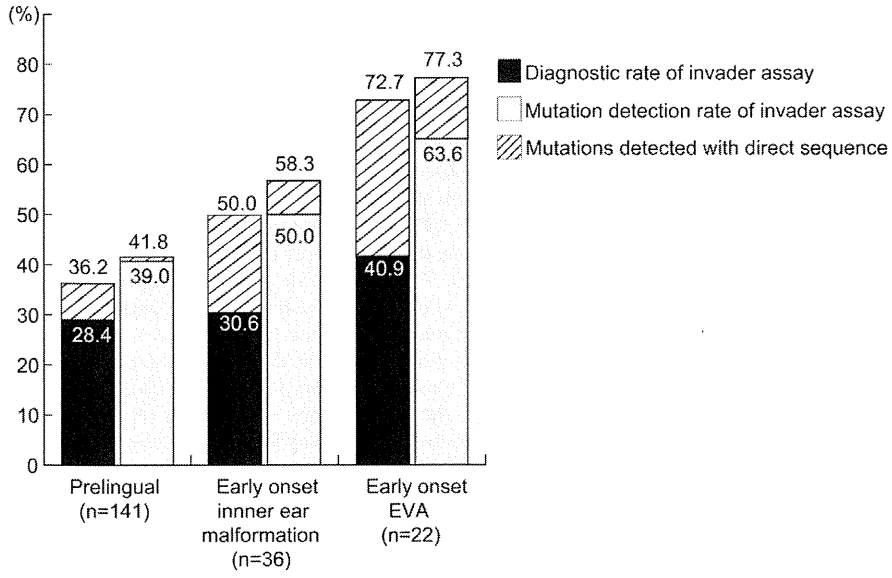

Figure 2. Radiographic findings and detection rate. Detection rate was elevated when subjects were restricted to those with inner ear anomaly or EVA. Combined direct sequence and invader screening enhanced the diagnostic rate but not the mutation detection rate.
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various 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

Mutations in the *NOG* gene are commonly found in congenital stapes ankylosis with symphalangism, but not in otosclerosis

Usami S, Abe S, Nishio S, Sakurai Y, Kojima H, Tono T, Suzuki N. Mutations in the *NOG* gene are commonly found in congenital stapes ankylosis with symphalangism, but not in otosclerosis. Clin Genet 2012. © John Wiley & Sons A/S, 2012

Human noggin (*NOG*) is a responsible gene for multiple synostosis syndrome (SYNS1) and proximal symphalangism (SYM1), two conditions that are recently known to be within a wider range of clinical manifestations of stapes ankylosis with symphalangism. This study was performed to determine the range of phenotype caused by *NOG* mutations, using Japanese patients with various phenotypes including sporadic inherited SYM1, dominantly inherited SYM1, stapes ankylosis with broad thumb and toes (Teunissen and Cremer syndrome). In addition, 33 patients with typical otosclerosis (without symphalangism) were studied. Direct sequencing analysis disclosed three novel mutations of the *NOG* gene in three SYM1 families. None of the otosclerosis patients without symphalangism had *NOG* mutations, indicating that *NOG* mutations may be restrictively found within patients with various skeletal abnormalities. These results together with the literature review indicated that there are no clear genotype–phenotype correlations for NOG mutations. With regard to surgical outcome, most of the patients in these three families with *NOG* mutations showed remarkable air–bone gap recovery after stapes surgery. Molecular genetic testing is useful to differentiate syndromic stapes ankylosis from otosclerosis, and even mild skeletal anomalies can be a diagnostic indicator of NOG-associated disease.

Conflict of interest

The authors declare no conflict of interest.

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Key words: – otosclerosis – stapes ankylosis – stapes ankylosis with broad – SYM1 – symphalangism – Teunissen and Cremer syndrome – thumb and toes

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Human noggin (*NOG*) is a responsible gene for a wide range of clinical manifestations of stapes ankylosis with symphalangism. Proximal symphalangism (SYM1: MIM #185800) (1) is known as an autosomal dominant disorder with high penetrance. The most common clinical features are the immobility of the proximal interphalangia (PIP) joints of the hands and toes, and congenital conductive hearing loss due to stapes ankylosis. Multiple synostosis syndrome (SYNS1: MIM#186500) (1) is characterized by a more severe phenotype of the proximal symphalangism, such as progressive and expanded bony fusion of joints and unique facial manifestations. In addition, mutations in NOG have been identified in Tarsal–Carpal Coalition syndrome (TCC: MIM#186570) (2), stapes ankylosis with broad thumb and toes (MIM#184460) (Teunissen and Cremer syndrome) (3), and Brachydactyly type B2 (BDB2: MIM#611377) (4).

Otosclerosis (MIM#166800) is known as the single most common cause of progressive conductive hearing loss, characterized by abnormal bone remodeling in the otic capsule. Although there are a small number of familial cases that are likely to be monogenic, the majority of cases are sporadic. A series of studies has suggested that this condition involves both genetic and environmental factors (5).

Typical otosclerosis was included in this study because it is an interesting question as to whether some of the typical otosclerosis is a continuum of a category of disease caused by *NOG* mutations. We thought this may be true because (i) within SYM1, *NOG* mutations were found in patients with minor skeletal anomalies without symphalangism (3), and (ii) stapes ankylosis is an important phenotype of the animal model for $NOG^{+/-}$ mice (6).

To date, no detailed survey was available for *NOG* mutations in the stapes ankylosis patients with symphalangism in Asian populations. Therefore this study was undertaken to address whether *NOG* mutations are also causative and commonly found in those populations and if so, whether there is a different mutation spectrum.

In addition, previously reported *NOG* mutations were reviewed to determine their spectrum as well as whether there are any particular genotype–phenotype correlations caused by *NOG* mutations.

