backgrounds, environmental factors and mitochondrial haplotypes are necessary for the phenotypic manifestation of the mutation. The degree of hearing loss from mtDNA mutation can be similar within individual families but varied among different family groups, probably due to the modifier effect by nuclear genes.[22]

CONCLUSION

Nineteen known mitochondrial mutations were found predominantly in the maternally inherited group. Among them, frequencies of 1555A>G and 3243A>G mutations were significantly high, indicating that these two mutations are important causes of maternally inherited hearing loss. In addition to the previously reported mitochondrial mutations, we detected 10 novel homoplasmic mutations in the mitochondrial genes related to hearing loss by direct sequencing of whole mitochondrial genomes in Japanese patients. Two of them, 3595A>G and 6204A>G, are possibly associated with hearing loss.

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ORIGINAL ARTICLE

Mutation spectrum and genotype–phenotype correlation of hearing loss patients caused by *SLC26A4* mutations in the Japanese: a large cohort study

Maiko Miyagawa[1], Shin-ya Nishio[1], Shin-ichi Usami[1] and The Deafness Gene Study Consortium[2]

Mutations in *SLC26A4* cause a broad phenotypic spectrum, from typical Pendred syndrome to nonsyndromic hearing loss associated with enlarged vestibular aqueduct. Identification of these mutations is important for accurate diagnosis, proper medical management and appropriate genetic counseling and requires updated information regarding spectrum, clinical characteristics and genotype–phenotype correlations, based on a large cohort. In 100 patients with bilateral enlarged vestibular aqueduct among 1511 Japanese hearing loss probands registered in our gene bank, goiter data were available for 79, of whom 15 had Pendred syndrome and 64 had nonsyndromic hearing loss. We clarified the mutation spectrum for the *SLC26A4* mutations and also summarized hearing levels, progression, fluctuation and existence of genotype–phenotype correlation. *SLC26A4* mutations were identified in 82 of the 100 patients (82.0%). Of the Pendred syndrome patients, 93% (14/15) were carriers, as were 77% (49/64) of the nonsyndromic hearing loss patients. Clinical characteristics of patients with *SLC26A4* mutations were congenital, fluctuating and progressive hearing loss usually associated with vertigo and/or goiter. We found no genotype–phenotype correlations, indicating that, unlike in the case of *GJB2* mutations, the phenotype cannot be predicted from the genotype. Our mutation analysis confirmed the importance of mutations in the *SLC26A4* gene among hearing loss patients with enlarged vestibular aqueduct and revealed the mutation spectrum, essential information when performing genetic testing.
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Keywords: congenital hearing loss; DFNB4; enlarged vestibular aqueduct; goiter; Pendred syndrome; *SLC26A4*

INTRODUCTION

Based on our genetic screening, *SLC26A4* is the second most common responsible gene in Japanese deafness patients.[1] Mutations in the *SLC26A4* gene are known to be responsible for a broad phenotypic spectrum, from typical Pendred syndrome to nonsyndromic hearing loss with enlarged vestibular aqueduct (EVA). The prevalent association of *SLC26A4* mutations in these patients (90% in Pendred syndrome and 78.1% in nonsyndromic hearing loss associated with EVA) indicates the importance of this gene in the pathophysiology of this category of hearing impairment.[2] More than 160 mutations have been found in *SLC26A4* (Pendred/BOR Homepage, http://www.healthcare.uiowa.edu/labs/pendredandbor/), and different mutational spectrums among different ethnic groups have been reported.[2] The identification of *SLC26A4* mutations enables more appropriate genetic counseling and proper medical management for these patients. For such clinical application, updated information regarding mutation spectrum, clinical characteristics and genotype–phenotype correlations based on a large cohort is needed. In addition to our previous reports,[1–7] the present study was performed using a large cohort of patients to collect updated data and summarize these data to enable more precise decision making by ear, nose and throat clinicians.

MATERIALS AND METHODS

Subjects

Data on 1511 independent probands and 1545 family members were collected from 33 ear, nose and throat departments nationwide in Japan and registered in our gene bank. All subjects or next of kin, caretakers or guardians on behalf of the minors/children gave prior written informed consent for participation in the project, and the Shinshu University Ethical Committee as well as the respective Ethical Committees of the other participating institutions of the Deafness Gene Study Consortium (Hokkaido University, Hirosaki University, Iwate Medical University, Tohoku University, Yamagata University, Fukushima Medical University, Jichi Medical University, Gunma University, Nihon University, Nippon Medical School, Nippon Medical School Tama Nagayama

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Hospital, Jikei University, Toranomon Hospital, Kitasato University, Hamamatsu Medical University, Mie University, Shiga Medical Center for Children, Osaka Medical College, Hyogo College of Medicine, Kobe City Medical Center General Hospital, Wakayama Medical University, Okayama University, Yamaguchi University, Ehime University, Kyushu University, Fukuoka University, Nagasaki University, Kanda ENT Clinic, Miyazaki Medical College, Kagoshima University and Ryukyus University) approved the study.

Computerized tomography scan was used to diagnose EVA (according to the criteria of EVA: a diameter of >1.5 mm at the midpoint between the common crus and the external aperture), and they were clinically well characterized by repeated auditory examinations.

The 100 subjects (51 males and 49 females) from among the 1511 probands who met the criteria of bilateral EVA and who ranged in age from 0 to 59 years with a mean age of 13.9 years at the time of examination were enrolled in the current study. Fifteen subjects had Pendred syndrome and 64 had nonsyndromic hearing loss.

The controls were 192 unrelated Japanese healthy individuals with normal hearing evaluated by auditory testing.

Mutation analysis

To identify *SLC26A4* mutations, a DNA fragment containing all the exons of *SLC26A4*, including flanking intronic sequences, was sequenced as described elsewhere.[4] New variants were tested in 192 unrelated normal hearing controls.

Possible pathologic mutations were defined as (1) mutations found to be homozygotes or compound heterozygotes (and determined by segregation study); (2) variants that were not found, or were very few, in the 192 control subjects; and (3) amino acids that were well conserved among various species.

Clinical evaluations

Hearing levels were determined by pure-tone audiometry in adults. For the young patients, conditioned orientation response audiometry or auditory steady-state response was used. Clinical data, including hearing loss progression, fluctuation, episodes of tinnitus and vestibular dysfunction (vertigo, dizziness) and goiter, were collected by anamnestic evaluation. For genotype–phenotype correlation analysis, one-way analysis of variance (Tukey's honest significant difference (HSD) test), Kruskal–Wallis test and multivariate statistics (multiple regression analysis and logistic regression analysis) were used.

RESULTS

SLC26A4 mutation spectrum

There were a total of 39 *SLC26A4* mutations found in the probands with bilateral EVA (Table 1). These mutations were either homozygous, compound heterozygous or heterozygous with no other mutations being detectable. There were two nonsense mutations (p.S610X, p.L727X), three deletion frameshift mutations (c.322delC, c.917delT, c.1219delCT) and three insertion frameshift mutations (c.139insC, c.1652insT, c.2111ins GCTGG). Seven splice site mutations were found (c.416-1G>A, c.600+1G>T, c.601-1G>A, c.919-2A>G, c.1001+1G>A, c.1002-9A>G and c.1707+5G>A).

There were 24 missense mutations (p.P76S, p.T94I, p.P123S, p.M147V, p.P297Q, p.K369E, p.A372V, p.N392Y, p.G396E, p.T410M, p.A434T, p.G439R, p.S448L, p.T527P, p.I529S, p.S532I, p.C565Y, p.R581S, p.S657N, p.V659L, p.S666F, p.T721M, p.H723R and p.H723Y). To evaluate the evolutionary conservation of the amino acids affected by these missense mutations, we made an alignment of the *SLC26A4* amino acid sequence of four mammalian species: human, rat, cow and mouse. On the basis of this alignment, all missense mutations had changed evolutionary conserved amino acids. Of these mutations, nine variants had not been reported. We checked the 192 control subjects with normal hearing, but with the exception of p.H723R in 1 case, no mutations were detected.

Sequencing identified mutations in 82 of the 100 patients (82.0%). Mutations were detected in 93% of those with Pendred syndrome (14/15) and 77% (49/64) of those with nonsyndromic hearing loss. Of these, 15/100 (15.0%) were homozygous, 51/100 (51.0%) were compound heterozygous and 16/100 (16.0%) were heterozygous (Table 2).

The most frequent mutation was p.H723R that accounted for 36.0%, and the second was c.919-2A>G found in 7.0%, followed by c.1707+5G>A (4.0%). Frequency of the other 36 mutations was very low (0.5–2.0%).

Clinical findings

Table 2 shows the clinical details for the 100 subjects.

The subjects had an average hearing level of 80.9 dB (7.5–112.5 dB), with hearing loss that was mild in 5, moderate in 22, severe in 37, profound in 19 and unknown in 12. Regarding onset age of hearing loss, 45 patients were congenital, 18 were prelingual (1–3 years old), 20 were from 4 to 14 years and 17 were unknown. These results clearly indicated that early onset is dominant in patients with EVA. Also, 70 patients (70%) showed progressive hearing loss and 56 patients (56.0%) felt fluctuation of hearing. With regard to the 79 patients for whom data on vertigo were available, 41 patients complained of vertigo and 38 did not. Of the 79 patients for whom data on goiter were available, 15 had goiter and 64 did not, with an onset age from 12 to 33 years. As to family history, all families were recessive inheritance or sporadic cases.

Genotype–phenotype correlations for diagnostic age, fluctuation, vertigo, tinnitus and goiter are summarized in Figure 1.

We defined nonsense or frameshift mutations as truncating (T) and missense mutations as nontruncating (NT) and classified the genotypes as truncating/truncating (T/T), truncating/nontruncating (T/NT) or nontruncating/nontruncating (NT/NT). Significant differences were not found between the groups in any of the clinical features (Tukey's HSD test was used for diagnostic age and Kruskal–Wallis test was used for fluctuation, vertigo, tinnitus and goiter, all tests indicated $P>0.05$; Figure 1). Figure 2 shows the relationship between hearing loss severity and the mutation (T or NT) that also showed no significant differences (Tukey's HSD test, $P>0.05$). We also performed multivariate statistics (multiple regression analysis and logistic regression analysis) and we found that only the age of the patients correlated with the hearing loss severity while the genotype of *SLC26A4* mutations did not significantly affect the hearing loss severity ($P>0.05$).

