427C>T	4 (0.8%)	4 (1.5%)
GJB2	exon 2	p.H100fs	c.299_300del	5 (0.9%)	5 (1.9%)
GJB2	exon 2	p.T123N	c.368C>A	4 (0.8%)	4 (1.5%)
GJB2	exon 2	p.T86R	c.257C>G	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.F191L	c.570T>C	0	0
GJB2	exon 2	p.I71T	c.212T>C	0	0
GJB2	exon 2	p.A49V	c.146C>T	0	0
GJB2	exon 2	p.G12fs	c.35delG	0	0
SLC26A4	exon 19	p.H723R	c.2168A>G	22 (4.1%)	17 (6.4%)
SLC26A4	int 7/exon 8	splice site	c.919-2A>G	2 (0.4%)	2 (0.8%)
SLC26A4	exon 10	p.T410M	c.1229C>T	4 (0.8%)	3 (1.1%)
SLC26A4	exon 7	p.V306fs	c.917insG	0	0
SLC26A4	exon 19	p.T721M	c.2162C>T	0	0
SLC26A4	exon 8/int 8	splice site	c.1001+1G>A	0	0
SLC26A4	exon 9	p.A372V	c.1115C>T	0	0
SLC26A4	exon 5	p.M147V	c.439A>G	1 (0.2%)	1 (0.4%)
SLC26A4	int 5/exon 6	splice site	c.601-1G>A	0	0
SLC26A4	exon 9	p.K369E	c.1105A>G	1 (0.2%)	1 (0.4%)
SLC26A4	exon 15	p.S551fs	c.1652insT	1 (0.2%)	1 (0.4%)
SLC26A4	exon 15	p.C565Y	c.1693G>A	0	0
SLC26A4	exon 17	p.S666F	c.1997C>T	0	0
SLC26A4	exon 19	p.E704fs	2111ins GCTGG	1 (0.2%)	1 (0.4%)
SLC26A4	exon 4	p.L108fs	c.322delC	0	0
SLC26A4	exon 4	p.P123S	c.367C>T	0	0
SLC26A4	exon 10	p.N392Y	c.1174A>T	0	0
SLC26A4	exon 17	p.S610X	c.1829C>A	0	0
SLC26A4	exon 17	p.S657N	c.1970G>A	0	0
EYA1	exon 12	p.D396G	c.1187A>G	0	0
EYA1	exon 8	p.R264X	c.790C>T	0	0
EYA1	exon 7	p.Y193X	c.579C>G	0	0
COCH	exon 5	p.A119T	c.441G>A	0	0
KCNQ4	exon 5	p.W276S	c.827G>C	0	0
MYO7A	exon22	p.A886fs	c.2656_2664del	0	0
TECTA	exon 16	p.R1773X	c.5318C>T	0	0
TECTA	exon 20	p.R2121H	c.6063G>A	0	0
Mitochondrial 12S rRNA			m.1555A>G	-	5 (1.9%)
Mitochondrial tRNALeu			m.3243A>G	-	6 (2.3%)
Mitochondrial tRNASer			m.7445A>G	-	0
Mitochondrial tRNALys			m.8296 A>G	-	0
CRYM	exon 8	p.K314T	c.941 A>C	0	0
CRYM	exon 8	p.X315Y	c.945 A>T	0	0

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Table 3. Mutation list found by direct sequencing analysis.

Gene	*Exon*	*Codon location*	*Nucleotide change*	*Frequency of mutant alleles (n = 528)*	*Number of patients with mutations (n = 264)*
GJB2	exon 2	p.T8M	c.23C>G	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.K12fs	c.35insG	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.F106Y	c.317T>A	1 (0.2%)	1 (0.4%)
GJB2	exon 2	p.A171fs	c.511insAACG	2 (0.4%)	2 (0.8%)
GJB2	exon 2	p.C174S	c.522G>C	1 (0.2%)	1 (0.4%)
SLC26A4	exon 14	p.S532I	c.1595G>T	2 (0.4%)	2 (0.8%)
SLC26A4	exon 16	p.R581S	c.1743G>C	1 (0.2%)	1 (0.4%)
SLC26A4	exon 17	p.V659L	c.1975G>C	2 (0.4%)	2 (0.8%)
SLC26A4	exon 10	p.L407fs	c.1219delCT	1 (0.2%)	1 (0.4%)
SLC26A4	exon 15/int 15	splice site	c.1931+5 G>A	5 (0.9%)	4 (1.5%)

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patient. p.F191L, p.I71T, p.A49V and c.35delG mutations were not found. One pair of p.[G45E; Y136X] mutations was detected among 10 persons in a heterozygous state. Subsequent parental DNA segregation study through direct sequencing indicated two mutations were in *cis*. The p.T123N mutation was found in 4 subjects but, based on our recent study, is not likely to be a pathologic mutation [5].

The second most frequent gene with mutations was the *SLC26A4* gene (23/264; 8.7%). Five cases were homozygotes of p.H723R, one was a homozygote of p.T410M, 3 were compound heterozygotes, and 14 had only one mutation of *SLC26A4* (Table 4). Of the 19 *SLC26A4* mutations, 12 (c.917insG, p.T721M, c.1001+1G>A, p.A372V, c.601-1G>A, p.C565Y, p.S666F, c.322delC, p.P123S, p.N392Y, p.S610X, and p.S657N) were not found in any samples, but the remaining 7 *SLC26A4* mutations were confirmed in more than one subject. Especially, the p.H723R mutation was found to be in high allele frequency (4.1%). All of the patients with *SLC26A4* mutations had EVA, which has been demonstrated to be a result of the mutations of this gene. *SLC26A4* mutations were detected by Invader assay in 63.6% of the patients with EVA.

Mitochondrial m.1555A>G mutations were found in 1.9% (5/264) of the patients and the m.3243A>G mutation was identified in 2.3% (6/264).

Mutations in nine deafness genes (*COCH*, *KCNQ4*, *MYO7A*, *TECTA*, *CRYM*, *POU3F4*, *EYA1*, mitochondrial tRNA(Lys) m.8296A>G, mitochondrial tRNA(Ser) m.7445A>G) were not identified in any patients (Table 2).

Notably, 4 subjects were found to have double gene mutations. Two cases were *SLC26A4* compound heterozygous or homozygous mutations with a *GJB2* heterozygous mutation. One case was a compound heterozygous of *GJB2* with a *SLC26A4* heterozygous mutation and the remaining case was a *GJB2*

Table 4. Diagnostic efficiency of Invader assay alone and Invader assay and direct sequencing.