Materials and methods

Subjects

We ascertained three Japanese families to be associated with conductive hearing loss and symphalangism, including an autosomal dominant SYM1 family, a sporadic SYM1 case with normal parents, and an autosomal dominant stapes ankylosis with broad thumb and toes (Teunissen and Cremer syndrome) family. Thirty-three Japanese otosclerosis patients, who underwent stapes surgery, were also screened for mutations in the *NOG* gene. Their clinical symptoms, including ages at surgery (36–77 years old: average 54.4 years old), onset age (15–57 years old: average 37.3 years old), gender (10 male and 23 female), laterality (9 unilateral and 24 bilateral), and hearing threshold (average 63.1 dB), are summarized in Table 1. Average onset age, was hearing threshold, was evaluated using pure-tone audiometry classified by a pure-tone average over 250, 500, 1000, 2000, and 4000 Hz. By detailed anamnestic and medical examination, no patients had any associated skeletal abnormalities. All of the patients were sporadic cases and no similar condition was observed within their familial members. Satisfactory outcomes after stapes surgery were obtained in all 33 subjects.

We obtained informed consent for participation in this project from each subject and also from 192 normal control subjects who were unrelated Japanese individuals without any noticeable hearing loss evaluated by auditory testing. Otologic examination, audiometric analysis, and radiologic imaging were carried out for each patient.

Family 1

As shown in the pedigree (Fig. S1a), patient #991 was diagnosed with symmetric conductive hearing loss of 50 dB (Fig. 1b) at the age of 6 years. Tympanography indicated type A sclerosis and absence of the stapedius reflex, whereas otomicroscopy results were normal. Temporal bone computed tomography (CT) scan revealed no inner or middle ear malformations. Her hearing level was stable and non-progressive, and she received hearing aids in both ears. At the age of 17, exploratory tympanotomy of the left ear showed bony fixation of the footplate without any other deformities in the middle ear and stapedotomy using a Teflon piston and wire was performed, resulting in a remarkable improvement in hearing. One year later, stapedotomy was undertaken in her right ear also. After the surgery, the postoperative hearing levels showed 20–30 dB and she did not use her hearing aids. The X-ray presented in Fig. 1 shows symphalangism in the PIP joints of the second to fifth fingers of both hands and in the distal interphalangeal (DIP) joints of the left second and fifth fingers and of the right fifth finger. There was symphalangism in both hands, resulting in limited mobility of the fingers. Symphalangism (fixation of the proximal interphalangeal joint) in both feet was also found. The ankylosis was confirmed by X-ray examination

Table 1. Clinical symptoms of Otosclerosis patients

Patient number	Age	Gender	Onset age	Affected side	Hearing threshold (right)	Hearing threshold (left)
1	48	M	36	Bilateral	68.3	56.3
2	59	F	45	Bilateral	56.3	56.3
3	43	F	38	Left	10.8	64.0
4	36	M	25	Bilateral	61.3	66.3
5	46	F	40	Right	38.0	19.0
6	44	F	33	Bilateral	59.0	66.0
7	65	F	49	Bilateral	81.0	63.0
8	77	F	57	Bilateral	104.0	105.0
9	45	F	41	Bilateral	56.0	69.0
10	44	F	30	Left	30.0	80.0
11	61	F	44	Bilateral	23.0	68.0
12	58	F	49	Right	76.0	35.0
13	43	M	25	Left	14.0	49.0
14	58	F	47	Bilateral	53.0	50.0
15	54	F	38	Bilateral	57.0	45.0
16	53	F	40	Right	58.0	6.0
17	44	F	25	Bilateral	53.0	56.0
18	62	F	48	Bilateral	42.5	52.5
19	43	F	33	Bilateral	27.0	45.0
20	57	F	40	Left	26.0	65.0
21	65	F	15	Bilateral	53.0	50.0
22	54	M	46	Bilateral	50.0	30.0
23	48	F	23	Bilateral	71.0	56.0
24	62	F	39	Bilateral	38.0	37.0
25	76	F	43	Bilateral	78.0	75.0
26	71	M	41	Bilateral	91.3	97.5
27	71	F	40	Bilateral	126.3	110.0
28	45	M	45	Left	42.5	73.75
29	41	F	30	Bilateral	98.8	95
30	44	M	30	Left	30.0	76.3
31	44	M	30	Right	58.8	38.8
32	64	M	35	Bilateral	51.0	46.0
33	70	M	30	Bilateral	48.8	53.8

F, female; M, male.

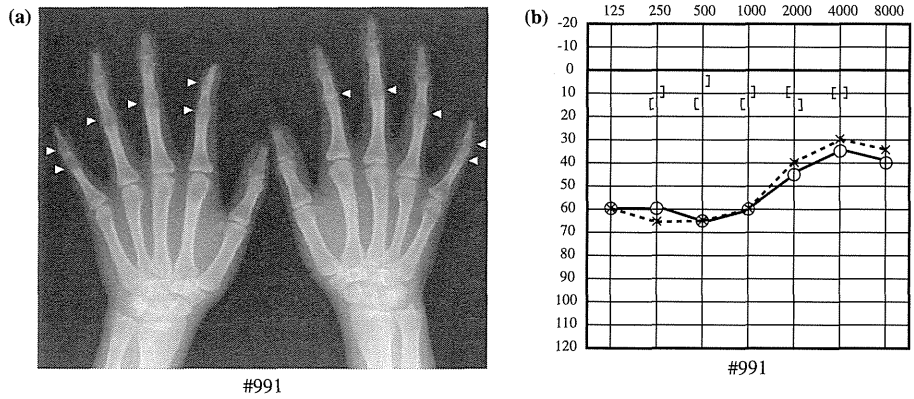

Fig. 1. (**a**) Photograph with arrowheads indicating symphalangism in the hands of patient #991. (**b**) Audiograms from patient #991 showing conductive hearing loss.