DISCUSSION

The present large cohort study revealed a high prevalence (82%; 82/100) of *SLC26A4* mutations in sensorineural hearing loss patients with EVA in Japanese. The frequency (8.7%) is the second most common next to *GJB2* that is found in 16.2% of overall and 25.6% of congenital hearing loss patients.[1]

Our mutation analysis results confirmed the previous reports that indicated the importance of this gene among hearing loss patients with EVA. This study also added novel mutations and summarized updated data for the precise molecular diagnosis.

First, the high prevalence (82%) of *SLC26A4* mutations in EVA patients is compatible with the high prevalence of *SLC26A4* mutations reported in eastern Asians; that is, 97.9% in Chinese,[8] and 92% in Koreans.[9] These frequencies are higher than those reported in Caucasoid populations (20% in Americans,[10] 40.0% in French[11] and 28.4% in Spanish[12]). It is still an open question whether other genes are involved in the EVA patients without *SLC26A4* mutations.

Table 1 Possible pathogenic variants found in enlarged vestibular aqueduct (EVA) subjects ($n = 100$)

			Frequency (n = 100)				
Nucleotide change	*Amino acid change*	*Exon*	*Homozygote*	*Compound heterozygote*	*Heterozygote*	*Allele frequency (in 200 alleles)*	*References*
c. 139insC		1		1		0.50	This study
c. 266C>T	p. P76S	2		1		0.50	Suzuki *et al.*[5,6]
c. 281C>T	p. T94I	3		1		0.50	Wang *et al.*[7,8]
c. 322delC		4		1		0.50	Tsukamoto *et al.*[2,4]
c. 367C>T	p. P123S	4		1		0.50	Tsukamoto *et al.*[2,4]
c. 416-1G>A		Intron 4		2		1.00	Tsukamoto *et al.*[2,4]
c. 439A>G	p. M147V	5		2		1.00	Tsukamoto *et al.*[2,4]
c. 600+1G>T		Intron 5		1		0.50	This study
c. 601-1G>A		Intron 5		1		0.50	Tsukamoto *et al.*[2,4]
c. 890C>A	p. P297Q	7		1		0.50	This study
c. 917delT		7			1	0.50	Tsukamoto *et al.*[2,4]
c. 919-2A>G		Intron 7	1	11	1	7.00	Coucke *et al.*[21]
c. 1001+1G>A		Intron 8		2		1.00	Coyle *et al.*[22]
c. 1002-9A>G[a]		Intron 8		1		0.50	This study
c. 1105A>G	p. K369E	9		1		0.50	Usami *et al.*[2,3]
c. 1115C>T	p. A372V	9		1		0.50	Usami *et al.*[2,3]
c. 1174A>T	p. N392Y	10		3		1.50	Park *et al.*[14,16]
c. 1187G>A	p. G396E	10		1		0.50	This study
c. 1219delCT		10		1		0.50	This study
c. 1229C>T	p. T410M	10	1	1		1.50	Coyle *et al.*[22]
c. 1300G>A	p. A434T	11			1	0.50	This study
c. 1315G>A	p. G439R	11		1		0.50	Suzuki *et al.*[5,6]
c. 1343C>T	p. S448L	11		1		0.50	Wang *et al.*[7,8]
c. 1579A>G	p. T527P	14		2		1.00	Suzuki *et al.*[5,6]
c. 1586T>G	p. I529S	14		1		0.50	Wang *et al.*[7,8]
c. 1595G>T	p. S532I	14		2		1.00	Usami *et al.*[3,17]
c. 1652insT		15		3	1	2.00	Tsukamoto *et al.*[2,4]
c. 1694G>A	p. C565Y	15		1		0.50	Tsukamoto *et al.*[2,4]
c. 1707+5G>A		Intron 15	1	6		4.00	Park *et al.*[8,9]
c. 1743G>C	p. R581S	16		2		1.00	Iwasaki *et al.*[5,18]
c. 1829C>A	p. S610X	17		1		0.50	Tsukamoto *et al.*[2,4]
c. 1970G>A	p. S657N	17		1		0.50	Tsukamoto *et al.*[2,4]
c. 1975G>C	p. V659L	17		3		1.50	Wang *et al.*[7,8]
c. 1997C>T	p. S666F	17		1		0.50	Tsukamoto *et al.*[2,4]
c. 2111ins GCTGG		19		1	1	1.00	Usami *et al.*[2,3]
c. 2162C>T	p. T721M	19		1	1	1.00	Usami *et al.*[2,3]
c. 2168A>G	p. H723R	19	11	40	10	36.00	Usami *et al.*[2,3]
c. 2168C>T	p. H723Y	19	1			1.00	This study
c. 2180T>A	p. L727X	19		1		0.50	This study

[a]c. 1002-9A>G, uncertain pathogenicity.

Mutations in *FOXI1*,[13] a modulatory gene of *SLC26A4*, were not found in our series of patients (data not shown). As seen in previous mutation screening reports, we encountered a significant number of heterozygous cases without a second mutation even after direct sequencing of the coding region of the gene. It is highly likely that there is one more occult mutation somewhere because patients with heterozygous mutation are associated with EVA.

Second, it is evident that the mutation spectrum found in the Japanese population is quite different from that in Caucasoid populations, but similar to the mutation spectrum reported in the Asian populations, especially Koreans.[8–12,14] There are two frequent mutations in east Asians, namely p.H723R and c.919-2A>G. p.H723R is most prevalent in the Japanese and Korean populations,[8] whereas c.919-2A>G is most common in the Chinese.[7]

The existence of a genotype–phenotype correlation is still controversial.[6,12,15] Mutations in *SLC26A4* can cause a broad phenotypic spectrum, from typical Pendred syndrome to nonsyndromic hearing loss associated with EVA. In the present study, various features of the phenotype were compared with the genotypes. We defined nonsense or frame shift mutations as truncating (T) and missense mutations as non-truncating (NT) and classified the genotypes as truncating/truncating (T/T), truncating/non-truncating (T/NT), or non-truncating/non-truncating (NT/NT). However, statistical differences were not found between the groups in any of the clinical features (χ^2 tests, $P > 0.05$; Figure 1).

Concerning the relationship between the severity of hearing loss and individual *SLC26A4* mutations, several functional studies have demonstrated the property of transporter function.[16–18] Furthermore,

Table 2 Phenotypes and genotypes of affected EVA subjects

ID	*Age*	*Mutation allele 1/allele 2*	*Age of awareness*	*Progression*	*Fluctuation*	*Tinnitus*	*Vertigo*	*Goiter*	*Threshold (Rt) (dB)*[a]	*Threshold (Lt) (dB)*[a]	*Hearing level in the low frequencies*[b]
77	12	p. [917delT];[=]	12	+	+	+	+	−	58.75	45	49.375
237	7	p. [T721M];[H723R]	0	+	−	−	+	−	112.5	68.75	83.75
334	23	p. [A372V];[H723R]	0	NA	NA	+	NA	NA	96.25	83.75	81.9
695	4	p. [K369E];[H723R]	0	+	−	NA	NA	−	100	90	89.4
752	18	p. [1652insT];[=]	1	−	−	+	+	+	98.75	102.5	96.3
1045	25	p. [H723R];[H723R]	0	+	NA	−	+	+	78.75	90	85.6
1306	3	p. [919-2A>G];[H723R]	0	NA	NA	NA	NA	NA	NA	NA	NA
1365	20	p. [T721M];[=]	2	NA	NA	NA	NA	NA	96.25	105	96.9
1379	10	p. [1001+1G>A];[H723R]	0	+	+	−	−	NA	66.25	46.25	57.5
1432	6	p. [H723R];[=]	0	+	−	−	−	NA	102.5	105	100.0
1625	16	p. [919-2A>G];[H723R]	0	+	+	NA	+	NA	100	95	88.1
1795	NA	p. [H723R];[=]	NA	NA	N/A	NA	NA	NA	NA	NA	NA
1820	12	p. [H723R];[H723R]	5	+	+	−	−	NA	72.5	73.75	61.3
1957	7	p. [S666F];[H723R]	3	+	+	NA	NA	−	95	101.25	93.8
1961	12	p. [C565Y];[H723R]	0	+	N/A	NA	NA	NA	108.75	110	103.8
2010	12	p. [416-1G>A];[H723R]	9	+	+	−	−	+	80	91.25	81.3
2202	4	p. [P297Q];[T527P]	3	+	−	−	−	−	77.5	76.25	73.8
2331	31	p. [H723R];[H723R]	0	+	+	+	+	+	90	100	87.5
2449	1	p. [139insC];[322delC]	0	NA	NA	−	+	−	100	85	92.5
2462	52	p. [M147V];[H723R]	2	+	+	−	−	−	98.75	95	88.1
2498	0	p. [919-2A>G]; [1001+1G>A]	0	+	+	NA	−	−	86.25	86.25	83.8
2538	10	p. [H723R];[H723R]	3	+	+	−	−	+	81.25	55	66.9
2621	3	p. [R581S];[H723R]	0	+	+	−	−	−	91.25	91.25	90.0
2695	13	p. [T527P];[H723R]	2	+	+	+	+	−	62.5	61.25	63.1
2728	3	p. [919-2A>G];[H723R]	1	+	+	−	−	−	97.5	97.5	93.8
2798	15	p. [H723R];[H723R]	4	+	+	NA	+	+	52.5	96.25	66.3
2804	2	p. [1707+5G>A];[H723R]	0	+	+	−	−	−	78.75	78.75	82.5
3072	44	p. [G439R];[H723R]	6	+	+	+	+	−	110	108.75	105.0
3074	21	p. [H723R]; [=]	2	+	+	+	+	+	105	106.25	99.4
3298	6	p. [919-2A>G];[H723R]	0	+	+	+	+	−	73.75	110	86.9
3301	4	p. [416-1G>A];[H723R]	0	+	+	+	+	−	65	72.5	68.1
3442	6	p. [919-2A>G];[H723R]	NA	+	NA	+	+	−	81.25	50	60.0
3450	14	p. [H723R];[H723R]	0	+	+	+	+	−	110	73.75	87.5
3561	6	p. [H723Y];[H723Y]	4	NA	NA	NA	NA	NA	83.75	65	71.3
3994	59	p. [601-1G>A];[H723R]	10	+	+	+	+	+	96.0	94	91.3
3996	8	p. [H723R];[1652insT]	0	+	−	+	−	−	100	110	98.1
3999	8	p. [H723R];[1652insT]	0	+	+	−	+	−	30	50	40.0
4050	5	p. [M147V];[H723R]	1	+	+	+	+	−	107.5	85	93.8
4097	3	p. [N392Y];[1002-9A>G]	0	−	−	−	−	−	106.25	85	93.1
4098	26	p. [N392Y];[919-2A>G]	2	−	+	+	+	−	110	37.5	71.3
4102	5	p. [N392Y];[H723R]	0	+	+	+	+	−	95	78.75	83.1
4131	10	p. [H723R];[=]	8	+	+	−	−	−	81.25	60	70.6
4144	21	p. [H723R];[H723R]	4	+	NA	+	+	−	93.75	105	95.6
4232	15	p. [V659L];[H723R]	NA	−	+	+	+	−	60	92.5	69.4
4299	4	p. [S532I];[2111ins GCTGG]	3	−	+	−	+	−	17.5	70	42.5
4305	14	p. [A434T];[=]	0	+	−	+	−	−	110	110	105.0
4320	10	p. [G396E];[S532I]	NA	+	+	+	−	−	72.5	80	72.5
4338	6	p. [R581S];[H723R]	0	+	+	+	+	−	78.75	52.5	64.4
4380	10	p. [1707+5G>A];[H723R]	2	+	+	−	−	−	96.25	81.25	84.4
4386	21	p. [H723R];[H723R]	NA	+	+	+	+	+	77.5	93.75	85.0
4398	4	p. [1652insT];[H723R]	2	+	+	+	+	−	70	97.5	86.9
4434	8	p. [T410M];[1707+5G>A]	1	+	+	−	+	−	92.5	100	91.3
4469	11	p. [H723R]; [=]	0	+	NA	−	−	−	20	21.25	16.9
4485	40	p. [H723R]; [=]	10	+	+	+	+	−	56.25	65	58.8

