図６　日本人難聴患者 1845 例を対象に実施した、CDH23 遺伝子変異 4 種類のアリル頻度 P240L 変異、R2029W 遺伝子変異の頻度が高く、founder effect によるものであることが推定される。(Miyagawa et al., submitted)

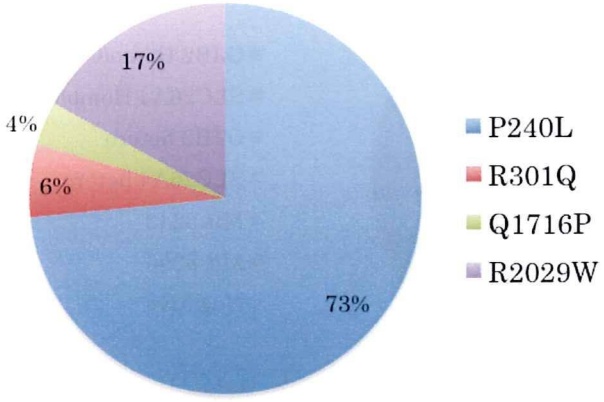


図７　保存乾燥臍帯を用いた先天性 CMV 感染症の検査の結果
1,2,3 はそれぞれサイトメガロウイルス上に設計した CMV_1、CMV_2、CMV_S プライマーの増幅産物、M はマーカーを示す。＋はポジティブコントロール、―はネガティブコントロールの結果を示す。(Furutate et al., submitted)

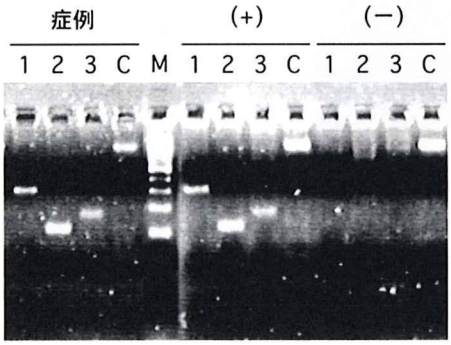


図８　信州大学医学部附属病院において期間中に先進医療「先天性難聴の遺伝子診断」を受診した患者（52 例）の結果。52 例中 23 例（44.2%）より変異が検出された。