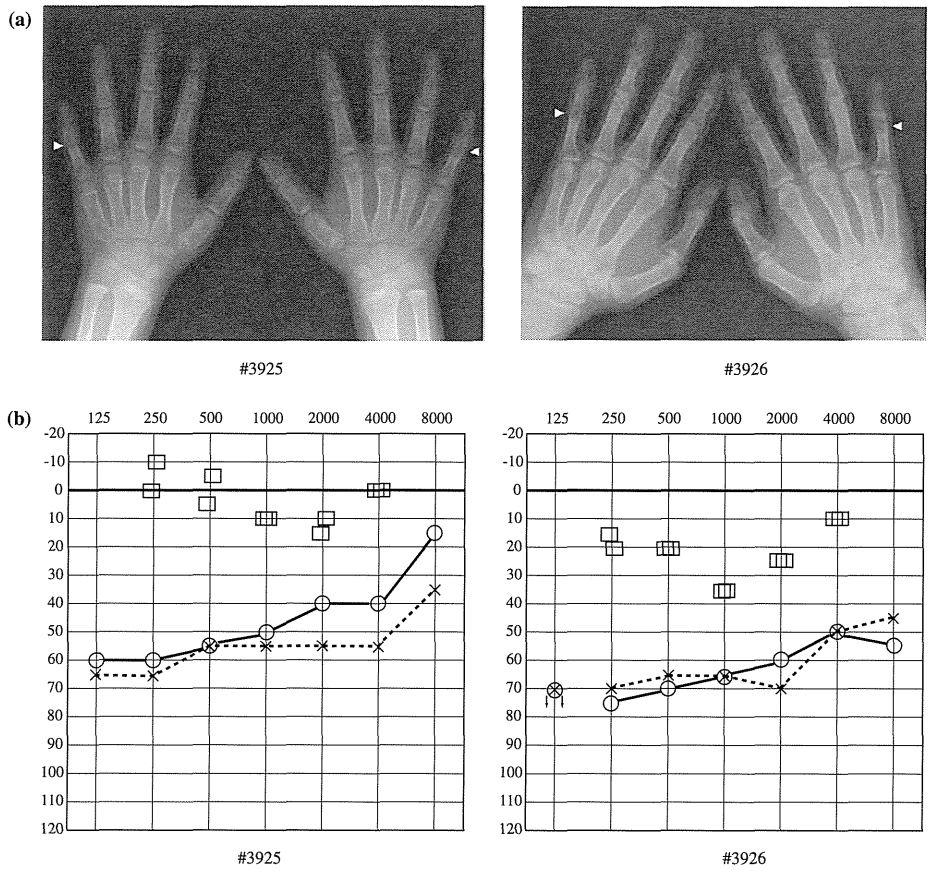

Fig. 2. (**a**) Photograph with arrowheads indicating symphalangism in the hands of patients #3925 and #3926. (**b**) Audiograms from patients #3925 and #3926 showing conductive hearing loss.

(Fig. 1a). Congenital hyperopia (only in this patient within the family) was also present.

Family 2

As shown in the pedigree (Fig. S2a), a girl and her father (patients #3925 and #3926) visited our hospital due to bilateral hearing loss. Audiograms indicated bilateral mixed hearing loss (Fig. 2b). Anamnestic evaluation suggested that the hearing loss was non-progressive without any associated symptoms such as ear fullness, tinnitus or vertigo. Patient #3926 underwent stapedotomy at the age of 42, achieving significant recovery of his hearing. There was symphalangism in the PIP joint of both fifth fingers, resulting in limited mobility of the fingers. Fixation of the proximal interphalangeal joint was not found in either foot. The ankylosis was confirmed by X-ray examination

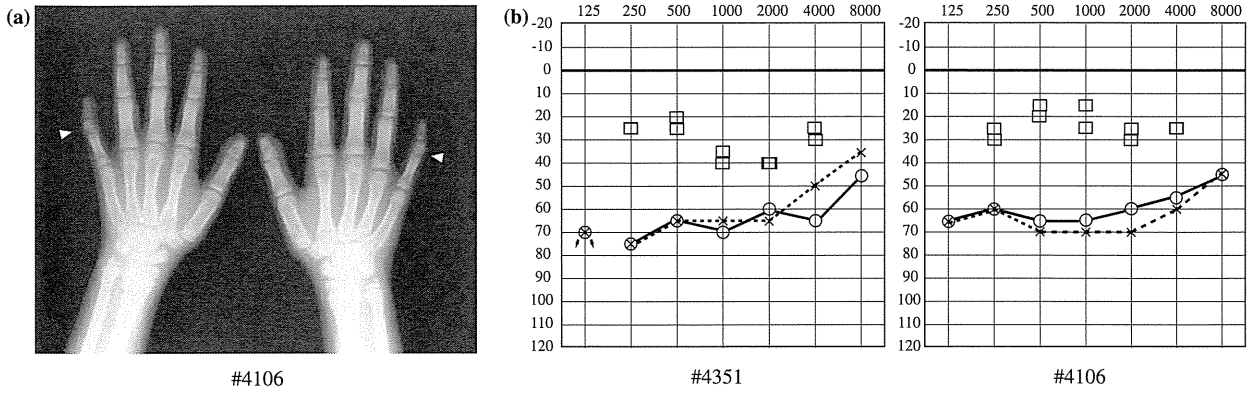

Fig. 3. (**a**) Photograph with arrowheads indicating symphalangism in the hands of patients #4106. (**b**) Audiograms from patients #4106 and #4351 showing conductive hearing loss.

(Fig. 2a). Congenital hyperopia was not present in this family.