Table 2 (Continued)

ID	Age	Mutation allele 1/allele 2	Age of awareness	Progression	Fluctuation	Tinnitus	Vertigo	Goiter	Threshold (Rt) (dB)[a]	Threshold (Lt) (dB)[a]	Hearing level in the low frequencies[b]
4486	20	p. [1707+5G>A]; [1707+5G>A]	4	+	+	+	+	+	72.5	95	78.1
4490	25	p. [T410M];[T410M]	0	−	−	+	+	+	87.5	92.5	90.0
4508	29	p. [H723R];[H723R]	5	+	+	−	−	−	85	110	91.9
4518	26	p. [H723R];[919-2A>G]	0	+	+	+	+	−	105	97.5	98.1
4530	5	p. [H723R];[919-2A>G]	0	+	+	−	+	−	67.5	86.25	71.9
4545	12	p. [1707+5G>A];[H723R]	4	+	+	+	+	+	86.25	28.75	53.1
4549	13	p. [V659L];[1219delCT]	NA	+	+	+	+	−	38.75	50	38.1
4663	0	p. [1707+5G>A];[H723R]	0	−	+	NA	NA	−	68.75	68.75	99.2
4696	0	p. [V659L];[H723R]	0	+	−	NA	NA	−	NA	NA	97.5
4362	26	p. [H723R]; [=]	6	+	−	−	−	−	70	68.75	63.8
4513	34	p. [H723R]; [=]	NA	+	+	+	NA	−	71.25	53.75	61.3
4645	23	p. [919-2A>G]; [=]	14	+	−	+	−	−	96.25	105	93.8
723	NA	p. [H723R]; [=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
724	NA	p. [2111ins5bp]; [=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
742	NA	p. [H723R]; [=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
1975	3	p. [H723R];[H723R]	0	NA	NA	NA	NA	NA	80	70	62.5
2082	2	p. [H723R];[H723R]	0	−	−	−	−	−	NA	NA	NA
4735	9	p. [H723R];[919-2A>G]	0	+	+	+	+	−	107.5	110	103.8
195	20	p. [=];[=]	2	+	+	+	+	−	83.75	83.75	81.9
670	8	p. [=];[=]	3	+	−	+	−	−	26.25	107.5	62.5
1755	16	p. [=];[=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
2607	5	p. [=];[=]	0	−	+	−	−	−	97.5	105	98.8
3851	33	p. [=];[=]	0	+	+	+	−	+	103.75	103.75	100.6
4194	11	p. [=];[=]	NA	+	+	−	−	−	67.5	80	76.3
4215	5	p. [=];[=]	0	+	+	−	−	−	98.75	93.75	93.8
4216	55	p. [=];[=]	NA	+	+	+	+	NA	51.25	78.75	68.8
4258	30	p. [=];[=]	28	NA	−	+	−	−	17.5	7.5	13.8
4281	6	p. [=];[=]	2	−	−	−	−	−	57.5	61.25	63.1
4324	37	p. [=];[=]	6	−	−	−	−	−	10	27.5	22.5
4352	3	p. [=];[=]	0	+	+	−	−	−	86.25	88.75	88.1
4357	6	p. [=];[=]	4	+	+	+	−	−	71.25	72.5	67.5
4397	5	p. [=];[=]	0	−	−	−	−	−	102.5	105	100.6
4402	8	p. [=];[=]	0	+	−	−	−	−	100	90	88.8
4450	12	p. [=];[=]	NA	+	+	+	−	−	NA	NA	NA
4462	8	p. [=];[=]	7	+	−	+	−	−	63.75	20	41.3
4488	1	p. [=];[=]	0	−	−	NA	−	−	97.5	97.5	95.0
4671	2	p. [H723R];[600+1G>T]	0	+	−	−	+	−	NA	NA	NA
3253	NA	p. [I529S];[H723R]	NA	NA	NA	NA	NA	NA	NA	NA	NA
4949	0	p. [L727X];[H723R]	0	+	−	−	−	−	NA	NA	51.7
J27	NA	p. [H723R];[S448L]	NA	NA	NA	NA	NA	NA	NA	NA	90.6
3309	5	p. [919-2A>G];[P76S]	0	+	+	+	+	−	106.25	106.25	101.3
J15	0	p. [P123S];[H723R]	0	NA	NA	NA	NA	NA	NA	NA	NA
FUK2004	1	p. [H723R];[T94I]	0	NA	NA	N/A	NA	NA	NA	NA	85.0
1299	NA	p. [S610X];[S657N]	0	NA	NA	NA	NA	NA	NA	NA	NA
SNS5500	42	p. [919-2A>G];[919-2A>G]	4	+	+	+	+	+	70	81.3	64
SNS5503	37	p. [H723R];[1707+5G>A]	5	+	+	+	+	+	67.5	70	NA

Abbreviation: EVA, enlarged vestibular aqueduct; Lt, left; NA, not available; Rt, right.
[a]Average of 500, 1000, 2000 and 4000 Hz.
[b]Average of 125, 250 and 500 Hz.

retention of improperly folded Pendrin mutants in the endoplasmic reticulum has been suggested as the major pathological mechanism for Pendred syndrome.[19,20] In this study, we compared not only the difference between the T and NT mutations, but also compared the individual mutations and severity of hearing. However, there were no correlations (data not shown). Indeed, there was great variation regarding hearing loss severity even with the same mutations. For example, in the patients homozygous for the most prevalent mutation, p.H723R, hearing level at low frequency varied from 61 to 99 dB (Table 2). In addition, many reports have described intrafamilial

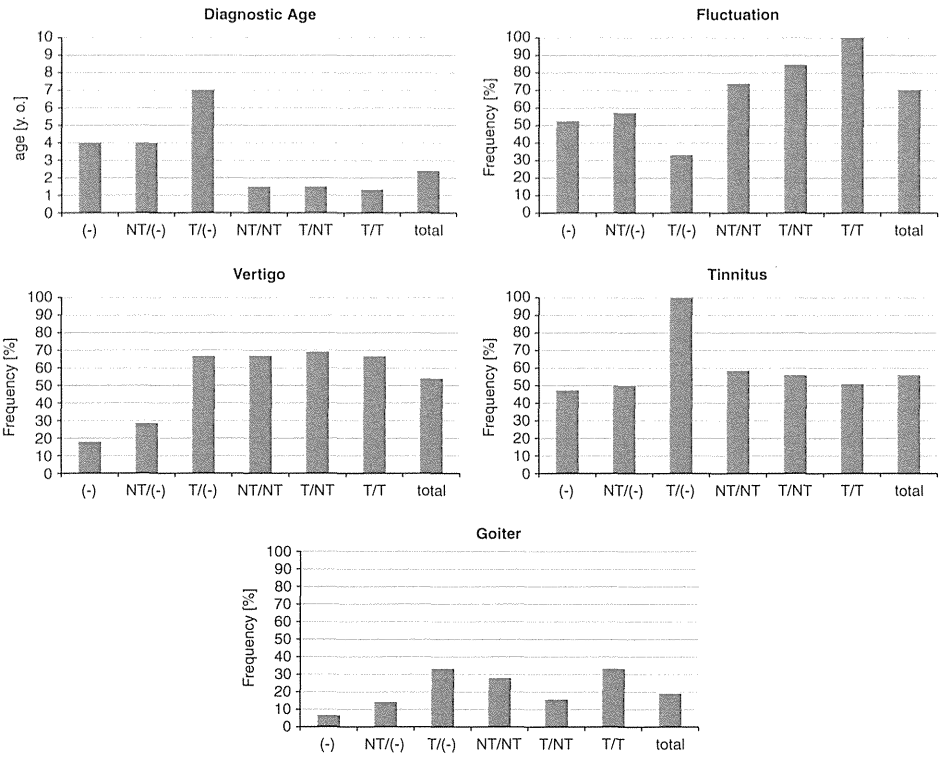

Figure 1 Genotypes and phenotypes (diagnostic age, fluctuation, vertigo, tinnitus and goiter) in the current study. NT/(−), heterozygote of nontruncating mutation; NT/NT, nontruncating/nontruncating; NT/T, nontruncating/truncating; T/(−), heterozygote of truncating mutation; T/T, truncating/truncating; (−), wild type.