	Total (n = 264)	*Early onset (n = 141)*	*Late onset (n = 100)*
Invader assay alone			
GJB2 homozygote/compound heterozygote	30 (11.4%)	29 (20.6%)	1 (1.0%)
GJB2 heterozygote	13 (4.9%)	7 (5.0%)	6 (6.0%)
SLC26A4 homozygote/compound heterozygote	9 (3.4%)	9 (6.4%)	0 (0%)
SLC26A4 heterozygote	14 (5.3%)	10 (27.1%)	2 (2.0%)
Mitochondria A1555G	5 (1.9%)	2 (1.4%)	2 (2.0%)
Mitochondria A3243G	6 (2.2%)	1 (0.7%)	5 (5.0%)
Total	74 (28.0%)*	55 (39.0%)*	16 (16.0%)
Invader assay and direct sequencing			
GJB2 homozygote/compound heterozygote	33 (12.5%)	31 (21.9%)	2 (2.0%)
GJB2 heterozygote	13 (4.9%)	7 (5.0%)	5 (5.0%)
SLC26A4 homozygote/compound heterozygote	18 (6.8%)	18 (12.7%)	0 (0%)
SLC26A4 heterozygote	7 (2.7%)	4 (2.8%)	2 (2.0%)
Mitochondria A1555G	5 (1.9%)	2 (1.4%)	2 (2.0%)
Mitochondria A3243G	6 (2.2%)	1 (0.7%)	5 (5.0%)
Total	78 (29.5%)**	59 (41.8%)**	16 (16.0%)

*Three cases carried double mutations (cases 1 to 3 in Table 5).
**Four cases carried double mutations shown in Table 5.
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Table 5. Double mutation cases found in simultaneous mutation screening.

Genotype	*Patients Number*
GJB2:p.[V37I];[V37I]; Mitochondria m.1555A>G	1 (0.4%)
GJB2:c.[235delC];p.[R143W]; *SLC26A4*:p.[M147V]	1 (0.4%)
GJB2:p.[V37I]; *SLC26A4*:p.[H723R];[H723R]	1 (0.4%)
GJB2:p.[F106Y]; *SLC26A4*:p.[H723R]; c.[1931+5G>A]	1 (0.4%)
Total	4 (1.5%)

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homozygous mutation with a mitochondrial 1555A>G mutation (Table 5).

The detection rate of mutations was 40.4% for the patients with congenital or early-onset hearing loss, i.e. in those with an awareness age of 0~6 years. The rate in congenital hearing loss patients also increased when restricting to the patients with moderate or more severe hearing loss (>50 dB; 40.7%) or severe hearing loss (>70 dB; 44.3%) (Fig. 1). In contrast, the detection rate was only 16.0% in the patients with an older age of onset/awareness (Fig. 1). Among the 13 included genes, mutations is *GJB2* and *SLC26A4* were mainly found in congenital patients or early-onset patients, in contrast with mitochondrial mutations, such as 12S rRNA m.1555A>G or tRNA(Leu(UUR)) m.3243A>G, which were predominantly found in older-onset patients (Table 4). The p.V37I mutation in the *GJB2* gene was also found in older-onset patients (data not shown).

With regard to the relationship between radiographic findings and genetic testing, the mutation detection rate was elevated when restricting to the patients with inner ear anomaly (50.0%) and EVA (63.6%) (Fig. 2).

Direct sequencing

Direct sequencing identified 9 mutations in 15 cases which were not included in the Invader assay panel and improved the mutation detection/diagnostic rate obtained by Invader assay analysis (28.0%/18.6%) to 29.5%/22.7%. (Fig. 1). Combining direct sequencing with invader screening enhanced the diagnostic rate notably but not the mutation detection rate. In detail, direct sequencing identified additional mutations in three cases with single *GJB2* mutations by Invader assay that were finally diagnosed as compound heterozygous mutations of *GJB2* (p.[T86R]; c.[511insAACG], p[T8M];[V37I] and c.[35insG];[235delC]).

In 7 cases only a single *SLC26A4* mutation was found by invader assay, and additional mutations were found by direct sequencing (two cases of p.[H723R];c[1931+5G>A] and one each cases of p.[R581S];[H723R], p.[V659L];[H723R], p.[S532I]; c.[2111insGCTGG], p.[T410M]; c.[1931+5G>A] and p.[K396E];[S532I]). Two cases carried EVA but without any mutations found in Invader assay, c[1931+5G>A]; [1931+5G>A] and p.[V659L];c[1219 delCT] compound heterozygous mutations were found by direct sequencing. With the combination of Invader assay and direct sequencing, and restriction to patients with EVA, the mutation detection rate was elevated to 17/22 cases (77.3%, Fig. 2). Fifteen of them carried homozygous or compound heterozygous *SLC26A4* mutations.

Discussion

We previously reported that simultaneous detection of common deafness gene mutations has excellent sensitivity and accuracy [2]. In this study, using samples from patients at 33 institutions nationwide from northern to southern Japan, we confirmed that the Invader assay based on the Japanese deafness gene mutation database works efficiently in the clinical base to detect the responsible gene mutations from the patients with