Family 3

The pedigree shows hearing loss was inherited in four generations, indicating autosomal dominant inheritance (Fig. S3a). In addition to conductive hearing loss (Fig. 3b), the family members were associated with the following clinical phenotype in various degrees: hyperopia, broad thumbs and first toes, symphalangism, syndactyly, and fused cervical vertebrae (Fig. S3a). The clinical diagnosis was therefore stapes ankylosis with broad thumb and toes (3) or Teunissen and Cremer syndrome (7). Patient #4351 had conductive hearing loss, hyperopia, broad thumbs and first toes, symphalangism, and syndactyly. She noted her hearing loss around age 10. Stapedotomy was performed when she was 37 (right) and 38 (left) years old, achieving significant recovery of hearing. Patient #4106 had conductive hearing loss, hyperopia, broad thumbs and first toes, and fused cervical vertebrae. His hearing loss was noted around age 3 and was diagnosed at the age of 8. Stapedotomy was performed when he was 9 (right) and 10 (left) years old, achieving significant recovery of hearing.

Mutation identification

Human *NOG* gene coding is constituted of one single exon, in which an open reading frame of 696 nucleotides encodes a NOG polypeptide of 232 amino acids. A sequence obtained from GeneBank U31202 was used to design primers containing the entire coding region of *NOG*. Two fragments to entirely cover the coding region of NOG were amplified with polymerase chain reaction (PCR) and two specific primer pairs, as follows: F1, 5′-CTTGTGTGCCTTTCTTCCGC-3′; R1, 5′-TACTGGATGGGAATCCAGCC-3′; and F2, 5′-TACGACCCAGGCTTCATGGC-3′; R2, 5′-TAGCACG AGCACTTGCACTC-3′.

PCR reactions were carried out in 25 μl total volume containing 40 ng of genomic DNA, 10 pmol of each primer, 2 mM dNTPs, ×10 PCR buffer and 0.2 U of ExTaq polymerase (Takara, Tokyo, Japan). PCR conditions were denaturing at 94°C for 2 min; 35 cycles at 96°C for 30 s, 60°C for 30 s, 72°C for 1 min extension, with a final extension step at 72°C for 5 min in a Perkin-Elmer 9600 thermal cycler (Perkin-Elmer, Foster City, CA). PCR products were purified with a Suprec filter (Takara) and sequenced directly, using four primers (F1, R1, F2, and R2) and ABI BigDye terminators, on an ABI 3100 sequencer (Perkin-Elmer).

Results

Three novel mutations of the *NOG* gene were found by direct sequencing analysis in three families, whose common clinical features were compatible with SYM1, i.e. immobility of the PIP joints of the hands and toes, and congenital conductive hearing loss due to stapes ankylosis. Patient #991 of family 1 had a heterozygous G>T transversion at nucleotide 551 (Fig. S1b), predicting a cysteine (C) for phenylalanine (F) substitution at amino acid 184 (C184F) in the coding region of NOG. Since the C184F mutation was not found in either parent and was found only in the proband (patient #991), it was suggested that the mutation arose *de novo* in only the affected individual. Patients #3925 and #3926 of family 2 had a heterozygous T>A transversion at nucleotide 463 (Fig. S2b), predicting a cysteine (C) for serine (S) substitution at amino acid 155 (C155F) in the coding region of NOG. Patients #4106 and #4351 had a heterozygous C215X mutation.

None of the otosclerosis patients had *NOG* mutations. These three mutations were not observed in any of the other family members nor in the 192 unrelated Japanese controls (384 chromosomes).

Discussion

This study identified three novel mutations in the *NOG* gene in families with symphalangism, being consistent with the previous work showing that *NOG* is the responsible gene for SYM1 and stapes ankylosis with

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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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)