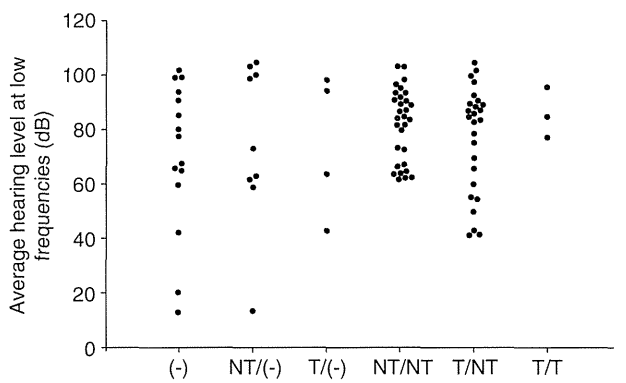

Figure 2 The relationship between hearing level at the lower frequencies and genotype. Hearing level was the average of 125, 250 and 500 Hz. NT/(−), heterozygote of nontruncating mutation; NT/NT, nontruncating/nontruncating; NT/T, nontruncating/truncating; T/(−), heterozygote of truncating mutation; T/T, truncating/truncating; (−), wild type.

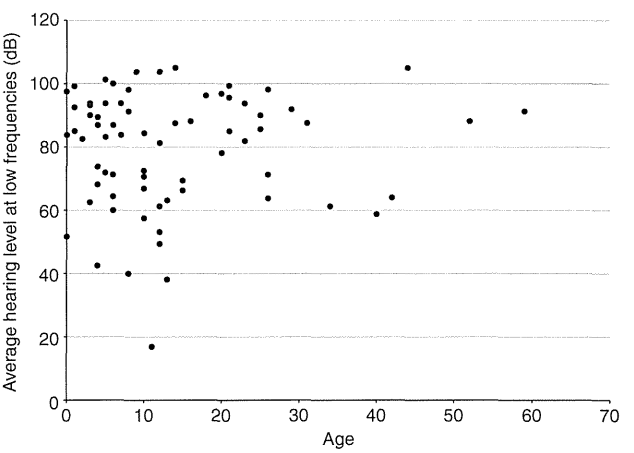

Figure 3 The relationship between hearing level and age in subjects with biallelic *SLC26A4* mutations. Hearing level was calculated as the average of 250, 500, 1000 and 2000 Hz in both sides.

phenotypic variation.[8–12] Therefore, phenotype may be determined not only by *SLC26A4* mutations but also other factors (genetic as well as environmental), contributing to such variability (Figure 2).

Unlike in the case of *GJB2*, phenotype cannot be predicted from the genotype;[6] however, the clarification of clinical features will enable more appropriate genetic counseling and proper medical management for these patients.

The present study confirmed clinical characteristics of 66 patients with EVA caused by biallelic *SLC26A4* mutations. These included congenital (5/63, 7.9%), fluctuated (42/52, 80.8%) and progressive (49/56, 87.5%) hearing loss usually associated with vertigo (35/52, 67.3%) and/or goiter (12/53, 22.6%) during long-term follow-up, in accordance with our previous study.[6] It is known that goiter sometimes becomes apparent between 10 and 20 years of age. The present cohort included young children, and therefore the frequency of goiter may be underestimated. As seen in Figure 3, in 66 patients with biallelic mutations for whom data were available, onset of hearing loss was likely to be early onset, and progressive with age.

CONCLUSIONS

Pendred syndrome and nonsyndromic hearing loss associated with EVA are a continuum of disease characterized as being associated with congenital, fluctuating and progressive hearing loss, and most patients have vertigo and/or goiter. However, in the present study, no genotype–phenotype correlation was found. The results obtained from the present study will facilitate accurate molecular diagnosis and better genetic counseling.

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ORIGINAL ARTICLE

A Japanese family showing high-frequency hearing loss with *KCNQ4* and *TECTA* mutations

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Abstract

Conclusions: We describe a Japanese family with high-frequency sensorineural hearing loss (SNHL) harboring a c.211delC mutation in the *KCNQ4* gene. Families showing progressive high-frequency SNHL should be investigated for mutations in the *KCNQ4* gene. *Objective:* To determine the responsible deafness gene in a Japanese family with dominantly inherited high-frequency SNHL of unknown etiology. *Methods:* We performed hearing tests for five members of the family, and the three affected with hearing loss underwent further audiological and vestibular examinations. Genetic analysis was performed to identify any possible causative mutations, as well as analysis of detailed clinical findings to determine the phenotype. *Results:* The three affected subjects showed high-frequency SNHL. Extensive audiologic evaluation suggested cochlear involvement and progressive hearing loss. As for bilateral caloric testing, two of the three affected subjects showed hyporeflexia with recurrent vestibular symptoms. We identified the c.211delC mutation in the *KCNQ4* gene and the c.2967C>A (p.H989Q) mutation in the *TECTA* gene. Based on the genotype–phenotype correlation, the c.211delC mutation in the *KCNQ4* gene was associated with high-frequency SNHL in this family.

Keywords: *Progressive hearing loss, c.211delC mutation, hyporeflexia, deafness gene*

Introduction

There are over 100 loci associated with nonsyndromic sensorineural hearing loss (SNHL) in humans [1]. To date, more than 60 loci of DFNA, the gene locus responsible for autosomal dominant deafness, have been identified and 27 genes were defined as DFNA-causative (Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage: http://hereditaryhearingloss.org). The *KCNQ4* and *TECTA* genes are frequently associated with autosomal dominant nonsyndromic SNHL [2]. *KCNQ4* is a member of the voltage-gated potassium channel family localized in inner and outer hair cells and plays a role in potassium recycling in the inner ear. *KCNQ4* is composed of 695 amino acids with 6 transmembrane domains and a hydrophobic P-loop region that forms a channel pore containing a potassium ion-selective filter located between the transmembrane domains S5 and S6 (residues 259–296) [3]. *KCNQ4*-associated hearing loss has been reported to be typically late-onset high-frequency-involved and progressive over time [4]. More than 20 pathologic mutations have been identified in *KCNQ4* and they are mostly missense mutations with a dominant-negative mechanism that causes progressive, predominantly high-frequency hearing impairment [3,5]. Recently, Naito et al. reported a novel recurrent deletion mutation, c.211delC, in 13 Japanese patients with high-frequency-involved hearing loss [5]. This

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deletion mutation located in the N-terminal site causes truncation of *KCNQ4* protein product, and might have insufficient function for inner ear potassium recycling [5]. In contrast, the *TECTA* gene encodes α-tectorin, the major component of noncollagenous glycoprotein of the tectorial membrane, and has a role in intracochlear sound transmission [6]. Mutations of the *TECTA* gene cause ultrastructural defects of the tectorial membrane, in turn causing hearing loss [7]. 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 [7]. Missense mutations affecting the ZP domain are associated with mid-frequency hearing loss, whereas mutations in the ZA domain are associated with hearing impairment primarily affecting the high frequencies [8].

We investigated the genetic cause in a Japanese family carrying nonsyndromic high-frequency SNHL with an autosomal dominant inheritance pattern. In addition, we analyzed their detailed audiological and vestibular findings.

Material and methods

Medical history and otological examination

One proband, as well as two other affected and two unaffected family members, from one autosomal dominant inherited SNHL family participated in this study. A complete history concerning hearing loss and symptoms potentially related to syndromic hearing loss was taken from all subjects and they all underwent otoscopic examination. Pure-tone audiometry was conducted in an acoustically isolated room using an AA-78 audiometer (Rion, Tokyo, Japan). Air- and bone-conduction thresholds were measured as decibel hearing level.

Detailed audiological and vestibular examination

Two of the three affected subjects underwent self-recording audiometry and evoked and distortion-product otoacoustic emissions (EOAE and DPOAE) examinations. All three underwent speech discrimination testing and caloric testing. In caloric testing, electronystagmography was recorded by cold water irrigation (20°C, 5 ml, 20 s). The details of the methods used for these evaluations, including self-recording audiometry, EOAE and DPOAE, speech discrimination testing, and caloric testing have been described previously [9].

Sequencing analysis of the KCNQ4 gene and TECTA gene

All 14 exons and flanking intronic sequences of the *KCNQ4* gene and all 23 exons and flanking intronic sequences of the *TECTA* gene were amplified by polymerase chain reaction (PCR). Primers were designed to flank all of the exon–intron boundaries through use of the Primer3Plus web-based server (http://primer3-plus.com). Each genomic DNA sample (40 ng) was amplified using a Multiplex PCR Assay Kit (Takara, Shiga, Japan) for 5 min at 95°C, followed by 40 three-step cycles of 94°C for 30 s, 60–67.6°C for 90 s, and 72°C for 90 s, with a final extension at 72°C for 10 min, ending with a holding period at 4°C in a Perkin-Elmer thermal cycler. The PCR products varied in size at about 100–400 bp, and they were treated with ExoSAP-IT (GE Healthcare Bio, Santa Clara, CA) 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). Computer analysis to predict the effect of missense variants on the protein function was performed with wANNOVAR [10] (http://wannovar.usc.edu) including the following functional prediction software: PhyloP (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/phyloP44way/), Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/), likelihood ratio test (LRT; http://www.genetics.wustl.edu/jflab/lrt_query.html), and MutationTaster (http:/www.mutationtaster.org/).

Ethics statement

All subjects gave prior written informed consent for participation in the project, and the Ethical Committee of Jichi Medical University approved the study.