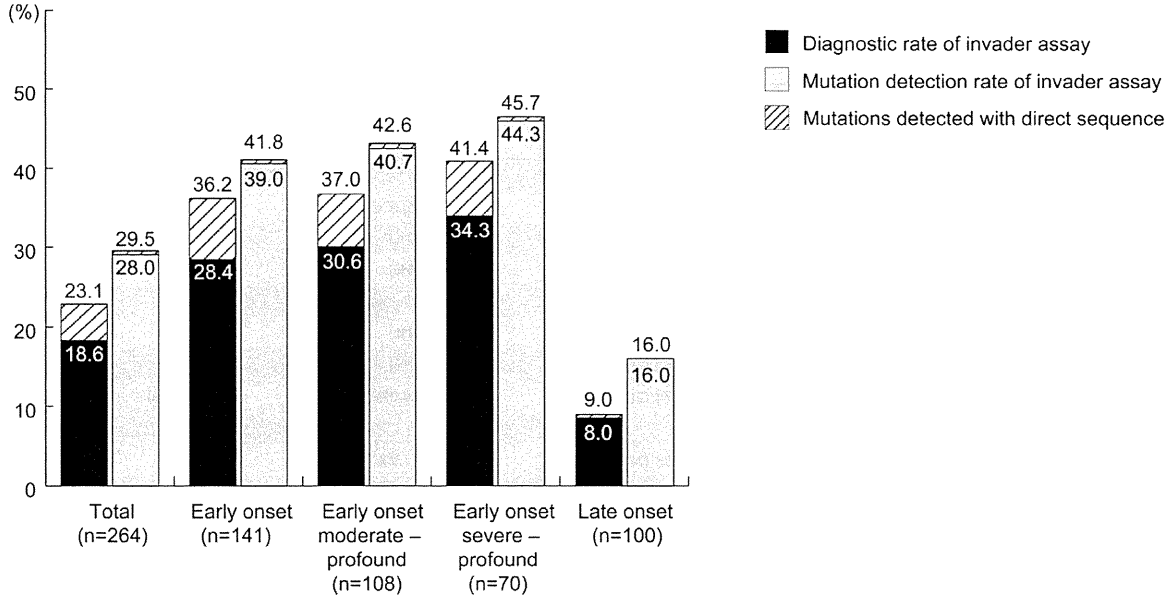

Figure 1. Detection rate by onset/awareness age and severity of hearing loss. Diagnostic rates and detection rates of this simultaneous multiple mutations screening and direct sequencing for biallelic mutations in autosominal recessive genes or mitochondrial mutations increased when restricted to congenital/early-onset hearing loss, and moderate or severe hearing loss. Combined direct sequence and invader screening enhanced the diagnostic rate but not the mutation detection rate.
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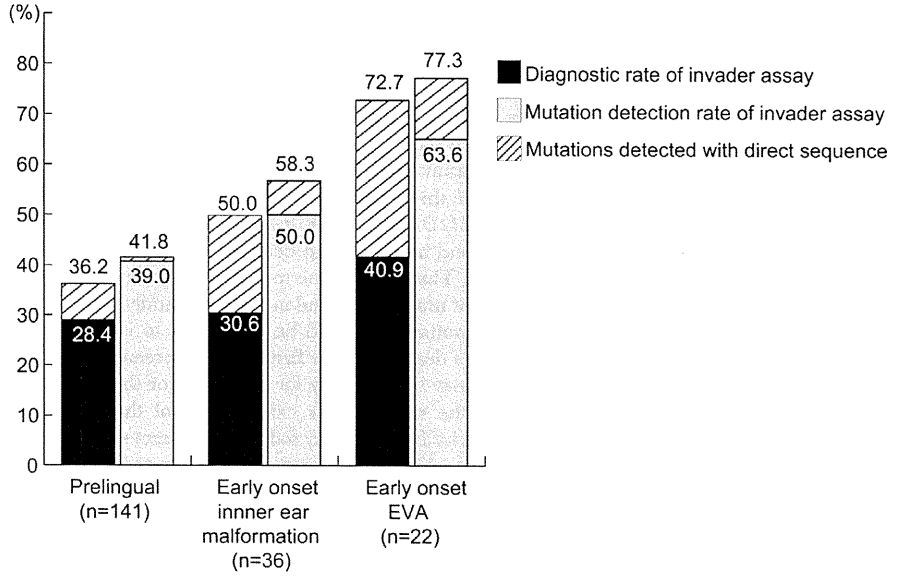

Figure 2. Radiographic findings and detection rate. Detection rate was elevated when subjects were restricted to those with inner ear anomaly or EVA. Combined direct sequence and invader screening enhanced the diagnostic rate but not the mutation detection rate.
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various onset/awareness ages. We detected mutations in 29.5% overall, and the 41.8% detection rate for congenital or early onset sensorineural hearing loss was especially remarkable. A series of epidemiological studies have demonstrated that genetic disorders are common causes of congenital deafness and it is estimated that 60–70% of the etiology may be caused by genetic factors [1]. Genetic testing is crucial to diagnose the etiology, but more than 100 genes are estimated to be involved and such genetic heterogeneiety has hampered the genetic testing for deafness as a routine clinical test. The present detection rate; i.e., 41.8%, is a strikingly good rate for a clinical application, and it is expected that clinical deafness mutation screening will greatly improve medical management and facilitate extensive genetic counseling for hearing impairment. Additional direct sequencing, as well as a new version of the screening panel which includes novel identified mutations, will likely improve the detection rate. For the older ages of onset, the detection rate was comparatively low (16.0%). Probably this is due to the panel mainly including responsible genes for congenital deafness but not the responsible genes for late onset hearing loss. An alternative explanation may be that environmental factors may be involved in this group of deafness patients.

The present study confirmed that mutations in three genes, *GJB2*, *SLC26A4*, and the mitochondrial 12 s rRNA, are so far the major known causes of hereditary hearing loss nationwide in Japanese [6], and thus much attention should be paid to these genes when performing genetic testing of hearing loss patients.

The most frequently found were mutations in the *GJB2* gene. This gene is so far the most common responsible gene for congenital deafness worldwide [7]. The detection rates (17.4% for all, 27.0% for congenital) are in accordance with our previous data of 15% in the overall deafness population and 25% in congenital deafness patients [5]. The mutation spectrum found in this study is also in accordance with our previous results [2,4,5]. In *GJB2* screening, 46 (17.4%) samples from deafness subjects had mutations of one or both alleles of the *GJB2* gene. As expected from the above reports, the c.235delC mutation was found to be the most prevalent mutation in our screening, accounting for 10.9% (29 of 264) of the hearing-impaired persons. Fourteen patients were c.235delC homozygotes and 11 were compound heterozygotes having c.235delC, confirmed by segregation analysis, and 4 patients were c.235delC heterozygotes without a second mutation. Direct sequencing identified novel mutations (p.T8M, c.35insG, p.F106Y, p.C174S and c.512insAACG) in the patients with a single mutation detected by Invader assay (Table 3).

Many benefits of *GJB2* gene genetic testing have been pointed out. There have been general rules that inactivating mutations (deletion mutations and stop mutations) show more severe phenotypes compared to those caused by non-inactivating mutations (missense mutations) [5,8,9]. As well as a highly accurate diagnosis, these genotype-phenotype correlation data could provide prognostic information to help decide the strategy of intervention with hearing, i.e., whether a child should receive cochlear implantation or hearing aids. For the patients with severe phenotypes who have *GJB2* mutations, genetic information would aid decision-making regarding cochlear implantation, because their hearing loss is of cochlear origin and they therefore are good candidates for implantation. In fact, cochlear implantation has resulted in remarkable improvement in auditory skills and development of speech production for patients with profound hearing loss associated with *GJB2* mutations [10].

In the *SLC26A4* gene, 7 cases were homozygotes, 11 cases were compound heterozygotes, and 7 cases had only one mutation (Table 4). Of the 19 *SLC26A4* mutations, 12 were not found in any samples, but the remaining 7 mutations were all confirmed in more than one patient. Especially, the p.H723R mutation was found to be in high allele frequency (4.1%). Direct sequencing identified novel mutations (c.1931+5G>A, p.S532I, p.R581S, p.V659L) in the patients with a single mutation by Invader assay and c.1219delCT mutation in a patient with EVA (Table 3).

As in our previous study [2], *SLC26A4* mutations were found only in the patients with EVA, suggesting a phenotype of hearing loss with EVA can be a diagnostic indicator of this category of disease.

Fluctuation and progressiveness of hearing loss are characteristic of hearing loss associated with EVA [11,12] and the early detection of *SLC26A4* mutations enables prediction of these clinical symptoms. Genetic testing is also useful in estimating associated abnormalities (goiter), selection of appropriate habilitation options, and better genetic counseling. In some cases, goiter is evident during the teen years [12]. In this study, 8 patients had hearing loss and goiter and 4 of them carried homozygous or compound heterozygous *SLC26A4* mutations.

In recessive mutations such as *GJB2* and *SLC26A4*, detection of two mutations in the paternal and maternal alleles is a hallmark. In the present "two step" screening method Invader assay is first performed followed by direct sequencing. As seen in Figs. 1 and 2, most of the mutations were successfully detected by the first Invader screening and the additional direct sequencing improved the "diagnostic" rate. This is very important to find the first mutation for identifying the responsible gene and the results indicate this screening is technically efficient. Difficult cases of a heterozygous state without a second mutation are also seen [4,5,13,14]. As previously reported, in a substantial proportion of patients our Invader techniques and additional direct sequencing revealed only one mutant *GJB2* or *SLC26A4* allele causing deafness by recessive pattern. We believe that there is one more occult mutation somewhere because the frequency of heterozygous patients was much higher than that of mutation frequency in the control population. Another explanation may be the high frequency of carriers in the population. But given the carrier frequency in normal controls, the number of heterozygous deafness cases was greater than would be expected. Second mutations may be present in the same gene or genes in the same chromosomal region. Recent statistical analysis has shown that one allele mutation of *GJB2* and *SLC26A4* is more likely to be a pathological status than a carrier status [15] and indeed, patients with one *SLC26A4* mutation are associated with EVA, therefore it is strongly likely that there is a second mutation within this gene. Another possibility is that mutations in the regulatory region may be involved in phenotypic expression [16].