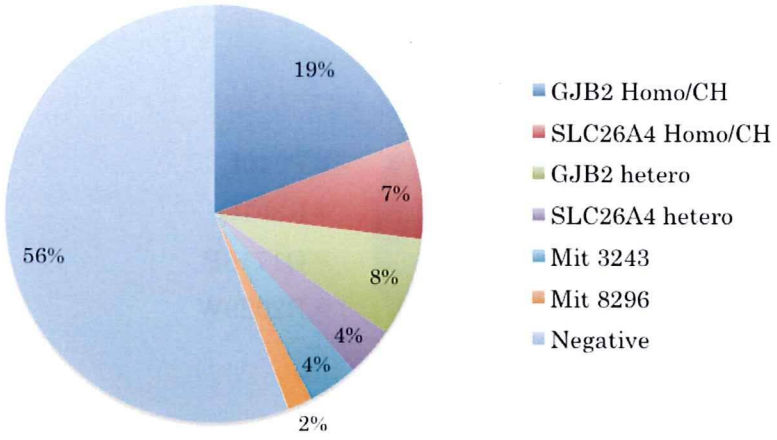


図９　虎の門病院において期間中に先進医療「先天性難聴の遺伝子診断」を受診した患者（33 例）の結果。33 例中 15 例（45.5%）より変異が検出された。

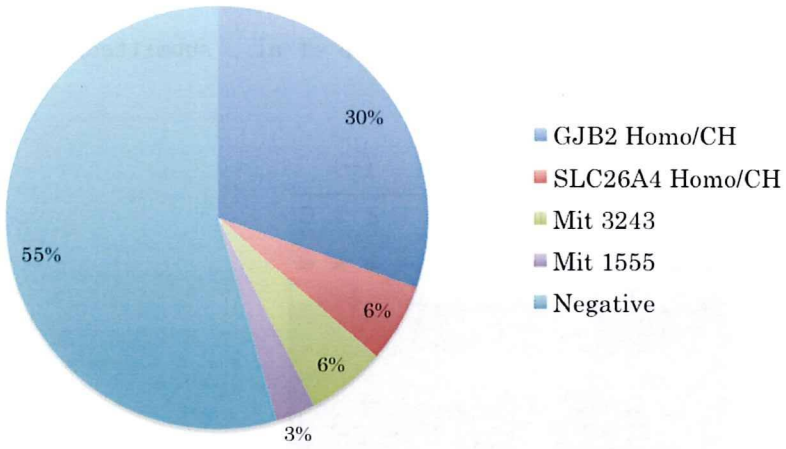


図 10　難聴の原因遺伝子変異の特定された群（特定群）とその他の群（非特定群）の WISC-Ⅲの結果の比較。動作性 IQ に関しては健聴群の平均値 100 とほぼ同等の結果であるが、言語性 IQ に関しては健聴群と比較して有為に低いことが明らかとなった。また、特定群と非特定群の比較では非特定群の方が有為に言語性 IQ の成績が低いことが示された（Maeda et al., submitted）

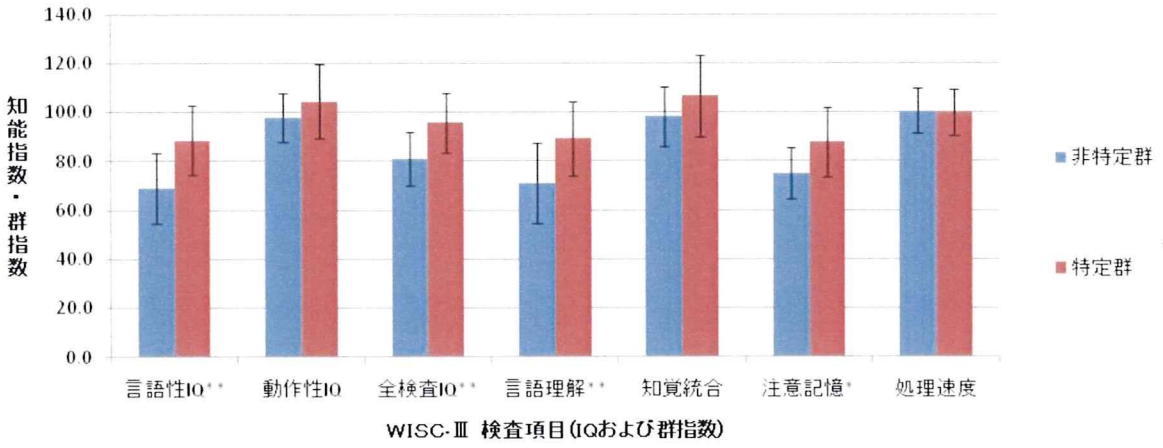
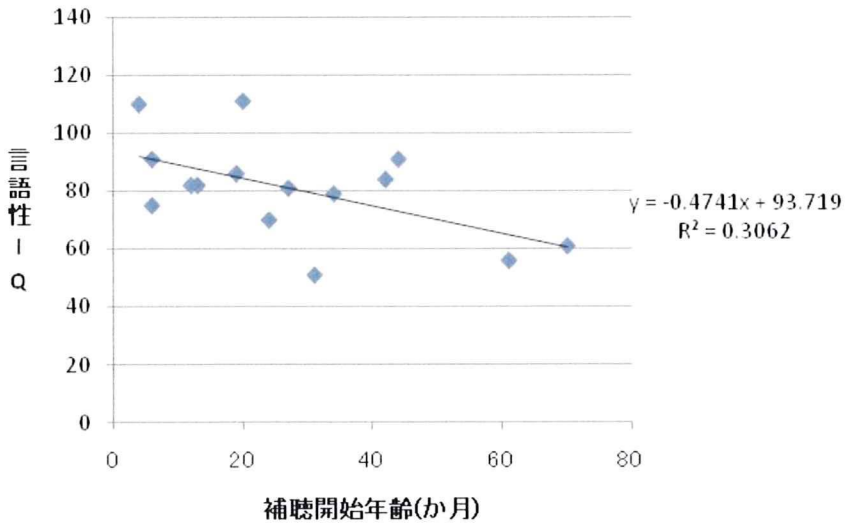