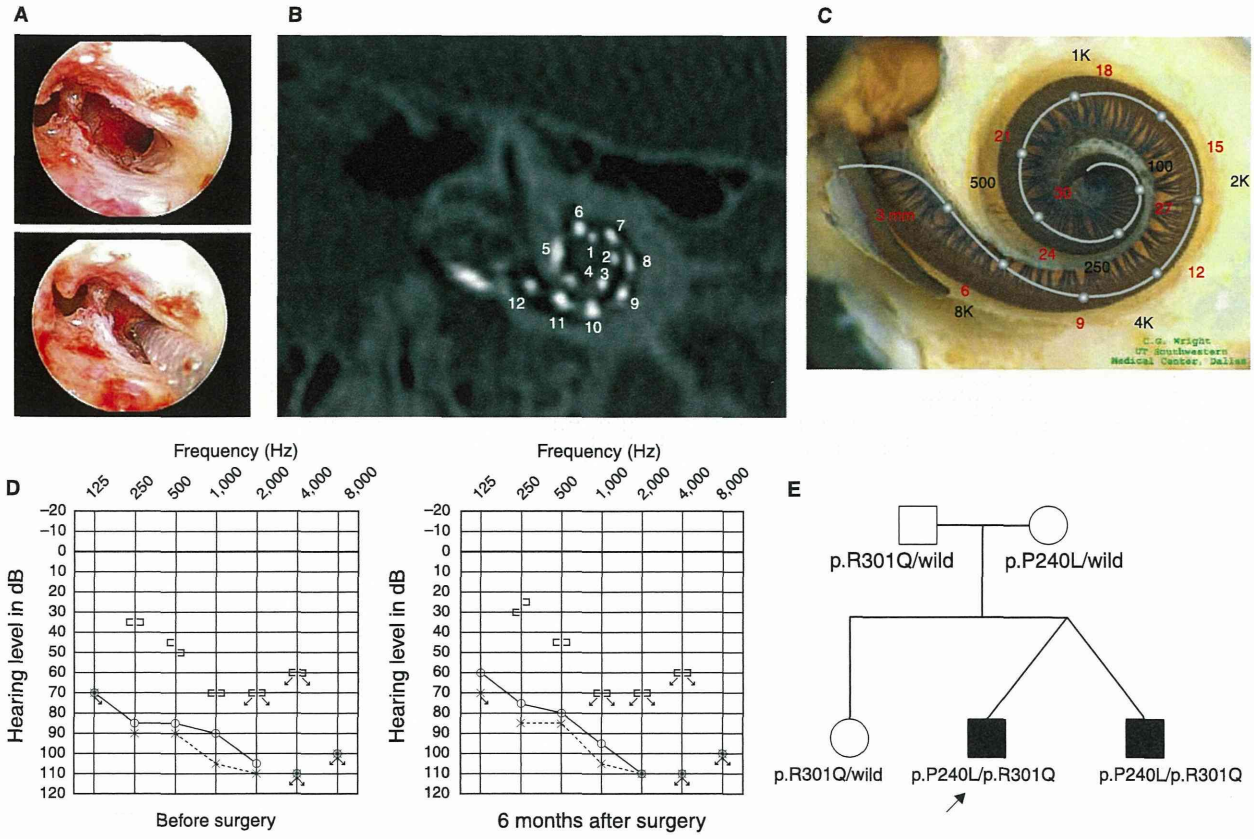

Figure 1. Case no. 16. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative and postoperative audiograms. The image of human cochlea neural tissues stained by osmium tetroxide used in Figures 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,

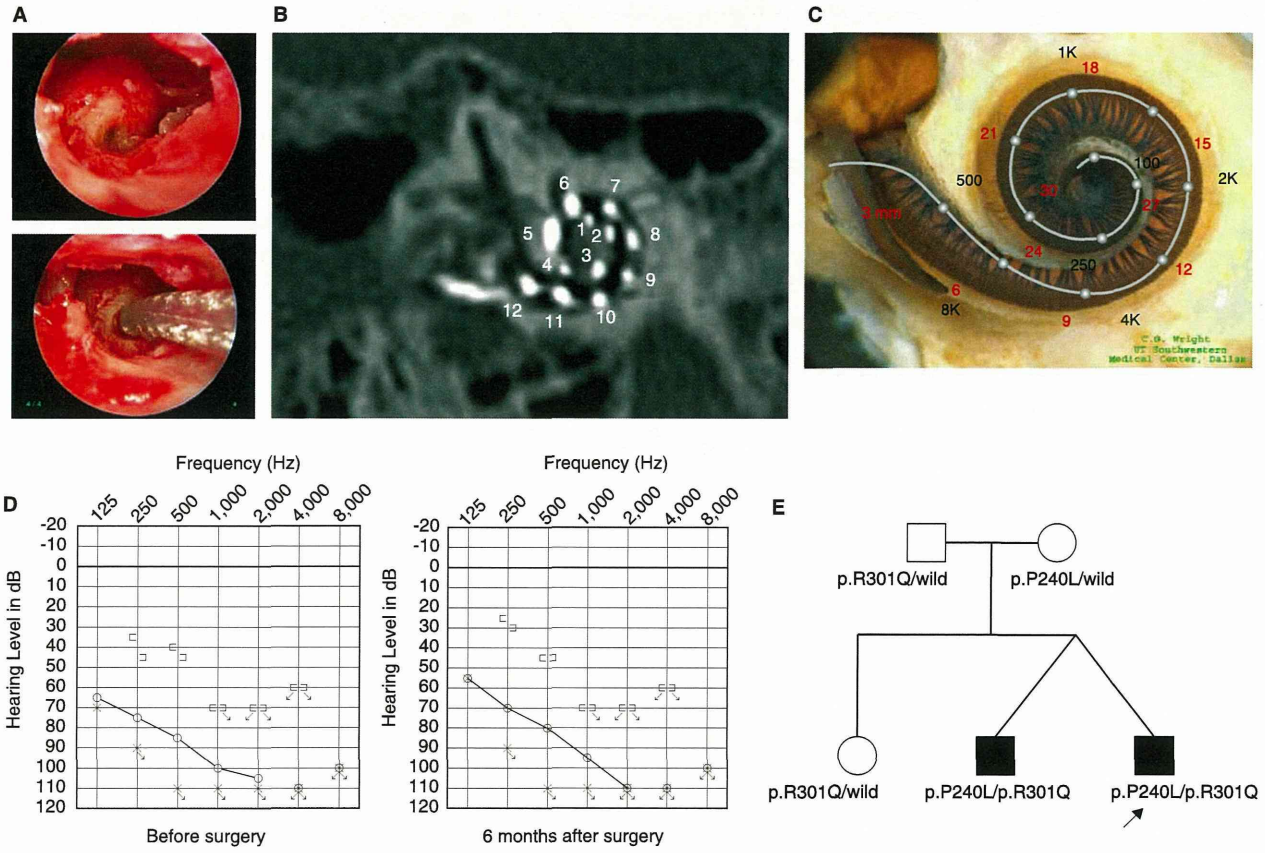

Figure 2. Case no. 17. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative and postoperative audiograms. (E) Pedigree and the mutations found in the *CDH23* gene.