The m.1555A>G mutation in the mitochondrial 12SrRNA gene, which was found in 5 4 subjects, was mainly found in those with older onset age. This mutation has been reported to be associated with aminoglycoside injection and found in 3% of the patients who visited the outpatient clinic [17,18]. The current findings are compatible with our previous report that this mutation is a frequently encountered cause for postlingual deafness in patients who received cochlear implantation [18]. This mutation was also found in the congenital or early onset age group as well, in line with our previous study [2]. It is likely that there is a considerably large high-risk population worldwide and a rapid screening method as well as careful counseling should be established to prevent aminoglycoside-induced hearing loss in this group.

The m.3243A>G mutation in the tRNA(Leu (UUR)) gene was found in 6 patients in the older-onset group. This mutation was first reported at a high frequently in the patients with clinical manifestations of MELAS [19], and has also been found in diabetes mellitus patients [20]. It is known to be commonly associated with hearing loss patients (especially with diabetes mellitus) [21]. The hearing loss is adult onset, symmetric high frequency involved [22]. In this study, all 6 patients with this mutation were associated with diabetes mellitus and progressive hearing loss. Five patients had maternally inherited hearing loss (the mother also had hearing loss), but one subject was a sporadic case (the mother did not have hearing loss from the anamnestic evaluation) and therefore is unlikely to be a mitochondrial candidate from clinical evaluation. The present multigene screening is also unexpectedly efficient for such atypical cases.

Heteroplasmy is one of the significant factors determining the expression of mitochondrial disease. The Invader assay is comparatively accurate at detecting the heteroplasmic rate [2], and the present two patients with the 3243 mutation showed 3% and 24% heteroplasmic rates.

In contrast to the three genes discussed above, mutations of the *COCH, KCNQ4, MYO7A, TECTA, CRYM, POU3F4 and EYA1* genes were not found in the present deaf subjects in line with our previous study [2]. This is likely due to them being very rare and usually independent mutations found in only one family. Although analysis for these mutations should be performed to identify the molecular nature of deafness as the first deafness screening step, a different strategy may be necessary for screening for them.

In conclusion, the simultaneous examination of the multiple deafness mutations by Invader assay followed by direct sequencing if necessary, will enable us to detect deafness mutations in an efficient and practical manner for clinical use. This screening strategy will facilitate more precise clinical genetic diagnosis, appropriate genetic counseling and proper medical management for auditory disorders. Against this background, since 2008 the Ministry of Health and Welfare of Japan has allowed this screening to be performed as an advanced medical technology.

A Japanese summary of this article has been provided as Supporting Information (Japanese summary S1).

Supporting Information

Japanese Summary S1 Simultaneous Screening of Multiple Mutations by Invader Assay. The present method of simultaneous screening of multiple deafness mutations by Invader assay followed by direct sequencing will enable us to detect deafness mutations in an efficient and practical manner for clinical use.
(PDF)

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Author Contributions

Conceived and designed the experiments: SU. Performed the experiments: SN MN SA TY. Analyzed the data: SN MN SA TY. Contributed reagents/materials/analysis tools: SN MN SA TY. Wrote the paper: SU. Collection of DNA samples and clinical data: The Deafness Gene Study Consortium.

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ORIGINAL ARTICLE

Patients with *CDH23* mutations and the 1555A>G mitochondrial mutation are good candidates for electric acoustic stimulation (EAS)

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Abstract

Conclusions: CDH23 mutations and the 1555A>G mitochondrial mutation were identified among our series of electric acoustic stimulation (EAS) patients, confirming that these genes were important in hearing loss with involvement of high frequency. Successful hearing preservation as well as good outcomes from EAS indicated that patients with this combination of mutations are good candidates for EAS. *Objectives:* Screening for gene mutations that possibly cause hearing loss involving high frequency was performed to identify the responsible genes in patients with EAS. In addition to a review of the genetic background of the patients with residual hearing loss, the benefit of EAS for patients with particular gene mutations was evaluated. *Methods:* Eighteen patients (15 late-onset, 3 early-onset) with residual hearing who had received EAS were included in this study. Genetic analysis was performed to identify *GJB2, CDH23, SLC26A4,* and the 1555 mitochondrial mutations. *Results:* Three early-onset patients had *CDH23* mutations. One late-onset patient had the 1555 A>G mitochondrial mutation.

Keywords: *Residual hearing, hearing preservation, gene, mitochondria, 12S rRNA*

Introduction

Hearing loss in the majority of patients with residual hearing at lower frequencies is more or less progressive, although the speed of progression, i.e. rapid or rather stable, may be dependent on the etiology. An unresolved issue is the prediction of progressiveness based on the etiology of individual hearing loss. We have recently reported at least four genes that are responsible for the candidates for electric acoustic stimulation (EAS), and therefore there is not a single etiology but rather a great genetic heterogeneity involved in this particular type of hearing loss [1]. In this study, screening for mutations of four genes (*GJB2, CDH23, SLC26A4,* and the 1555 mitochondrial mutations), which possibly cause high frequency hearing loss, was performed to identify the responsible genes for 18 patients with EAS.

Material and methods

Eighteen patients (8 males and 10 females, aged 1–68 years) were included in this study. Clinical features of the subjects are summarized in Table I. As regards onset of hearing loss, 15 patients were late-onset (10–50 years old) and 3 patients were early-onset (most probably congenital). Anamnestic evaluation and/or serial audiogram indicated that all of the patients had progressive sensorineural hearing loss. No patients had any anomalies such as enlarged vestibular aqueduct. All patients had some residual hearing in the lower frequencies, and therefore received EAS. The round window approach was applied for all the patients, and intraoperative and postoperative intravenous administration of dexamethasone was used as described in a previous report [2]. For genetic analysis, direct sequencing for *GJB2, SLC26A4, CDH23,* and

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Table I. Clinical features of subjects in study.