Results

Mutation analysis

We identified the c.211delC mutation in the *KCNQ4* gene in four of the subjects (three with high-frequency SNHL and one without SNHL), and the c.2967C>A (p.H989Q) mutation in the *TECTA* gene in two subjects with high-frequency SNHL (Figure 1).

Medical history and clinical findings

Otoscopic examination demonstrated a normal tympanic membrane in both ears of all five subjects.

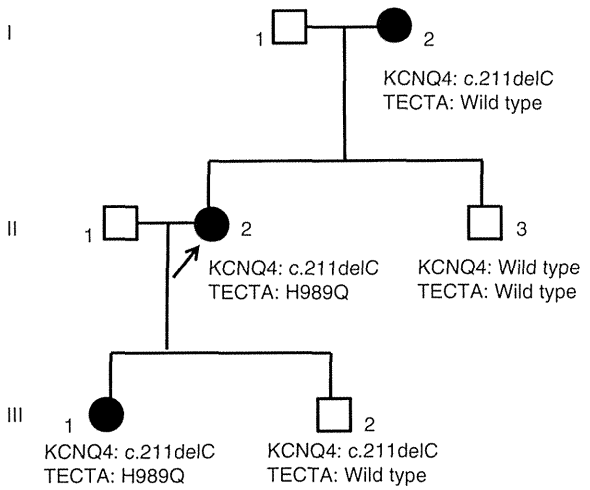

Figure 1. Pedigree of the family and the detected mutations in the *KCNQ4* and *TECTA* genes. The arrow indicates the proband.

Audiometric examination confirmed high-frequency SNHL in three of the five subjects (I-2, II-2, and III-1 in Figure 2). Self-recording audiometry showed Jerger type I [11] hearing loss in both ears of subject III-1, indicating that they had normal hearing. On the other hand, subject I-2 showed Jerger type II [11] hearing loss in the high-frequency area in both ears, indicating that this subject's hearing loss was of cochlear origin (Figure 3). Maximum speech discrimination scores in the three subjects with hearing loss showed mild to moderate defects, with subject I-2 having the lowest scores (Table I). Subject I-2 had no detectable DPOAE, but in two subjects (II-2 and III-1), DPOAE were were detected only in the lower frequency area. Subject III-2 carried the c.211delC mutation but did not have SNHL and showed normal DPOAE (Figure 4). As for bilateral caloric testing, subjects II-2 and III-1 showed hyporeflexia in the right ear with recurrent vestibular symptoms, while subject I-2 showed normal response without vestibular symptoms (Figure 5).

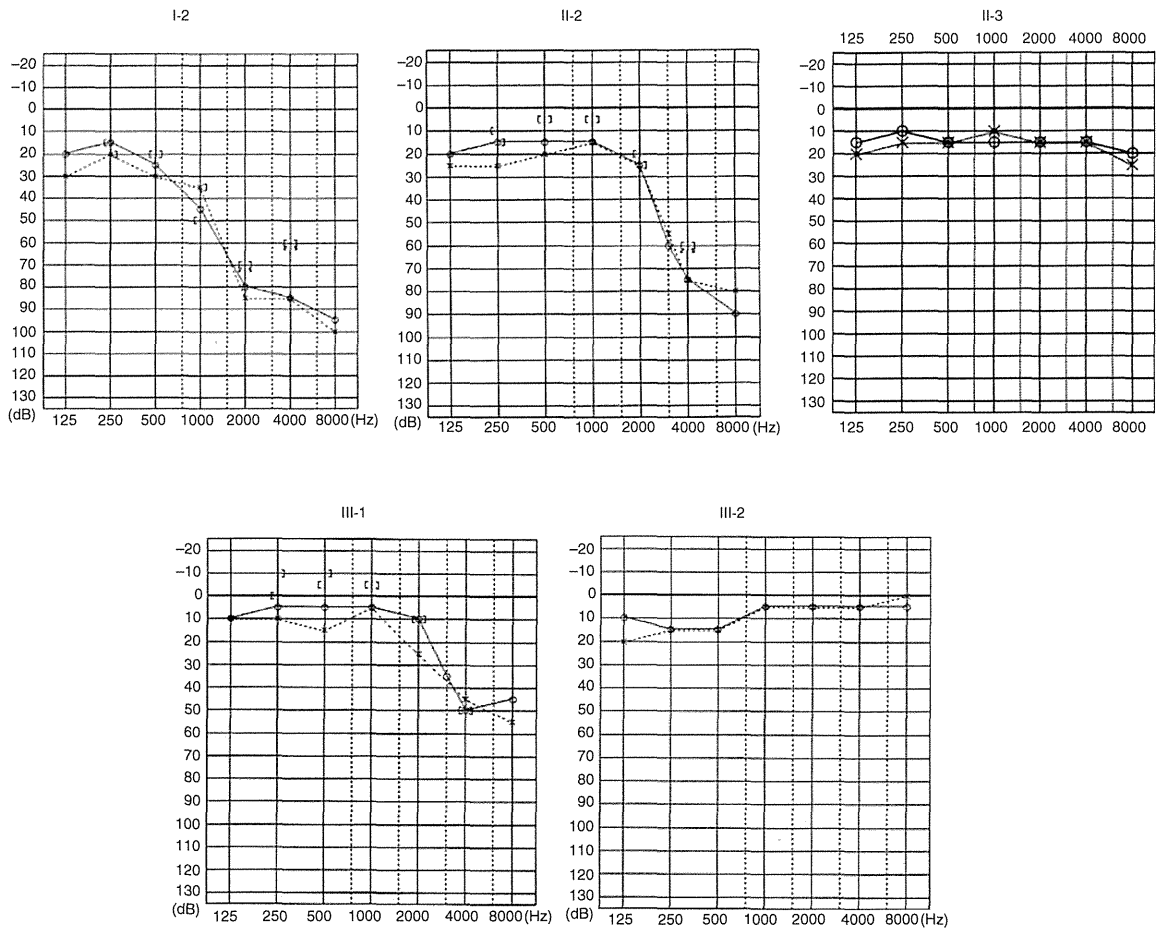

Figure 2. Pure-tone audiograms of the five family members shown in the Figure 1 pedigree.

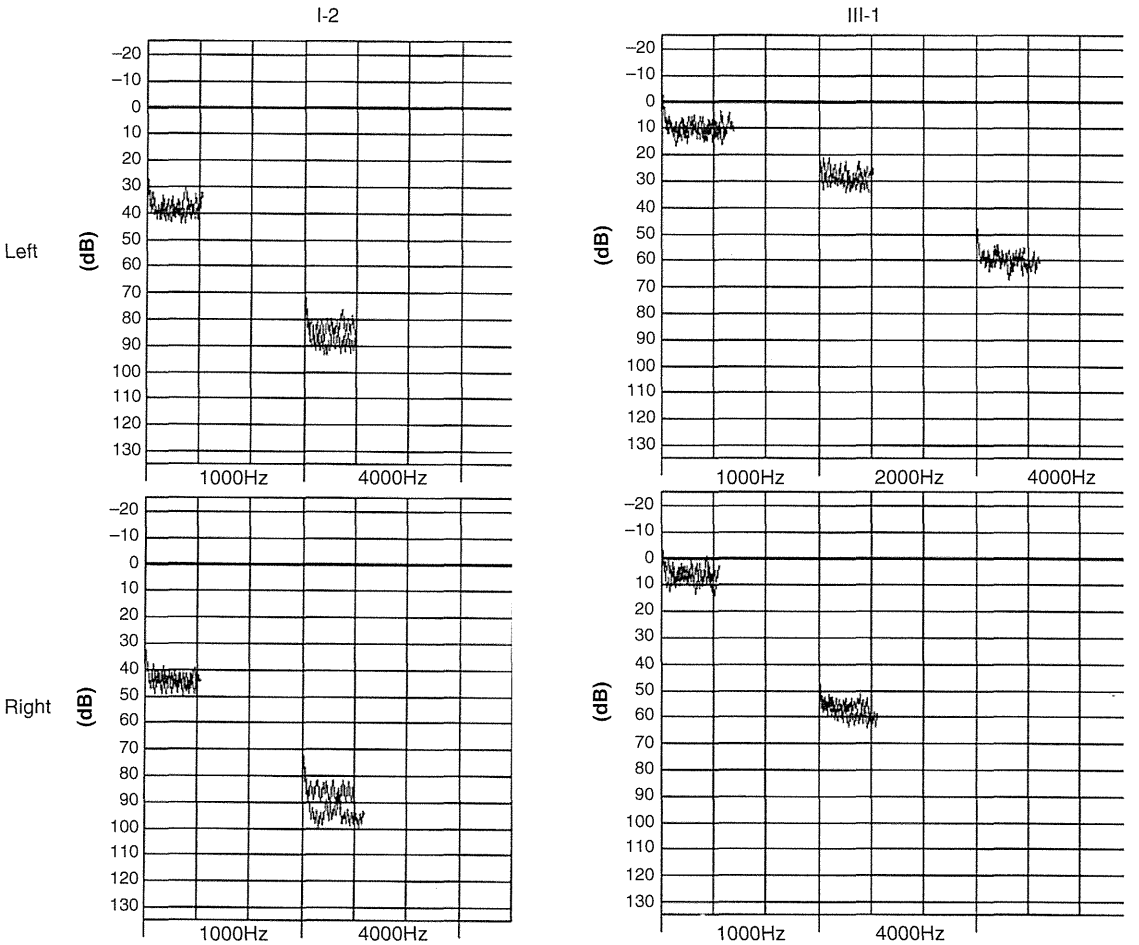

Figure 3. Self-recording audiometry results of two of the three subjects with high-frequency sensorineural hearing loss (SNHL).

Discussion

In the present study, we found a c.211delC mutation in the *KCNQ4* gene, as well as a c.2967C>A (p.H989Q) mutation in the *TECTA* gene, in an autosomal dominant inherited Japanese family with nonsyndromic high-frequency SNHL. The pathogenicity of the c.211delC mutation is strongly supported by the occurrence of the same mutation in several independent families with progressive nonsyndromic high-frequency SNHL [5,12]. Naito et al. reported that SNHL associated with the c.211delC mutation showed significant progression in only high frequencies by detailed progression analysis [5]. One subject (III-2), aged 6 years, carried the c.211delC mutation but did not have SNHL, suggesting that he may develop progressive high-frequency hearing loss in future. We explained this to the family, as it is the type of important information that we impart to patients during genetic counseling in our hospital.