図 11　補聴器装用開始年齢と言語性 IQ の相関

装用開始年齢と言語性 IQ の間には不の相関が認められ、早期に補聴器を装用開始（早期介入開始）することで言語性 IQ の伸びをある程度担保できることが明らかとなった。



Ⅱ．　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tsukada K, Nishio S, Usami S	A large cohort study of GJB2 mutations in Japanese hearing loss patients.	Clin. Genet.		In press	2010
Usami S, Miyagawa M, Suzuki N, Moteki H Nishio S Takumi Y Iwasaki S.	Genetic background of candidates for EAS (electric acoustic stimulation).	Audiological Medicine.		in press	2010
Lu SY, Nishio S, Tsukada K, Oguchi T, Kobayashi K, Abe S, Usami S.	Factors that affect hearing level in individuals with the mitochondrial 1555A>G mutation.	Clinical Genet.	75 (5)	480-484	2009
宇佐美真一	難聴とウイルス感染	MB ENTONI	99	8-16	2009
宇佐美真一	先天性難聴	小児科	50	1182-1185	2009
武市紀人、柏村正明、中丸裕爾、津府久崇、福田諭、鈴木美華、宇佐美真一	難聴□伝子診断が有用であった人工内耳一症例	Audiology Japan	52	214-219	2009
宇佐美真一	薬剤と□伝子	耳鼻咽喉科・頭頸部外科	81	759-767	2009
宇佐美　真一	予防医学からみた□伝性難聴	JOHNS	25	1719-1723	2009

Ⅳ. 研究成果の刊行物・別刷

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Short Report

A large cohort study of *GJB2* mutations in Japanese hearing loss patients

Tsukada K, Nishio S, Usami S-i. A large cohort study Of *GJB2* mutations in Japanese hearing loss patients.
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GJB2 is the gene most frequently associated with hereditary hearing loss, and the *GJB2* mutation spectrums vary among different ethnic groups. In this study, the mutation spectrum as well as clinical features of patients with *GJB2* mutations as found in more than 1000 Japanese hearing loss families are summarized. The present results show that the frequency of *GJB2* mutations in the Japanese population with hearing loss is 14.2% overall and 25.2% in patients with congenital hearing loss. c.235*del*C was the most frequent allele (49.8%), was associated with a more severe phenotype, and was mainly found in patients who were diagnosed at the age of 3. In contrast, the second most frequent was p.V37I (16.5%), which has a milder phenotype and was mainly found in patients diagnosed at a higher age. Additional clinical features in hearing loss patients with *GJB2* mutations in this study were the near absence of tinnitus, vestibular dysfunction and inner ear malformations.

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Key words: clinical features – genotype – phenotype correlations – *GJB2* – hearing loss – mutation

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Mutations in the *GJB2* gene have recently been of particular interest because *GJB2* is the commonest causative gene for hereditary hearing loss in all populations. To date, more than 100 variations have been reported worldwide (see the Connexin-deafness homepage: http://www.davinc.crg.es/deafness) and the mutation spectrums vary among different ethnic groups. There have been many papers describing the frequency of *GJB2* mutations among hearing loss populations, but most studies have been based on small numbers of patients from a single center. A large cohort study may prevent bias and provide a more precise estimate of mutation frequencies. Therefore, with the goal of establishing a database of the mutations found in the East Asian populations, we estimated the *GJB2* mutation frequency and spectrum as well as associated clinical features using more than 1500 Japanese hearing loss families collected from multiple centers.

Subjects and methods

Subjects

Data on 3056 Japanese subjects of 1511 independent families were collected from 33 ENT departments nationwide in Japan. All subjects gave prior informed consent for participation in the project, which was approved by the ethical committee of each hospital. Of the 1511 probands, 1343 had bilateral sensorineural hearing loss and 168 had unilateral sensorineural hearing loss. The control group consisted of 252 unrelated Japanese individuals without any noticeable hearing loss evaluated by auditory testing.

Mutation analysis

To identify *GJB2* mutations, a DNA fragment containing the entire coding region was sequenced as described elsewhere (1). Screening for the known large DFNB1 deletions was performed

in the patients with a single heterozygous allele without the presence of a second pathogenic mutant allele, but none were detected (data not shown).

Computational analysis

To evaluate the importance of each amino acid affected by novel missense mutations found in this study, we used a computational analysis program for identification of functionally and structurally important residues in protein sequences: CONSEQ (http://conseq.tau.ac.il/index.html).