Case no.	Gender	Age (EAS)	Onset (age)	Progressiveness	Inheritance mode	Responsible gene	Implant	Insertion depth (mm)
1	F	59	Late (43)	+	Sporadic	N/I	PULSAR FLEXeas	24
2	F	71	Late (30)	+	AD	N/I	PULSAR FLEXeas	24
3	F	45	Late (25–30)	+	Sporadic	N/I	PULSAR FLEXeas	24
4	F	38	Late (34)	+	Sporadic	N/I	PULSAR FLEXeas	24
5	F	46	Late (30)	+	AD	N/I	PULSAR FLEXeas	24
6	M	29	Late (10)	+	AD	N/I	PULSAR FLEXeas	24
7	M	39	Late (20)	+	AD	N/I	PULSAR FLEXeas	24
8	F	35	Late (25)	+	Sporadic	N/I	PULSAR FLEXeas	24
9	M	52	Late (25)	+	Mitochondrial	*Mit. 1555A>G*	PULSAR FLEXeas	24
10	F	51	Late (30)	+	AD	N/I	PULSAR FLEXeas	24
11	M	39	Late (6)	+	Sporadic	N/I	PULSAR FLEXeas	24
12	F	45	Late (25)	+	Sporadic	N/I	PULSAR FLEXeas	24
13	F	38	Late (10)	+	AR	N/I	PULSAR FLEXeas	24
14	F	60	Late (40)	+	AD	N/I	Combi 40+ standard	31.5
15	M	68	Late (50)	+	Sporadic	N/I	PULSAR FLEXsoft	31.5
16	M	12	Early (3)*	+	AR	*CDH23*	PULSAR FLEXsoft	31.5
17	M	12	Early (1 year 8 months)*	+	AR	*CDH23*	PULSAR FLEXsoft	31.5
18	M	1	Early (0)†	NA	Sporadic	*CDH23*	PULSAR FLEXsoft	31.5

N/I, not identified within four genes.
*Most probably congenital.
†Newborn hearing screening.

the 1555 mitochondrial mutation was performed. Detailed methods are described elsewhere [3-6].

Results

All three early-onset patients had *CDH23* mutations (case nos 16, 17, and 18; Figures 1,2,3). One post-lingual patient had the 1555 A>G mitochondrial mutation (case no. 9; Figure 4). Hearing in the low frequencies after cochlear implantation was well preserved in all 18 cases including these 4 cases.

Case nos 16 and 17 (Figures 1 and 2)

The patients were 12-year-old twins, had the same mutations in the *CDH23* gene, and showed similar audiograms and a slowly progressive nature confirmed by serial audiograms. Both had some residual hearing in the lower frequencies and used hearing aids, but due to the progression of their hearing loss, they received cochlear implants (Nucleus CI24M device, with complete insertion of a straight array through cochleostomy) for the left ear at the age of 5 (no. 16) and 6 (no. 17). In one of the twins (no. 16) residual hearing was successfully preserved (Figure 1D), but the other (no. 17) lost his air-conduction thresholds after cochlear implantation even though the bone-conduction threshold remained stable (Figure 2D). Their audiological performance was good with the cochlear implantation (electric stimulation only). They wanted to have cochlear implants on the other sides, considering their residual hearing and the progressive nature of the hearing loss, and we decided to use a longer atraumatic electrode (MEDEL PULSAR CI100/FLEXsoft electrode) to cover the low frequencies (Figure 1A, B, C; Figure 2A, B, C). Hearing was well preserved 6 months postoperatively (Figures 1D and 2D). Both had compound heterozygous mutations (p.P240L/p.R301Q), and their parents were found to be carriers for these mutations (Figure 2E). After identification of the *CDH23* mutations, they were referred for ophthalmologic examination including electroretinography (ERG) and visual field analysis. Both had normal ERG response and no visual field deficits, confirming the nonsyndromic phenotype (DFNB12). Furthermore, they did not have any vestibular problems and showed normal responses in caloric testing. Their hearing thresholds improved to 30 dB and 35 dB (nos 16 and 17, respectively)

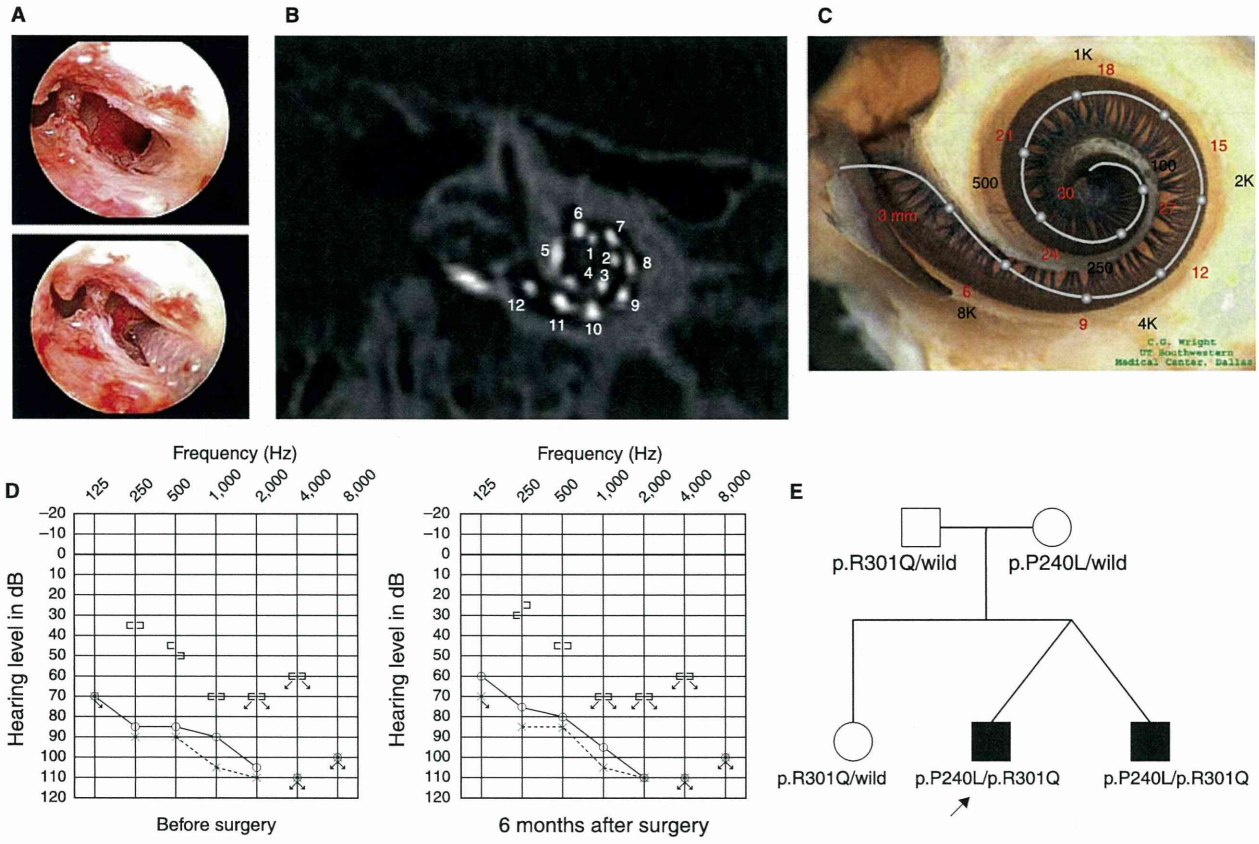

Figure 1. Case no. 16. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative and postoperative audiograms. The image of human cochlea neural tissues stained by osmium tetroxide used in Figures 1,2,3,4 was kindly provided by Dr C.G. Wright, USWT, Dallas, USA (red, mm from round window; black, corresponding frequency). (E) Pedigree and the mutations found in the CDH23 gene.

(average for all frequencies from 125 to 8000 Hz) 1 year after cochlear implantation. Their word recognition scores in quiet improved from 64% to 76% (no. 16) and from 60% to 76% (no. 17) at 1 year postoperatively.