Table I. Maximum speech discrimination scores of the three subjects with high-frequency sensorineural hearing loss (SNHL).

Subject	Age (years)	Side	Maximal speech discrimination (%)
I-2	55	Right	56
		Left	42
II-2	34	Right	74
		Left	78
III-1	14	Right	80
		Left	72

In the present family, subject I-2 (aged 55) showed the worst speech discrimination compared with II-2 (aged 34) and III-1 (aged 14), consistent with progressive hearing loss. Because subject I-2 also retained a nearly normal hearing level in low frequencies, it is highly likely that the c.211delC mutation does not cause profound deafness. This speculation is

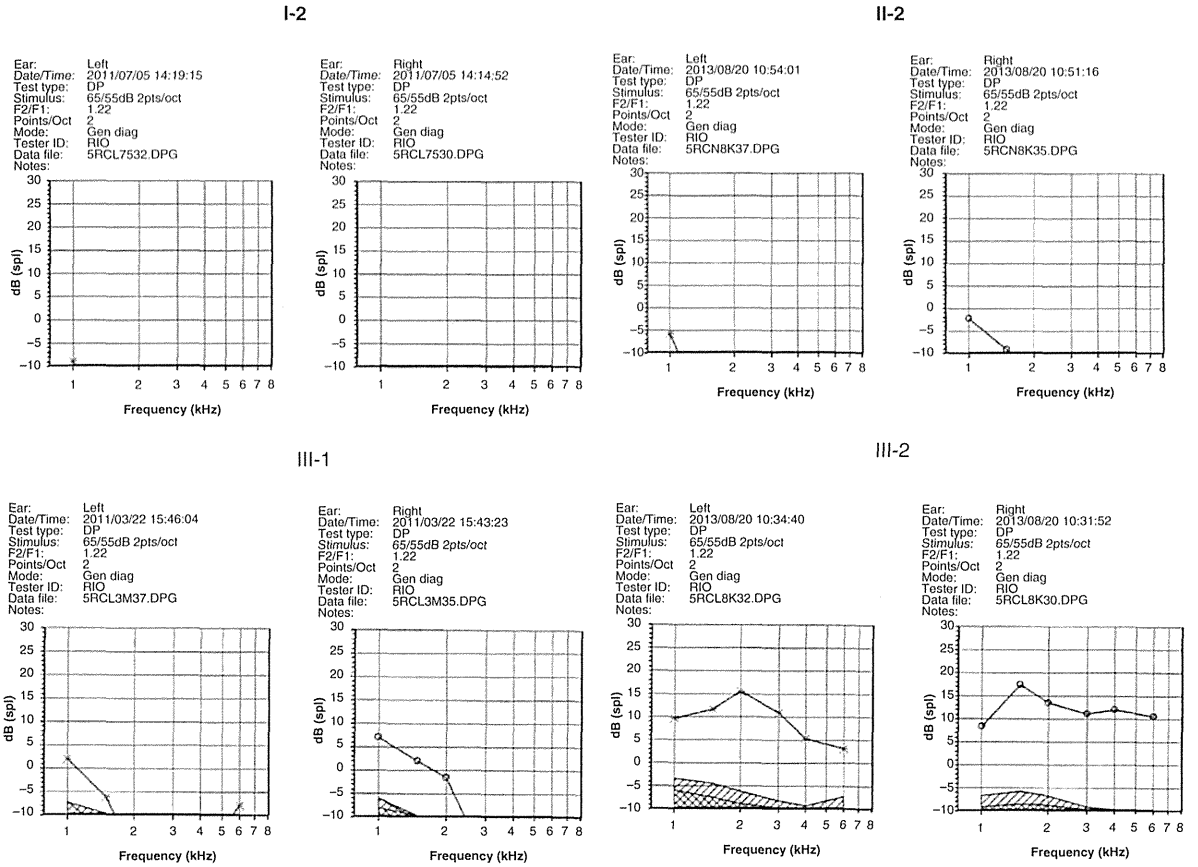

Figure 4. Distortion-product otoacoustic emissions (DPOAE) graphs of four subjects with the c.211delC mutation in the *KCNQ4* gene.

supported by the finding of Naito et al. that 20 patients with a maximum age of 73 who carried this mutation did not have progressive hearing loss. The detailed estimation of progression also does not suggest that development of profound hearing loss will occur.

Our findings from extensive audiological examination suggested that cochlear impairment induced by c.211delC mutation of the *KCNQ4* gene might start from the basal turn of the cochlea and progress to the middle turn. DPOAE in the middle frequency area was detectable in the youngest subject with high-frequency SNHL (III-1). Subject II-2 had detectable DPOAE only in the 1 kHz, at least in the right ear. The oldest subject (I-2) did not have detectable DPOAE in any frequency areas. These findings indicate that dysfunction of outer hair cells progressed from the basal turn to middle turn of the cochlea along with aging. In addition, this is supported by the results of self-recording audiometry, in which subject III-1 showed Jerger type I, indicating normal hearing, and subject I-2 showed Jerger type II (cochlear origin) hearing loss in the high-frequency area.

It is unclear whether vestibular symptoms are associated with the *KCNQ4* gene mutation. In the present study, two subjects carrying the c.211delC mutation showed recurrent vertigo and hyporeflexia in the right ear on caloric testing. However, one subject with this mutation showed normal caloric test responses without vestibular symptoms. In the two patients with vertigo, there was a unilateral decline of caloric response. Therefore, this vestibular dysfunction may not be due to the *KCNQ4* mutations, because if it were such a genetically determined vestibular response, it would usually be symmetric. Naito et al. also reported that in 20 patients carrying the c.211delC mutation, the majority did not have apparent vestibular symptoms, suggesting that this mutation is not associated with vestibular dysfunction [5].

We detected a novel missense mutation, p.H989Q, in the *TECTA* gene in two subjects with SNHL. This mutation is located in the TIL region of the zonadhesin-like domain D2 and is highly conserved in many species (from humans to fish). Alasti et al.

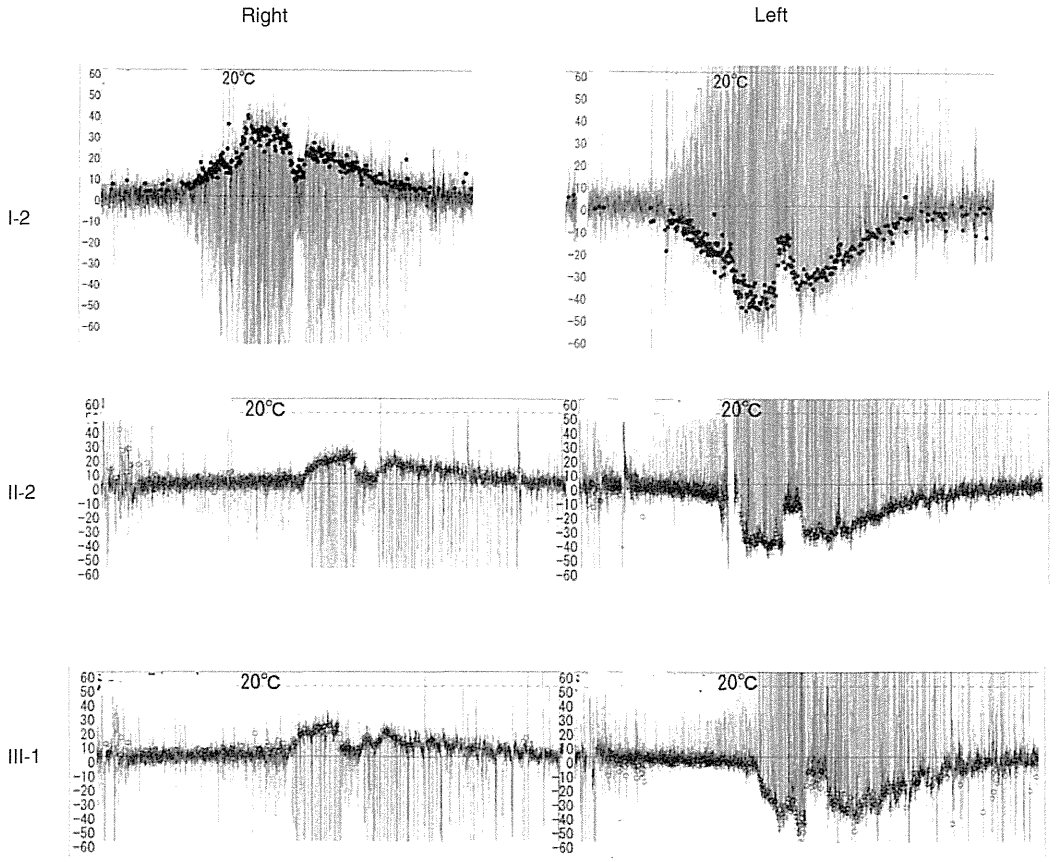

Figure 5. Caloric testing results of the three subjects with high-frequency sensorineural hearing loss (SNHL).

reported that ZA domain mutations cause progressive and high-frequency hearing loss [13]. However, our subject I-2 with high-frequency SNHL did not carry this mutation. Therefore we suspect that it was not associated with hearing loss in this family.

As technology develops and wide genome searches become more commonly performed, the detection of cases with two or more gene mutations is predicted to increase. It will be increasingly important to consider genotype–phenotype correlation of each mutation detected and to exercise due caution in determination of the causative mutation and selection of appropriate treatment.

Conclusion

In the present study, we found a c.211delC mutation in the *KCNQ4* gene in a Japanese family with autosomal dominant inherited progressive high-frequency SNHL, therefore the existence of this mutation should be considered in such families.