Clinical evaluations

Hearing levels were determined by pure-tone audiometry. For the young patients, conditioned orientation response audiometry (COR) or auditory steady-state response (ASSR) were used. Clinical data, including hearing loss progression, episodes of tinnitus and vestibular dysfunction (vertigo, dizziness, faintness), were collected by anamnestic evaluation. Thin section temporal bone computed tomography (CT) was used to investigate inner ear malformations.

Results

GJB2 mutation spectrum in hearing loss probands

There were a total of 26 *GJB2* variants observed in the ascertained probands with bilateral hearing loss (Table 1). Fourteen of those were missense mutations. To evaluate the evolutionary conservation of the amino acids affected by these missense mutations, we used a computational alignment program CONSEQ (not shown). On the basis of this alignment program, all missense mutations had changed evolutionary conserved amino acids, except for p.T123N and p.Y68C. Because p.N54S and p.M195V were found in the compound heterozygous state, they are likely to be pathogenic. Eight of the mutations were found in the control group (Table 1). p.V27I, p.E114G, p.I203T (1, 2), and p.I123N (3), frequently found in both probands and controls, were thought to be non-pathological polymorphisms. The c.235*del*C and p.V37I mutations found in the control group most likely represent the detection of carriers.

Frequency of *GJB2* mutations in hearing loss probands

With regard to the frequency of *GJB2* mutations in the 1343 independently ascertained probands with bilateral hearing loss, 191 (14.2%) had at least one pathogenic *GJB2* mutant allele (Table 2). The most prevalent mutation was c.235*del*C (49.8% of all pathogenic mutant alleles) and the second most frequent was p.V37I (16.5%) (Fig. 1).

The frequency of *GJB2* mutations was significantly higher in probands with prelingual hearing loss: 25.7% (108/420) in the prelingual group, 14.9% (15/101) in those diagnosed at age 4–5, and 7.8% (49/627) in age 6 or over (Table 2). c.235*del*C was also significantly higher in prelingual hearing loss probands (58.5%) compared to those who were diagnosed at the age of 6 and over (19.6%) ($p < 0.001$; χ^2 test). In contrast, p.V37I was significantly more frequent in probands who were diagnosed at the ages of 4–5 (36.4%) or 6 and over (41.1%) than in prelingual hearing loss probands (6.9%) ($p < 0.001$; χ^2 test) (Fig. 1).

Audiologic studies

Of the total 3056 subjects, 134 with bilateral hearing loss and biallelic *GJB2* mutations were selected for audiologic studies. We excluded 22 subjects who were from a family with another subject who had the same mutation. In the remaining 112 subjects, audiometric results were available for 105 probands, of 23 different genotypes. Figure 2 shows a collection of overlapping audiograms from those 105 subjects. We compared the hearing levels in the six genotypes that were shared by five or more subjects. The subjects with the p.V37I allele had significantly milder hearing loss ($p < 0.027$; Mann–Whitney *U* test).

p.V37I/p.R143W showed a significantly worse hearing level than p.V37I/p.V37I ($p = 0.025$; Mann–Whitney *U* test) and also tended to be worse than p.V37I/c.235*del*C ($p = 0.076$; Mann–Whitney *U* test). Moreover, comparison of c.235*del*C/c.235*del*C ($n = 35$) and c.235*del*C/p.R143W ($n = 13$) revealed that subjects with the p.R143W allele had a significantly worse hearing level than homozygotes ($p = 0.025$; Mann–Whitney *U* test).

Twenty-six subjects with biallelic *GJB2* mutations were followed at least two years by audiometric testing with progression of hearing loss seen in four subjects (15%), two (7%) of those being unilateral progression and two (7%) being bilateral progression.