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

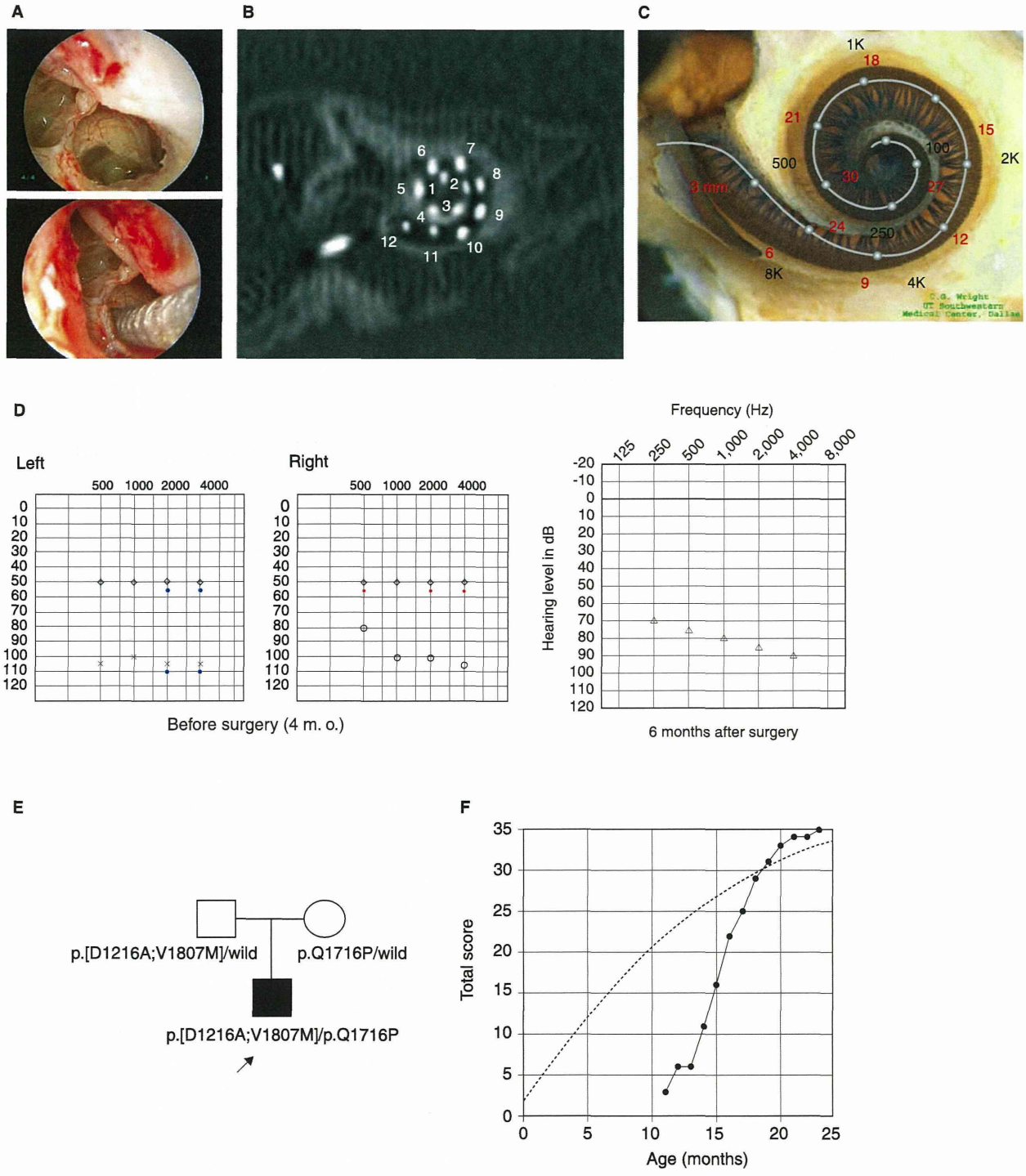

Figure 3. Case no. 18. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative ASSR findings (blue, left; red, right) and postoperative COR audiogram finding. (E) Pedigree and the mutations 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

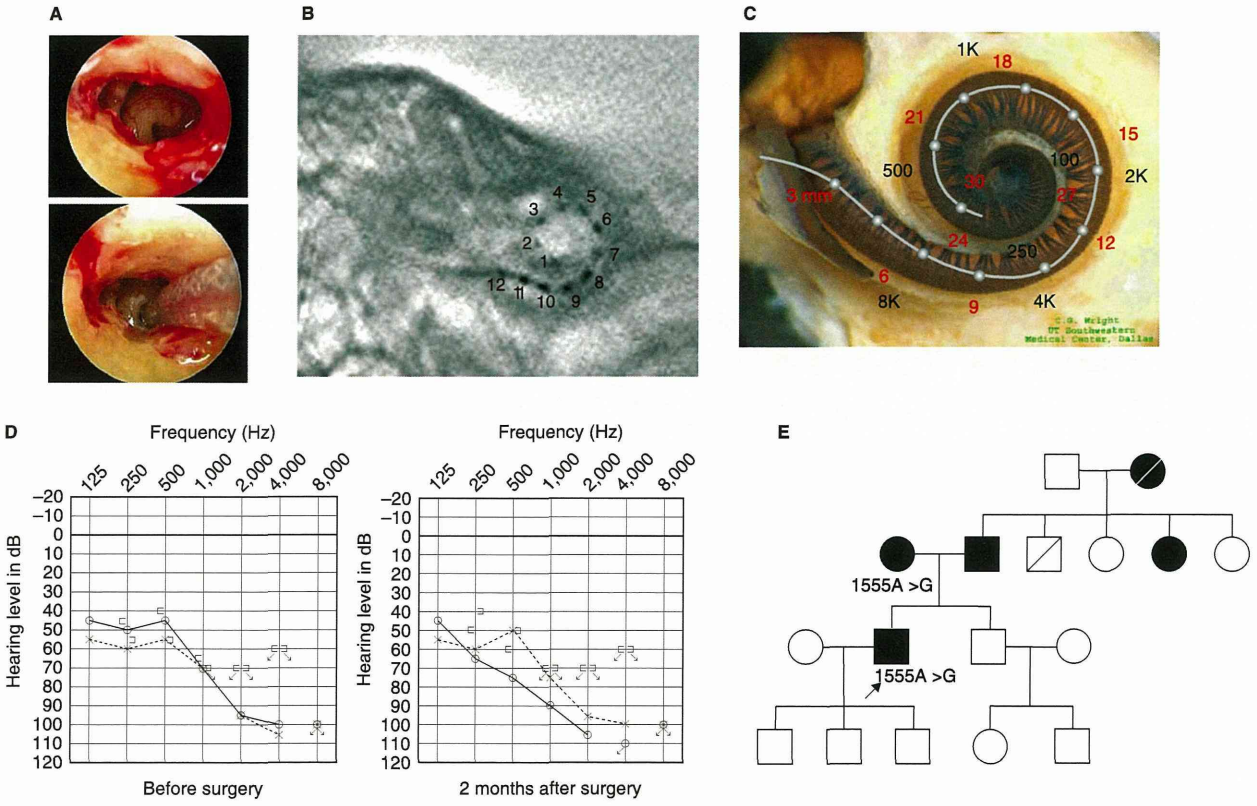

Figure 4. Case no. 9. (A) Endoscopic view of round window insertion, (B) postoperative X-ray finding, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative and postoperative audiograms. (E) Pedigree and the subjects with the mitochondrial 1555 mutations.