Acknowledgments

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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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ORIGINAL ARTICLES

Clinical Application of a Custom AmpliSeq Library and Ion Torrent PGM Sequencing to Comprehensive Mutation Screening for Deafness Genes

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Background: Congenital hearing loss is one of the most common sensory disorders, with 50–70% of cases attributable to genetic causes. Although recent advances in the identification of deafness genes have resulted in more accurate molecular diagnosis, leading to the better determination of suitable clinical interventions, difficulties remain with regard to clinical applications due to the extreme genetic heterogeneity of deafness. *Aim:* Toward more effective genetic testing, we adopted Massively Parallel DNA Sequencing (MPS) of target genes using an Ion PGM™ system and an Ion AmpliSeq™ panel to diagnose common mutations responsible for deafness and discover rare causative gene mutations. Before its clinical application, we investigated the accuracy of MPS-based genetic testing. *Results:* We compared the results of Invader assay-based genetic screening, the accuracy of which has already been verified in previous studies, with those of MPS-based genetic testing for a large population of Japanese deafness patients and revealed that over 99.98% of the results were the same for each genetic testing system. *Conclusion:* The Ion Personal Genome Machine system had sufficient uniformity and accuracy for application to the clinical diagnosis of common causative mutations and efficiently identified rare causative mutations and/or mutation candidates.

Introduction

CONGENITAL HEARING LOSS is one of the most common sensory disorders. It appears in one of 1000 newborns, with 50–70% of cases attributable to genetic causes (Morton and Nance, 2006). Approximately 100 genes are estimated to be involved in hereditary hearing loss, so there is a great need for effective genetic testing (Hereditary Hearing Homepage; http://webh01.ua.ac.be/hhh/). One-by-one gene screening is, however, time-consuming. By focusing on frequently recurring mutations with ethnic origin that are most likely to be encountered in a clinical setting, we developed the Invader assay-based genetic screening test for 46 mutations in 13 genes, which can identify ~30–40% of hearing loss patients (Abe *et al.*, 2007; Usami *et al.*, 2012). From 2012, genetic testing for hearing loss patients using the Invader assay has been covered by social health insurance in Japan. To improve the diagnostic rate of this genetic testing, additional genetic analysis for many rare genes was nevertheless required.

Massively Parallel DNA Sequencing (MPS) of target genes offers a useful method of identifying rare causative gene mutations and, thereby, improving the diagnostic rate. In our previous study, MPS analysis using an Ion PGM™ system and Ion AmpliSeq™ for the known 63 deafness-causing genes was able to identify rare gene mutations responsible for hearing loss in patients with cochlea implantation (Miyagawa *et al.*, 2013).

In the current study, we compared the results of Invader assay-based genetic screening with MPS-based genetic testing for a large population of Japanese hearing loss patients to investigate the accuracy of the MPS-based genetic test and its potential clinical application.

Subjects and Methods

Subjects

Three hundred eighty-four Japanese patients with bilateral sensorineural hearing loss from 53 ENT departments nationwide participated in this study. Informed written consent was obtained from all subjects, their next of kin, caretakers, or guardians (in the case of minors) before participation in the project. This study was approved by the Shinshu University Ethics Committee as well as the ethical committees of each of the other participating institutions listed in Acknowledgements.

Genetic analysis

We performed the Invader assay to screen for 46 known pathogenic mutations of 13 genes as a standard genetic test. This was followed by TaqMan genotyping assays for 55

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known mutations of six genes and the direct sequencing of the *GJB2* gene for all cases. Direct sequencing of the *SLC26A4* gene was also performed for patients with enlarged vestibular aqueduct (EVA). We also performed MPS analysis, as described below, for all cases and compared the results obtained from the Invader assay, TaqMan genotyping, and direct sequencing with the MPS results.

Invader assay

We first applied the Invader assay to screen for 46 known mutations of 13 known deafness genes listed previously (Usami *et al.*, 2012). These mutations were selected on the basis of a mutation/gene database established for the Japanese deafness population. The detailed protocol was described elsewhere (Usami *et al.*, 2012).

Direct sequencing

Direct sequencing of the *GJB2* gene was performed for all subjects, and the *SLC26A4* gene was analyzed for the subjects with EVA and for the patients with heterozygous *SLC26A4* mutations identified by the Invader assay. DNA fragments containing the entire coding region and splicing region were amplified and sequenced, as described elsewhere (Tsukada *et al.*, 2010; Miyagawa *et al.*, 2014).

TaqMan genotyping assay

For additional screening, TaqMan genotyping assays for 55 known mutations of six deafness genes were applied for all subjects using a custom TaqMan SNP Genotyping Assay (Applied Biosystems, Life Technologies), TaqMan genotyping master mix (Applied Biosystems, Life Technologies), and a StepOne Plus real-time PCR system (Applied Biosystems, Life Technologies) according to the manufacturer's instructions.

Amplicon library preparation

An Amplicon library of the target exons was prepared with an Ion AmpliSeq Custom Panel (Applied Biosystems, Life Technologies) and designed with an Ion AmpliSeq Designer (http://ampliseq.com) for 63 genes reported to cause nonsyndromic hearing loss (Hereditary Hearing loss Homepage; http://hereditaryhearingloss.org/) using an Ion AmpliSeq Library Kit 2.0 (Applied Biosystems, Life Technologies) and Ion Xpress™ Barcode Adapter 1–96 Kit (Applied Biosystems, Life Technologies) according to the manufacturer's instructions. The detailed protocol was described elsewhere (Miyagawa *et al.*, 2013).

Emulsion PCR and sequencing

The emulsion PCR was performed with the Ion OneTouch™ System and Ion OneTouch 200 Template Kit v2 (Applied Biosystems, Life Technologies) according to the manufacturer's instructions. After the emulsion PCR, template-positive Ion Sphere™ Particles were enriched with the Dynabeads® MyOne™ Streptavidin C1 Beads (Applied Biosystems, Life Technologies) and washed with the Ion OneTouch Wash Solution included in the kit. This process was performed using an Ion OneTouch ES system (Life Technologies).

After the Ion Sphere Particle preparation, MPS was performed with an Ion Torrent Personal Genome Machine (PGM) system using the Ion PGM 200 Sequencing Kit and Ion 318™ Chip (Life Technologies) according to the manufacturer's instructions.

Base call and data analysis

The sequence data were processed with standard Ion Torrent Suite™ Software ver 4.0 and the Torrent Server was used to successively map the human genome sequence (build GRCh37/hg19) with a Torrent Mapping Alignment Program optimized to Ion Torrent™ data. After the sequence mapping, the DNA variant regions were piled up with Torrent Variant Caller plug-in software set to run at high stringency. Selected variant positions were detected with the Hot Spot BED option. In conventional variant detection processes, only the mutation position is called; however, using the Hot Spot BED option, the variant positions specified in the BED file are always genotyped into wild type, heterozygous, or homozygous. After variant detection, variant effects were analyzed using the wANNOVAR website (Wang *et al.*, 2010; Chang and Wang, 2012).

Results

Uniformity of the MPS-based comprehensive mutation screening test

We first analyzed the uniformity of each MPS run and sample. In 64 sequence runs using the Ion torrent PGM sequencer with Ion 318-chips, the mean number (±standard deviation) of reads was 3.56±0.75 M. The distribution of the read numbers produced by each sequence run is shown in Figure 1. The uniformity of the read number for each MPS run was sufficiently high, with 41 of the 64 MPS runs (64%) providing 3–4 M reads. The mean number of sequenced bases of sufficient quality (>Q17) produced by each sequence run was 461±120 M.

The mean number of reads of the 384 samples analyzed by the 64 sequence runs was 580±168 thousand reads for each

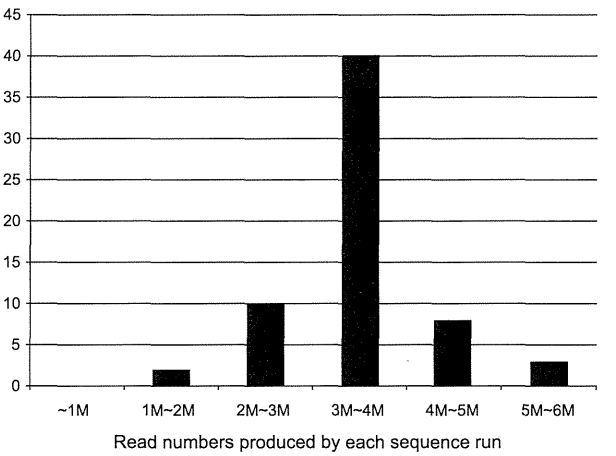

FIG. 1. The distribution of read numbers produced by each sequence run. In the 64 sequence runs, the average read number for each sequence was 3.56 M reads, and 41 massively parallel DNA sequencing (MPS) runs (64%) providing 3–4 M reads.

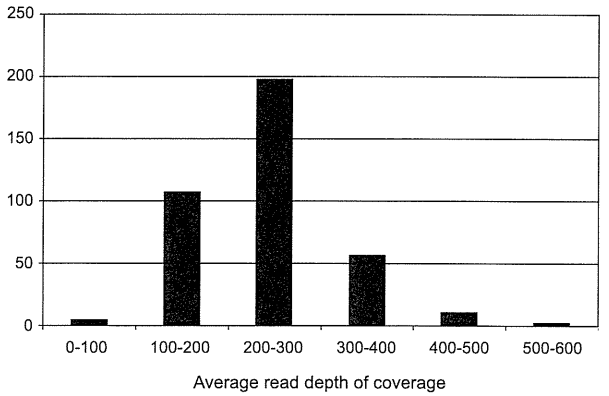

FIG. 2. The distribution of the average read depth of coverage of the target regions for the 384 samples. Among the 384 samples, only five samples (1.3%) had a depth of coverage of under 100×, with the other 379 samples (98.7%) showing a depth over 100×.

sample. The distribution of the average depth of coverage of the target region is shown in Figure 2. The mean depth of coverage of the target region of each of the 384 samples was 241±76×. Among the 384 samples, only five samples (1.3%) showed an average depth of coverage under 100×, with the other 379 samples (98.7%) all over 100×. The distribution of the average depth of coverage of the target region and the percentage of each region with over 20× coverage (indicating the percentage of each region sequenced 20 times or more by MPS) are shown in Figure 3. An average of 97.72±0.90% of each target region was sequenced with over 20×coverage. These data revealed that the MPS-based genetic testing has sufficient uniformity for clinical use. To reduce instances of incorrect genotyping and missed single-nucleotide polymorphism in poor coverage regions, we employed a minimum average depth of coverage of 100 and a minimum percentage of over 20× region coverage of 96%. Among the 384 samples, 14 samples (3.6%) did not fulfill these criteria, so we analyzed these samples again. After re-analysis, all of the samples fulfilled the above criteria.