Clinical findings

Based on the data availability, clinical findings were statistically evaluated. Episodes of tinnitus in patients with *GJB2* mutations were at a

Table 1. *GJB2* variants in deafness patients and controls

		Patients					Controls					
Amino acid change	Nucleotide change	Allele ($n = 2686$)	Allele frequency (%)	Homozygous (n)	Compound heterozygous (n)	Heterozygous (n)	Alleles ($n = 504$)	Allele frequency (%)	Controls ($n = 252$)	Carrier rate (%)	Evolutionary conservation	Reference
—	c.235*del*C	142	5.29	34	45	28	2	0.40	2	0.80	NA	Fuse et al., 1999
p.V37I	c.109G>A	47	1.75	3	11	30	3	0.60	3	1.20	Yes	Abe et al. (1)
p.G45E[a]	c.134G>A	34	1.27	1	22	10					Yes	Fuse et al., 1999
p.Y136X[a]	c.408C>A										Yes	Brobby et al., 1998
p.R143W	c.427C>T	18	0.67	0	16	2					NA	Abe et al. (1)
—	c.176_191*del*16bp	15	0.56	0	10	5					NA	Abe et al. (1)
—	c.299–300*del*AT	11	0.41	0	8	3					Yes	Ohtsuka et al. (2)
p.T86R	c.257C>A	8	0.30	0	5	3					NA	Hismi et al., 2006
—	c.512*ins*AACG	3	0.11	0	3	0					NA	Estivill et al., 1998
—	c.35*ins*G	2	0.07	0	2	0					Yes	Ohtsuka et al. (2)
p.I71T[b]	c.212T>C	2	0.07	0	0	2					Yes	Kenna et al., 2001
p.T8M	c.23C>T	1	0.04	0	0	1					Yes	This study
p.I33N[b]	c.98T>A	1	0.04	0	0	1					Yes	Ohtsuka et al. (2)
p.A49V[b]	c.146C>T	1	0.04	0	0	1					Yes	This study
p.N54S	c.161A>G	1	0.04	0	1	0					No	This study
p.Y68C[c]	c.203A>G	1	0.04	0	0	1					Yes	Wu et al., 2002
p.M93I	c.276G>A	1	0.04	0	1	0					Yes	This study
p.K112M[b]	c.335A>T	1	0.04	0	0	1					NA	This study
—	c.376-377*del*AA[d]	1	0.04	0	0	1					NA	Primignani et al.
p.W133X	c.398G>A	1	0.04	0	1	0					Yes	This study
p.K168R[b]	c.503A>G	1	0.04	0	0	1					Yes	This study
p.M195V	c.583A>G	1	0.04	0	1	0					NA	Yuge et al., 2002
—	c.605*ins*46bp	1	0.04	0	0	1					Yes	Feng et al., 2002
p.F191L	c.571T>C	0	0	0	0	0	1	0.20	1	0.40	yes	Seeman et al., 2002
p.R127H	c.380G>A	0	0	0	0	0	1	0.20	1	0.40		
Polymorphism												
p.V27I	c.79G>A	865	32.20	—	—	—	196	38.90	158	62.70	Yes	Kelley et al. (8)
p.E114G	c.341A>G	259	9.64	—	—	—	64	12.70	62	24.60	No	Fuse et al. 1999
p.T123N[e]	c.368C>A	18	0.67	0	3	15	2	0.40	2	0.80	No	Park et al. (3)
p.I203T	c.608T>C	112	4	—	—	—	21	4.10	21	8.30	No	Abe et al. (1)

[a]p.G45E and p.Y136X(c.134G>E) mutations are on the same parental allele.
[b]Variants with unproven pathogenic nature.
[c]Variant probably representing polymorphism, because no evolutionary conservation was observed.
[d]c.376–377delAA is thought to be a pathogenic mutation, but it was present as a single heterozygous allele without the presence of a second pathogenic mutant allele; therefore, it could not clearly be classified as pathogenic in this study.
[e]p.T123N was found with equal frequency in the probands and controls, and three out of eight subjects with compound heterozygous state did not have any hearing loss, suggesting the polymorphic nature of p.T123N.

Table 2. The frequency of GJB2 mutations and onset age of hearing loss

	GJB2 mutations	Homozygote	Compound heterozygote	Heterozygote
Total (n = 1343)	191 (14.2%)	38 (2.8%)	63 (4.7%)	90 (6.7%)
0–3 y.o. (n = 420)	108 (25.7%)	32 (7.6%)	47 (11.2%)	29 (6.9%)
4–5 y.o. (n = 101)	15 (14.9%)	1 (0.99%)	6 (5.9%)	8 (7.9%)
≥6 y.o. (n = 627)	49 (7.8%)	3 (0.48%)	4 (0.64%)	42 (6.7%)
Unknown (n = 195)	19	2	6	11