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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ORIGINAL ARTICLE

TECTA mutations in Japanese with mid-frequency hearing loss affected by zona pellucida domain protein secretion

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TECTA **gene encodes α-tectorin, the major component of noncollagenous glycoprotein of the tectorial membrane, and has a role in intracochlear sound transmission. The *TECTA* mutations are one of the most frequent causes of autosomal dominant (AD) hearing loss and genotype–phenotype correlations are associated with mutations of *TECTA* in exons according to α-tectorin domains. In this study, we investigated the prevalence of hearing loss caused by *TECTA* mutations in Japanese AD hearing loss families, and confirmed genotype–phenotype correlation, as well as the intracellular localization of missense mutations in the α-tectorin domain. *TECTA* mutations were detected in 2.9% (4/139) of our Japanese AD hearing loss families, with the prevalence in moderate hearing loss being 7.7% (4/52), and all patients showed typical genotype–phenotype correlations as previously described. The present *in vitro* study showed differences of localization patterns between wild type and mutants, and suggested that each missense mutation may lead to a lack of assembly of secretion, and may reduce the incorporation of α-tectorin into the tectorial membrane.**
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Keywords: autosomal dominant hearing loss; genotype–phenotype correlations; mid-frequency hearing loss; *TECTA*; zona pellucida domain

INTRODUCTION

Hearing loss affects about 1 in 500–1000 newborns in developed countries and genetic causes account for at least 50% of all childhood nonsyndromic sensory neural hearing loss (SNHL).[1] Most of these cases are affected with severe and congenital prelingual deafness and autosomal recessive inheritance represented by *GJB2* gene mutations predominates (80%) over autosomal dominant (AD) (20%).[2] Mild to moderate SNHL and/or late-onset SNHL, presenting with AD inheritance pedigree pattern, is commonly supposed to be of genetic causes. AD nonsyndromic hearing loss (ADNSHL) is represented by heterogeneity of genetic and clinical features, as 60 loci have been mapped, 24 genes have been cloned and correlation with audiological features have been reported (Van Camp and Smith, Hereditary Hearing Loss Homepage. WorldWide Web URL: http://hereditaryhearingloss.org). These types of SNHL can be characterized by age of onset, progression and pattern of audiogram.

As one cause of ADNSHL, *TECTA* mutations have been found in various types of hearing loss, age of onset, progression and frequency involvement in various populations.[3–10] This gene encodes α-tectorin, the major component of noncollagenous glycoprotein of the tectorial membrane that consists of an extracellular matrix overlying the organ of corti, contacting the outer cochlear hair cells, and having a role in intracochlear sound transmission.[11] The α-tectorin is composed of three distinct modules: the entactin G1 domain, the zonadhesin (ZA) domain with von Willebrand factor type D repeats and the zona pellucida (ZP) domain.[11] No nonsense mutations of *TECTA* have been reported in AD hearing loss. Missense mutations affecting the ZP domain are associated with mid-frequency hearing impairment, whereas mutations in the ZA domain are associated with hearing impairment primarily affecting the high frequencies.[12] Phenotypes of hearing loss can range from mild to severe and have pre or postlingual onset.[8]

In this study, (1) we examined the prevalence of hearing loss caused by *TECTA* mutations in Japanese ADSHNL and confirmed genotype–phenotype correlation, and (2) examined the impact of three missense mutations in the ZP domain on the cellular distribution of α-tectorin, known to cause mid-frequency hearing impairment. Many deafness-causing *TECTA* mutations have been reported, but the molecular mechanisms are unclear. To investigate the biological function of missense mutations in the ZP domain that were reported as causing ADSHNL GFP fusion proteins were generated and the effects of

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corresponding mutations on secretion patterns of the ZP domain of α-tectorin were examined.

MATERIALS AND METHODS

Subjects

A total of 139 Japanese AD (with two or more generations affected) sensorineural hearing loss families were screened for mutations in the *TECTA* gene. All probands were from independent families and none had any other associated neurological signs, visual dysfunction or diabetes mellitus. Hearing level was classified by a pure-tone audiometry average over 500, 1000, 2000 and 4000 Hz in the better hearing ears as follows: normal hearing, <20 dB; mild hearing loss, 21–40 dB; moderate hearing loss, 41–70 dB; severe hearing loss, 71–95 dB; and profound hearing loss, >95 dB (GENDEAF, 2004). Of the 139 probands, 4 (3%) had normal hearing (only limited frequencies involved), 40 (29%) had mild hearing loss, 52 (37%) had moderate hearing loss, 23 (17%) had severe hearing loss and 12 (9%) had profound hearing loss. Information on pure-tone audiometry was not available for eight (6%) of these subjects. The mean age at their participation (not onset of hearing loss) of the subjects were; normal hearing, 14.0 ± 10.6 years; mild hearing loss, 21.1 ± 15.5 years; moderate hearing loss, 25.3 ± 19.0 years; severe hearing loss, 32.1 ± 25.5 years; and profound hearing loss, 27.5 ± 19.2 years.

All subjects gave prior informed written consent for participation in this study and the Ethical Committee of Shinshu University approved the study.