Case no. 18 (Figure 3)

This case was a 1-year-old boy with the *CDH23* mutations. Auditory steady-state response (ASSR) evaluated at the age of 4 and 7 months showed some residual hearing at 500 Hz in the right ear (Figure 3D). He first received a left cochlear implant (MEDEL PULSAR CI100/standard electrode) at the age of 9 months. The parents wanted him to use a cochlear implant on the right side as well, and we decided to use a more atraumatic electrode (MEDEL PULSAR CI100/FLEXsoft electrode) because of the possible residual hearing in the low frequencies (Figure 3A, B, C). The second cochlear implant surgery was performed at the age of 12 months. Residual hearing measured by conditioned orientation reflex (COR) audiometry [7] was well preserved 1 year after cochlear implantation (Figure 3D). This patient had compound heterozygous mutations (p.[D1216A; V1807M]/p.Q1716P) and the parents were found to be carriers for these mutations (Figure 3E). Although the patient was too young to undergo ophthalmologic examination, he did not have any problems in vision or any vestibular problems, and there is no indicative evidence for Usher syndrome at this time.

In this very young case, auditory behavioral development was assessed by using the LittlEARS® Auditory Questionnaire, which has been designed for children under the age of 2 years [8,9]. The development curve showed a rapid increase in auditory behavior and reached the score seen in normally developed children (c 3F).

Case no. 9 (Figure 4)

This case was a 52-year-old male with the 1555A>G mitochondrial mutation. He noticed hearing loss around age 38 and used hearing aids, but his hearing loss was slowly progressive as evaluated by serial audiograms. Due to residual hearing in the lower frequencies,

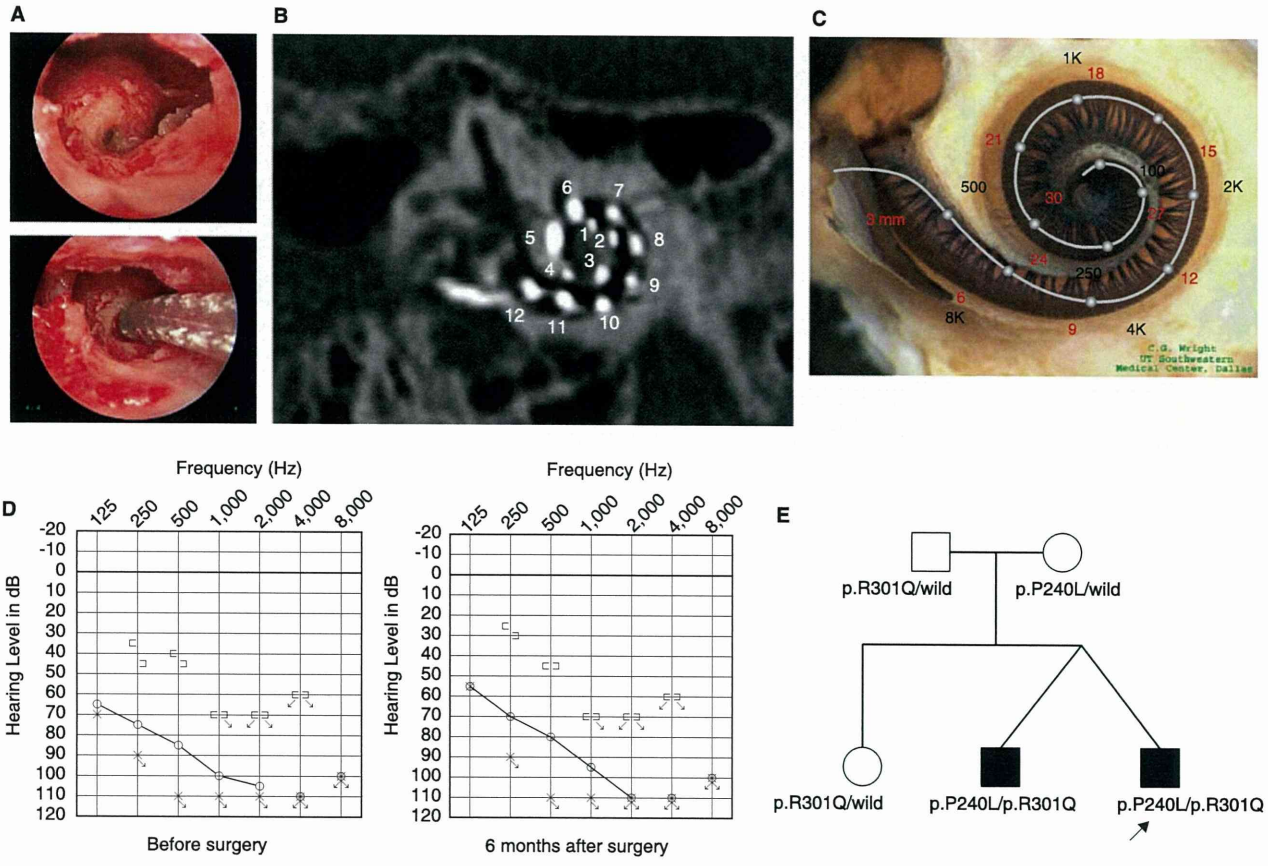

Figure 2. Case no. 17. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative and postoperative audiograms. (E) Pedigree and the mutations found in the *CDH23* gene.

an atraumatic electrode (MEDEL PULSAR CI100/FLEXeas electrode) was chosen (Figure 4A, B, C). Residual hearing was well preserved at 2 months postoperatively (Figure 4D). His parents had hearing loss, and the pedigree was consistent with mitochondrial inheritance (as well as autosomal dominant inheritance) (Figure 4E). Genetic screening detected the 1555 mitochondrial mutation in the patient and his mother. He had no history of exposure to aminoglycoside antibiotics. No vestibular symptoms were noted, and no abnormal findings were seen in vestibular testing including caloric response and vestibular evoked myogenic potential (VEMP). His hearing threshold improved to 30 dB (average for all frequencies from 125 to 8000 Hz) 2 months after cochlear implantation. Due to an insufficient follow-up period, his speech recognition score has not yet been evaluated.

Discussion

As predicted from our previous study [1] using patients who fulfilled the criteria for EAS, the *CDH23* mutations and the 1555A>G mitochondrial mutation were in fact found among our series of EAS patients.

Our previous study indicated that the *CDH23* mutations were frequently found in patients with recessive inheritance and the presence of residual hearing is one particular phenotypic feature of the patients with *CDH23* mutations [5], and actually all of the early-onset patients had the mutations in this gene.

The *CDH23* gene encodes cadherin 23, a protein thought to be a molecule that forms the lateral links between the stereocilia of hair cells [10]. One remarkable result in this study is that function of the lateral links remained stable even after deep insertion of the electrode of the cochlear implant. Such functional preservation enabled hearing preservation even in the presence of an electrode covering the corresponding frequency region.