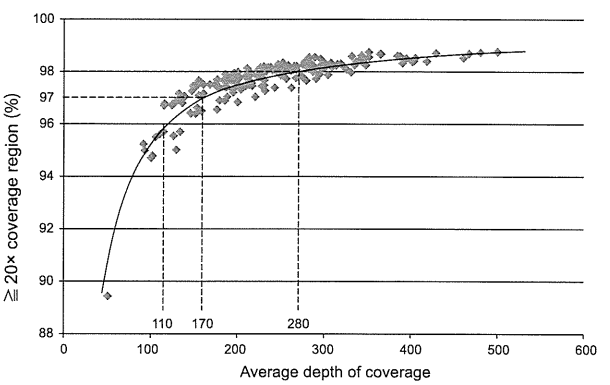

FIG. 3. The distribution of the average depth of coverage of the target regions and the percentage of regions with greater than 20× coverage. *Diamond shapes* indicate the average coverage depth of each sample and the ratio of regions with coverage depth over 20×. The results indicate that sufficient coverage was obtained for 96% of the target region.

Comparison of the Invader assay-based mutation screening and MPS-based comprehensive screening of deafness genes

To investigate the accuracy of the MPS-based comprehensive genetic screening, we compared the results of MPS-based genetic screening with those of Invader assay-based mutation screening and direct sequencing (Table 1).

From 384 patients, the Invader assay-based genetic screening detected 174 mutations (Table 1). According to our previous report, about 30% of patients (112 patients) carry one or more mutations, with *GJB2* mutations being the most frequent, followed by *SLC26A4* and Mitochondrial 1555A>G mutations. Among the invader assay results, one c.427C>T mutation was not detected in one case due to an unknown technical error (Usami *et al.*, 2012). The Invader assay was performed for the 46 variants in 384 samples with only one mutation not detected in the 17,664 SNVs examined, indicating that the accuracy of the Invader assay was over 99.99% (17,663/17,664). In the MPS-based screening, c.919-2A>G mutations of *SLC26A4* gene and mitochondrial mutations were not detected because these mutations are located in regions not covered by the AmpliSeq library primers. Misgenotyping of *GJB2* c.408C>A and c.427C>T heterozygous mutations as homozygous mutations was also observed in two cases (Table 1). This misgenotyping was caused by combined c.299_300del mutations located at the 3′ end of the AmpliSeq primer (Fig. 4). On the other hand, there were no false-positive results for the target mutations observed in the Invader assay. In this comparison, the MPS covered the 41 variants in the Invader assay in 384 samples, with only two mutations misgenotyped among the 15,744 SNVs, indicating that the accuracy of the MPS-based genetic screening test was 99.98% (15,742/15,744).

Comparison of the TaqMan genotyping assay-based mutation screening and direct sequencing with the MPS-based comprehensive screening of deafness genes

The TaqMan genotyping assay was performed, with the 58 mutations listed in Table 2 identified from the 384 patients. The c.211delC mutation of the *KCNQ4* gene and the c.2229_2301delGAA mutation of the *SLC26A4* mutation were not detected by the MPS-based genetic screening as these mutations were located in regions not covered by the AmpliSeq primers. The c.211delC mutation of *KCNQ4* was located in a GC-rich region with a GC content of about 80%, and we also found it difficult to detect this mutation by direct sequencing. In addition, *CDH23* c.4877A>C heterozygous mutations were not detected by MPS in one case. In this patient, the c.4877A>C mutation region had a depth of coverage of only 7×, which did not meet the filtering threshold of the variant caller software, resulting in a no call status. No false-positive cases were observed among the TaqMan genotyping assay target mutations.

Direct sequencing of the *GJB2* gene was performed for all patients and that of the *SLC26A4* gene for patients with EVA. As a result, a total of 27 mutations not identified by the Invader or TaqMan genotyping assays were detected (Table 3). Direct sequencing did not detect *GJB2* c.257C>T or c.511G>A mutations in one case each due to the low signal intensity of these nucleotide positions. Our comparison of

TABLE 1. COMPARISON OF THE INVADER ASSAY-BASED MUTATION SCREENING AND MASSIVELY PARALLEL DNA SEQUENCING-BASED COMPREHENSIVE SCREENING OF DEAFNESS GENES

Mutations	*Number of patients with mutations detected by Invader screening* (n = *384*)	*Variant alleles detected by Invader screening* (n = *768*)	*Variant alleles detected by MPS* (n = *768*)	*Variant alleles detected by direct sequencing* (n = *768*)
GJB2:NM_004004:c235delC:p.L79fs	42 (10.9%)	52 (6.8%)	52	52
GJB2:NM_004004:c.109G > A:p.V37I	19 (4.9%)	21 (2.7%)	21	21
GJB2:NM_004004:c.[134G > A; 408C > A]:p.[G45E; Y136X]	16 (4.2%)	17 (2.2%)	18[b]	17
GJB2:NM_004004:c.427C > T:p.R143W	13 (3.4%)[a]	13 (1.7%)[a]	15[b]	14
GJB2:NM_004004:c.176_191del16:p.59_64del	9 (2.3%)	10 (1.3%)	10	10
GJB2:NM_004004:c.257C > G:p.T86R	5 (1.3%)	6 (0.8%)	6	6
GJB2:NM_004004:c.299_300del:p.100_100del	6 (1.6%)	6 (0.8%)	6	6
SLC26A4:NM_000441:c.2168A > G:p.H723R	15 (3.9%)	20 (2.6%)	20	20
SLC26A4:NM_000441:c.1229C > T:p.T410M	4 (1.0%)	6 (0.8%)	6	6
SLC26A4:NM_000441:c.1174A > T:p.N392Y	1 (0.3%)	1 (0.1%)	1	1
SLC26A4:NM_000441:c.367C > T:p.P123S	1 (0.3%)	1 (0.1%)	1	1
SLC26A4:NM_000441:c.2162C > T:p.T721M	1 (0.3%)	1 (0.1%)	1	1
SLC26A4:NM_000441:c.601-1G > A:Splicing	1 (0.3%)	1 (0.1%)	1	1
SLC26A4:NM_000441:c.916dupG:p.I305fs	1 (0.3%)	1 (0.1%)	1	1
SLC26A4:NM_000441:c.1648dupT:p.R549fs	1 (0.3%)	1 (0.1%)	1	1
SLC26A4:NM_000441:c.919-2A > G:Splicing	1 (0.3%)	1 (0.1%)	0[c]	1
CRYM:NM_001888:c.941A > C:p.K314T	1 (0.3%)	1 (0.1%)	1	1
Mitochondria 1555A > G	5 (1.3%)	—	—	—
Mitochondria 3243A > G	8 (2.1%)	—	—	—
Mitochondria 8296A > G	1 (0.3%)	—	—	—

[a]c.427C > T mutation was not detected by Invader screening in one case (reason unknown).
[b]MPS misgenotyped heterozygous as homozygous mutations in one case each because of the other mutations located in the AmpliSeq primer region (see details in main text).
[c]c.919-2A > G mutation was located in the region not covered by AmpliSeq primers.
MPS, massively parallel DNA sequencing.

results showed that these mutations in the *GJB2* gene were identified by MPS. We, therefore, reanalyzed the direct sequencing data and finally confirmed these mutations by direct sequencing. On the other hand, c.107_120del and c.147C > G mutations of the *SLC26A4* gene (one case each) were not detected by MPS analysis. These results indicate that the accuracy of the MPS was equivalent to that of direct sequencing.

Advantage of the MPS-based comprehensive sequencing of deafness genes

The advantage of the MPS-based comprehensive sequencing of deafness genes lay in the improved diagnostic rate. When heterozygous pathogenic mutations are identified as autosomal recessive deafness causative genes by the

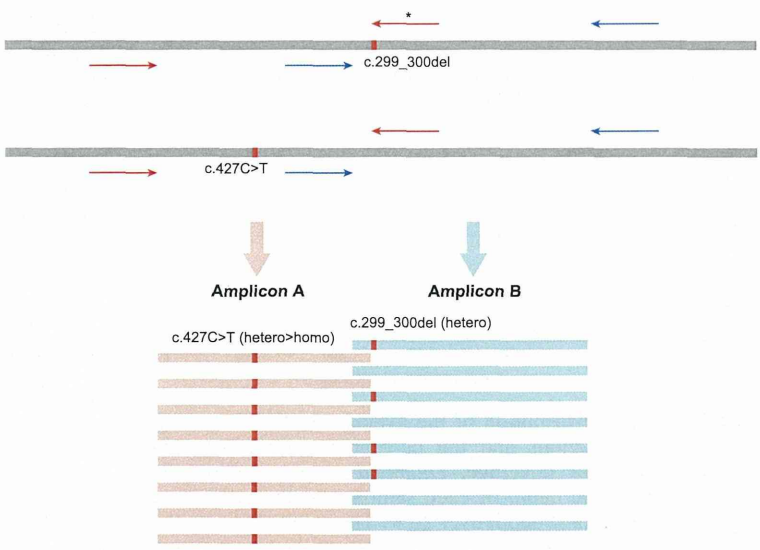

FIG. 4. Heterozygous c.427C > T (p.R143W) mutations were misgenotyped as homozygous by MPS because the c.299_300del mutations were located at the 3′ end of the amplicon. *Upper figure* indicated the position of c.299_300del, c.427C > T mutations and AmpliSeq primers. c.299_300del mutations were located in 3′ end of PCR primer of Amplicon A marked by *asterisk*. As a result, all of Amplicon A was produced from the allele with c.427C > T mutation and misgenotyped as a homozygous mutation illustrated in *lower figure*.