Mutation analysis

All 23 exons and flanking intronic sequences of the *TECTA* gene were amplified by PCR. Primers were designed to flank all of the exon–intron boundaries by use of the Primer3 web-based server (http://frodo.wi.mit.edu/). Each genomic DNA sample (40 ng) was amplified, using Ex-Taq polymerase (Takara, Otsu, Japan), for 5 min at 95 °C, followed by 37 three-step cycles of 95 °C for 30 s, 56–63 °C for 30 s and 72 °C for 1.5 min, with a final extension at 72 °C for 10 min, ending with a holding period at 4 °C in a Perkin–Elmer thermal cycler (Perkin-Elmer, Wellesley, MA, USA). The PCR products varied in size at about 200–700 bp, and they were treated with 0.1 μl exonuclease I (Amersham) and 1 μl shrimp alkaline phosphatase (Amersham, Princeton, NJ, USA) and by incubation at 37 °C for 30 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 3100 autosequencer (Applied Biosystems, Foster City, CA, USA).

cDNA products ZP domain expression plasmids

Full-length cDNAs of the ZP domains of α-tectorin genes were cloned by conventional PCR from the human fetal brain cDNA library (Invitogen, Carlsbad, CA, USA). Two pairs of primers for the entire coding regions of ZP domain including transmembrane domain of α-tectorin were used. PCR steps were denaturing at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min, and then processing with a final extension at 72 °C for 5 min. After amplification, expected sizes of PCR products were confirmed on 2% agarose gel, and the bands were visualized by ethidium bromide upon exposure to an ultraviolet transilluminator.

Produced cDNAs were digested with *Bam*HI/*Eco*RI and cloned into the *Bam*HI/*Eco*RI site of pEGFP-C2 vecter (Clontech, Palo Alto, CA, USA). Ligation reactants were transformed into *Escherichia coli* DH5a. A QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA) was used for purification of plasmid DNA according to the manufacturer's protocol.

Site-directed gene mutagenesis

Gene mutagenesis of the ZP domain cDNA was performed by mega primer PCR.[13] The mutatagenesis primers for 5509TG, 5876A>G and 6063G>A, which were previously reported from Spain,[4] Austria[14] and Japan,[5] respectively, were designed. The following reverse primers were used to produce the mutations for initial PCR reaction: 5509TG CCCCTCGATGCCGGTGCCC TGTCTGTCA, 5876AG TCCAGAGTGTGTTTTTACACATGATATG and 6063GA ACCGAGCTGGAAGAACTTGCACTTAGAT. Initial PCR reactions (20 μl) were prepared containing 0.1 μg of template DNA, 0.4 μM of mutation primer, 0.4 μM of ZP-*Eco*RI primer, 0.1 U of KOD pulse (TOYOBO, Osaka, Japan) and KOD buffer, 2.0 μM $MgSO_4$ and 0.8 μM dNTP. These PCR reactions were denatured at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and then processed with a final extension at 72 °C for 5 min. The subsequent mega primer reactions were prepared containing initial PCR products, which were diluted 50-fold each, 0.4 μM of ZP-*Bam*HI R primer, 2.0 U of Takara Ex-taq (Takara) and Ex-taq buffer (10 ×). These PCR products were inserted into a pEGFP-C2 vector with the same techniques as above. The sequences of all three cDNA constructs were confirmed by DNA sequencing using an ABI 3100 autosequencer.

Transfection and confocal microscopy

COS-7 cells grown in Dulbecco's modified Eagle medium (Mediatech, Herndon, VA, USA) supplemented with 10% fetal calf serum (Moregate, Bulimba, QLD, Australia) were transiently transfected with the indicated plasmids, using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. At 24 h after the transfection, cells were washed twice with phosphate-buffered saline. Cover slips were mounted onto glass slides and visualized under a Leica confocal microscope TCS SP2 AOBS (Leica Microsystems, Wetzlar, Germany).

RESULTS

Mutation screening of the *TECTA* gene

Direct DNA sequencing identified four pathogenic mutation alleles from AD families, including one family in which two mutations were found in one allele. Among those, the family with the c.6063G>A (p.R2021H) mutation was previously reported by Iwasaki *et al.*[5] Including those results, *TECTA* mutations were detected in 2.9% (4/139) of Japanese ADNSHL families, and the prevalence in moderate hearing loss was 7.7% (4/52).

The family F818 pedigree consisted of three generations and included nine affected members (four males and five females), four of whom participated in this study (Figure 1). This family had a p.R1773X (c.5318C>T) mutation affecting the ZA domain in exon 16, and had slowly progressive high frequency hearing loss. Segregation with hearing loss was confirmed in all cases for which DNA samples were available and none of the mutations were detected in controls.

The family F237 pedigree consisted of three generations and included five affected members (three males and two females), four of whom participated in this study (Figure 2). They demonstrated bilateral mild to moderate symmetric sensorineural hearing loss and showed a U-shaped audiogram, affected in the mid frequencies. Vestibular disorder symptoms were not observed, and inner ear abnormalities were not found with CT scans. Two missense mutations in one allele, p. [H1400W; T1866M] (c. [4198C>T; 5597C>T]), were detected in α-tectorin in this family. The mutation H1400W in exon 12 was in the ZA domain of α-tectorin, whereas T1866M was in the ZP domain.

The family F652 pedigree consisted of four generations and included 16 affected members (Figure 3). I1997T (c.5990T>C) mutations were detected in α-tectorin in this family. The mutation in exon 19 located in the ZP domain of α-tectorin. This missense mutation appeared in heterozygosity and was shown to segregate almost completely with the affected status in this family. The audiograms were symmetric and often showed a U shape, which indicates that predominantly the mid frequencies are affected. But, one member, a 11-year-old girl (Figure 3a(III-6)), although bearing the responsible mutation, had a normal audiogram and no demonstrable hearing loss.