As suggested by genotype–phenotype correlation study, USH1D, which has a more severe phenotype including severe to profound hearing loss, vestibular dysfunction, and retinitis pigmentosa, is usually associated with nonsense, splicing-site, and frameshift

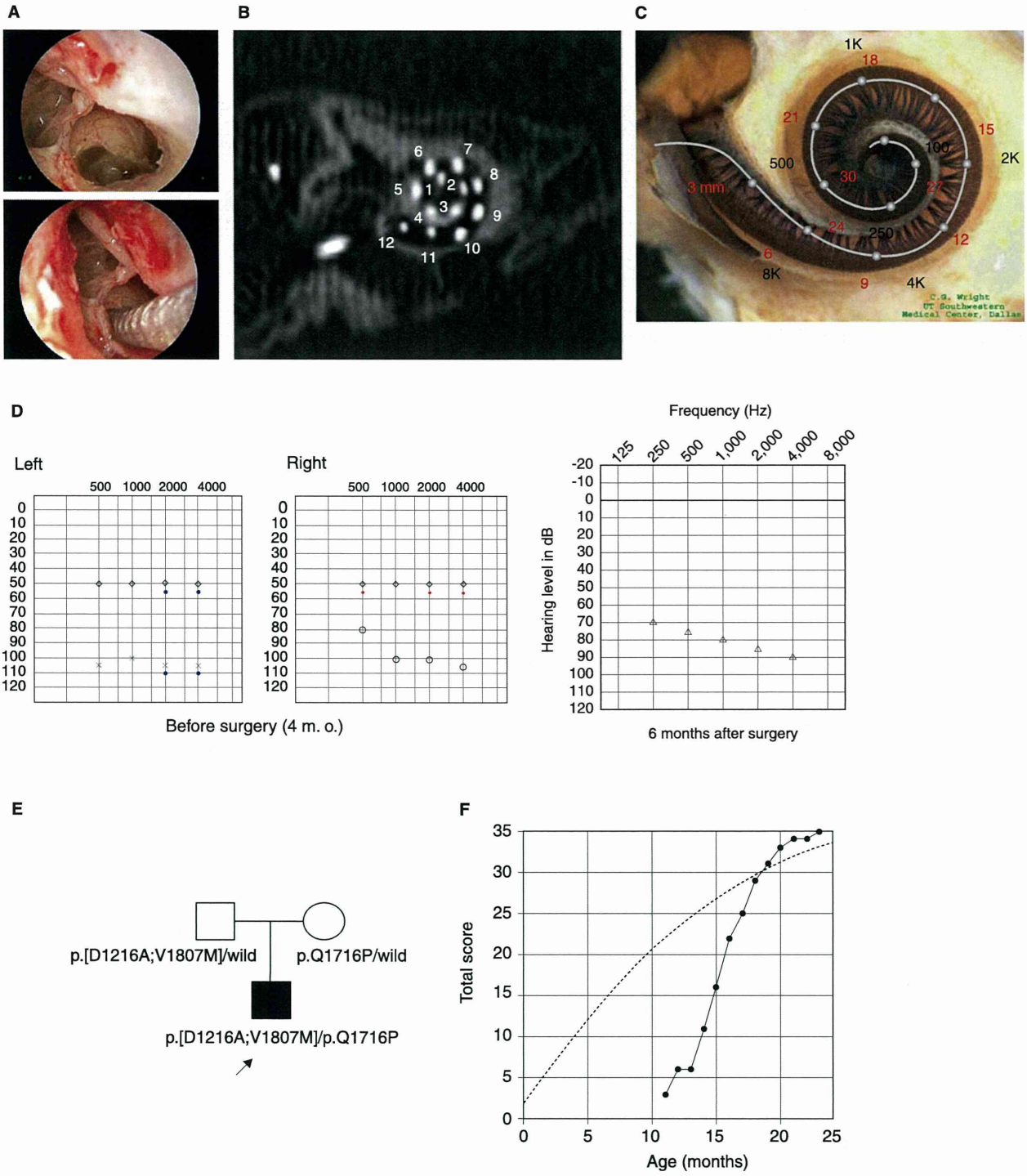

Figure 3. Case no. 18. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative ASSR findings (blue, left; red, right) and postoperative COR audiogram finding. (E) Pedigree and the mutations found in the *CDH23* gene. (F) Auditory behavioral development assessed by LittlEARS® Auditory Questionnaire. The development curve shows rapid improvement in auditory behavior reaching the curve of normally developed children.

mutations. In contrast, DFNB12, which has a milder phenotype, is associated with missense mutations [11,12]. The mutations found in the present three cases (we previously reported case nos 16 and 17 as family no. 3 [5]) are consistent with the general genotype–phenotype correlation rule.

In Usher type I patients, known to have the same etiology, improvement in sound detection as well as

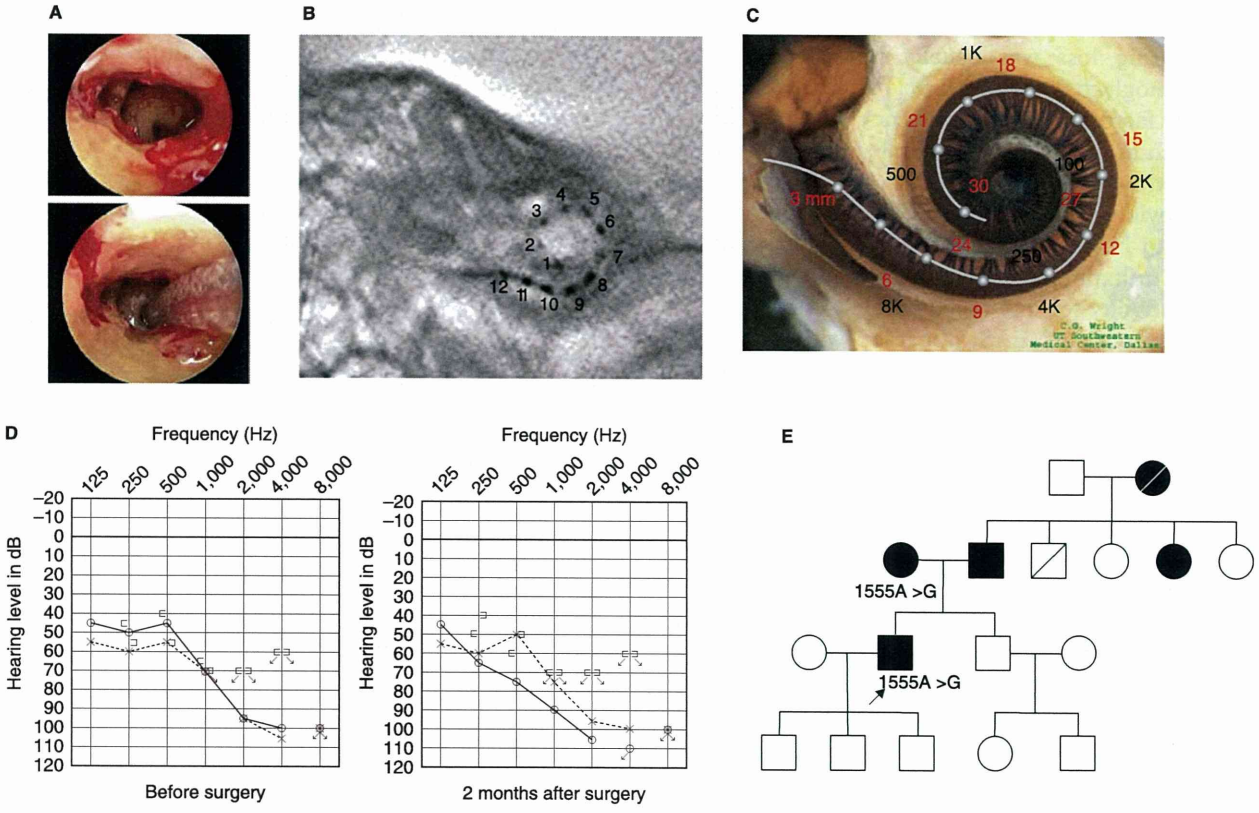

Figure 4. Case no. 9. (A) Endoscopic view of round window insertion, (B) postoperative X-ray finding, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative and postoperative audiograms. (E) Pedigree and the subjects with the mitochondrial 1555 mutations.

speech perception was seen in all patients, especially younger ones [13]. The present study clearly indicates that patients with the *CDH23* mutations are good candidates for EAS. The previous report together with the present cases indicates that progressiveness of hearing loss is a characteristic feature of the patients with this mutation [5,12]. Therefore, deep insertion with longer electrodes is recommended to prevent future deterioration. Successful hearing preservation and prediction of future hearing level by genetic diagnosis may facilitate decision making for early intervention.

It is interesting that *GJB2*, the most prevalent causative gene among the prelingual patients, was not found in the present series of patients. This is probably due to their more or less flat audiograms [1] and therefore they may be good candidates for conventional cochlear implantation.

In very young children, pure tone audiograms are not available. Acoustic brainstem response (ABR) is usually used to evaluate their hearing, but it is difficult to measure residual hearing in the low frequencies. Recently, acoustic steady-state response (ASSR) has been clinically available to measure hearing levels of 500 Hz or 250 Hz, but sometimes the low frequency part is not reliable or convincing [14]. In addition to such hearing testing, genetic testing is useful to predict the residual hearing at low frequencies. Especially for cases with *CDH23* mutations, predicted audiograms can be obtained for the very young patients. Based on this concept, together with consideration of their expected long life (which includes a risk of progression), we chose a longer atraumatic electrode (MEDEL PULSAR CI100/FLEXsoft electrode) for three patients with *CDH23* mutations.

It is known that patients with the 1555A>G mitochondrial mutation are susceptible to aminoglycoside antibiotics [15]. The 1555A>G mutation is one of the most important mutations among the hearing loss population in Japan, and approximately 3% of patients with sensorineural hearing loss possess this mutation [16]. Their hearing loss is known to be slowly progressive [6,17]. This mutation is an important cause in the post-lingual cochlear implant patients, found in 10% of them [16]. It has been reported that a patient with cochlear implantation showed excellent auditory performance [18], indicating that cochlear implantation is a valuable choice of therapy for patients with profound hearing loss caused by this mutation. This mutation was also found in

patients without any aminoglycoside exposure and their hearing loss was usually milder than those with aminoglycoside exposure [19]. Environmental causative factors other than aminoglycoside antibiotics – such as noise or mechanical stress – have been speculated, although not confirmed. The present study provided an important clinical experience that EAS could be safely performed even if the patients have this mutation and therefore possible association of susceptibility for any mechanical stress.

For outcome of EAS, together with successful hearing preservation, all four patients obtained 25–35 dB in average hearing threshold after implantation. Since EAS was implanted as a second cochlear implant for three cases with *CDH23* mutations, it is difficult to evaluate the independent benefit of EAS. However, improvement of word recognition scores after EAS was observed in case nos 16 and 17, indicating that additive benefit was clearly obtained even after a rather long period following the first implants (at 7 years and 6 years, respectively). For case no. 18, although it is also difficult to evaluate the independent benefit of EAS because of the very young age, the auditory behavioral development as assessed by the LittlEARS® Auditory Questionnaire was significantly improved after two consecutive implantations. Since the *CDH23* mutation will be potentially found in rather young candidates, this genetic marker could be available for the existence of residual hearing. For those patients, it is strongly suggested that the surgeon keep in mind the option of performing atraumatic surgery.

In the present series, there are many families with autosomal dominant hearing loss (6 of 18), suggesting that many other genes responsible for dominant hearing loss may be involved. It is also important to note that all of the patients showed progressive hearing loss. We are currently searching for the responsible genes for the patients with high frequency hearing loss.

In conclusion, the *CDH23* mutations and the 1555A>G mitochondrial mutation were identified among our series of EAS patients, confirming that these genes were important in high frequency hearing loss. Successful hearing preservation in these patients as well as good outcomes of EAS indicated that those with these mutations are good candidates for EAS. The present study indicates that genetic testing provides useful information regarding residual hearing and consequent therapeutic options.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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SHORT COMMUNICATION

Different cortical metabolic activation by visual stimuli possibly due to different time courses of hearing loss in patients with *GJB2* and *SLC26A4* mutations

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Abstract

Conclusion. We have demonstrated differences in cortical activation with language-related visual stimuli in patients who were profoundly deafened due to genetic mutations in *GJB2* and *SLC26A4*. The differences in cortical processing patterns between these two cases may have been influenced by the differing clinical courses and pathogenesis of hearing loss due to genetic mutations. Our results suggest the importance of hearing during early childhood for the development of a normal cortical language network. *Objectives.* To investigate the cortical activation with language-related visual stimuli in patients who were profoundly deafened due to genetic mutations in *GJB2* and *SLC26A4*. *Methods:* The cortical activity of two adult patients with known genetic mutations (*GJB2, SLC26A4*) was evaluated with fluorodeoxyglucose-positron emission tomography (FDG-PET) with a visual language task and compared with that of normal-hearing controls. *Results:* A patient with a *GJB2* mutation showed activation in the right auditory association area [BA21, BA22], and the left auditory association area [BA42] even with visual language task; in contrast, a patient with an *SLC26A4* mutation showed no significant activation in the corresponding area.

Keywords: *FDG-PET, visual language task, functional brain imaging*

Introduction

Functional brain imaging is an effective method for investigating the cortical processing of language, which has provided much evidence for the plasticity of the central auditory pathway following a profound loss of hearing [1–4]. Many previous studies showed that there is a capacity of the auditory cortex for cross-modal plasticity after auditory deprivation of the brain. Cerebral glucose metabolism in the primary auditory and related cortices in individuals with prelingual deafness was shown to decrease in younger patients, but to increase as they aged and, in fact, recover fully or even exceed the normal level of activation [5–7]. Children with prelingual deafness can acquire spoken language by cochlear implantation, but its efficacy decreases with age. The development of the auditory cortex is believed to depend on the patient's auditory experience within 'critical periods' in the early lifetime. Adults who had severe congenital hearing loss in their childhood may take advantage of hearing with cochlear implants if they had exploited residual hearing with hearing aids. It has been shown that low glucose metabolism in the temporal auditory cortex predicts a good cochlear implant outcome in prelingually deafened children, which suggests that low metabolism in the auditory cortex may indicate its potential of plasticity for spoken language acquisition [7].

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