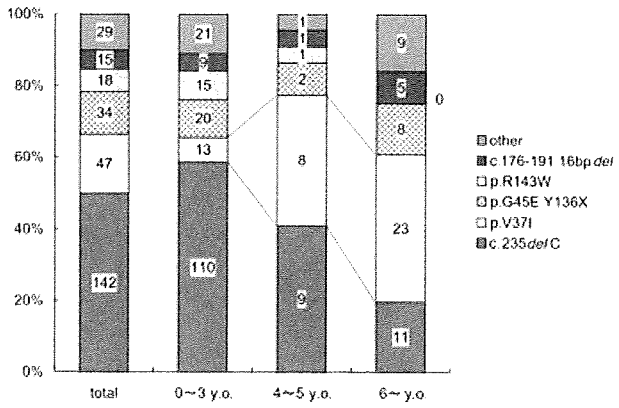

Fig. 1. Frequency of mutant GJB2 alleles in different onset age groups. c.235*del*C was mainly found in the onset age group of up to 3 years, where it was significantly higher than in age 6 and over ($p < 0.01$; χ^2 test). On the contrary, p.V37I was mainly found in the onset age groups of 4–5, and 6 and over, at a rate significantly higher than in up to age 3 ($p < 0.01$).

significantly lower rate (7/75: 9.3%) than in all bilateral hearing loss probands (520/1022: 50.9%) ($p < 0.001$; χ^2 test). Concerning episodes of vestibular dysfunction, only 4% (3/75) of those with biallelic *GJB2* mutations had vertigo, dizziness, or faintness, while 25.1% of all hearing loss probands (258/1029) had vertigo ($p < 0.001$; χ^2 test). Inner ear abnormalities were significantly lower in patients with biallelic *GJB2* mutations (5/62: 8.1%) than in all bilateral hearing loss probands (126/599: 21%) ($p = 0.014$; χ^2 test). In the five patients with biallelic *GJB2* mutations who had inner ear abnormalities, enlarged vestibular aqueduct (EVA) was found in three and the other two had hypoplasty of the cochlea and semicircular canals.

Discussion

GJB2 mutations were found in 14.2% of our bilateral hearing loss probands and 25.2% of the prelingual hearing loss patients. In previous studies in East Asia (1–6), frequency of *GJB2* mutations ranged from 10% to 38% in smaller cohorts. In the present large study using Japanese hearing loss patients collected from multiple centers, we could more accurately estimate the frequency of *GJB2* mutations in Japan and the mutation spectrum. We also found two novel mutation candidates, p.N54S and p.M195V, which cause non-conservative amino acid changes.

In Asian populations, c.235*del*C is the most common *GJB2* mutation, and its allele frequency in patients ranges from about 5% to 22% (1–7). The present study reconfirmed this mutation's high frequency in the Japanese hearing loss population. c.235*del*C accounted for 5.3% of the deafness alleles in all patients and 13.1% of those in prelingual patients.

The p.V37I mutation was originally reported as a polymorphism (8); however, recent reports tend to consider it pathogenic with a milder phenotype (9–12) and this was supported by our results.

Only four out of twenty-six probands showed progressive hearing loss, and bilateral progression was found in only two of those, with a deterioration of less than 20 dB. Therefore, our study supports the previously reported notion that hearing loss due to *GJB2* mutations is typically non-progressive (13–15). With regard to the milder phenotype of p.V37I, none of the five patients with this mutation showed progression. We conclude that this mutation causes milder congenital hearing loss which may not be noticed until age 4 or older.

However, even though it was the second most frequent allele in the hearing loss patients, the p.V37I allele was the most frequent in the control subjects. This may be due to the milder phenotype and non-progression of patients with p.V37I mutation, who therefore either do not visit ENT clinics or do not receive a recommendation for genetic testing from clinicians. Therefore, ENT clinicians should bear in mind the existence of the milder phenotype caused by the p.V37I mutation.

We found that patients with c.235*del*C/p.R143W were significantly more severely affected than those with other c.235*del*C-containing phenotypes. A recent study also reported that the hearing level of c.35*del*G/p.R143W is significantly worse than that of homozygous c.35*del*G (9). We compared homozygous for c.235*del*C with compound heterozygous with p.R143W (except

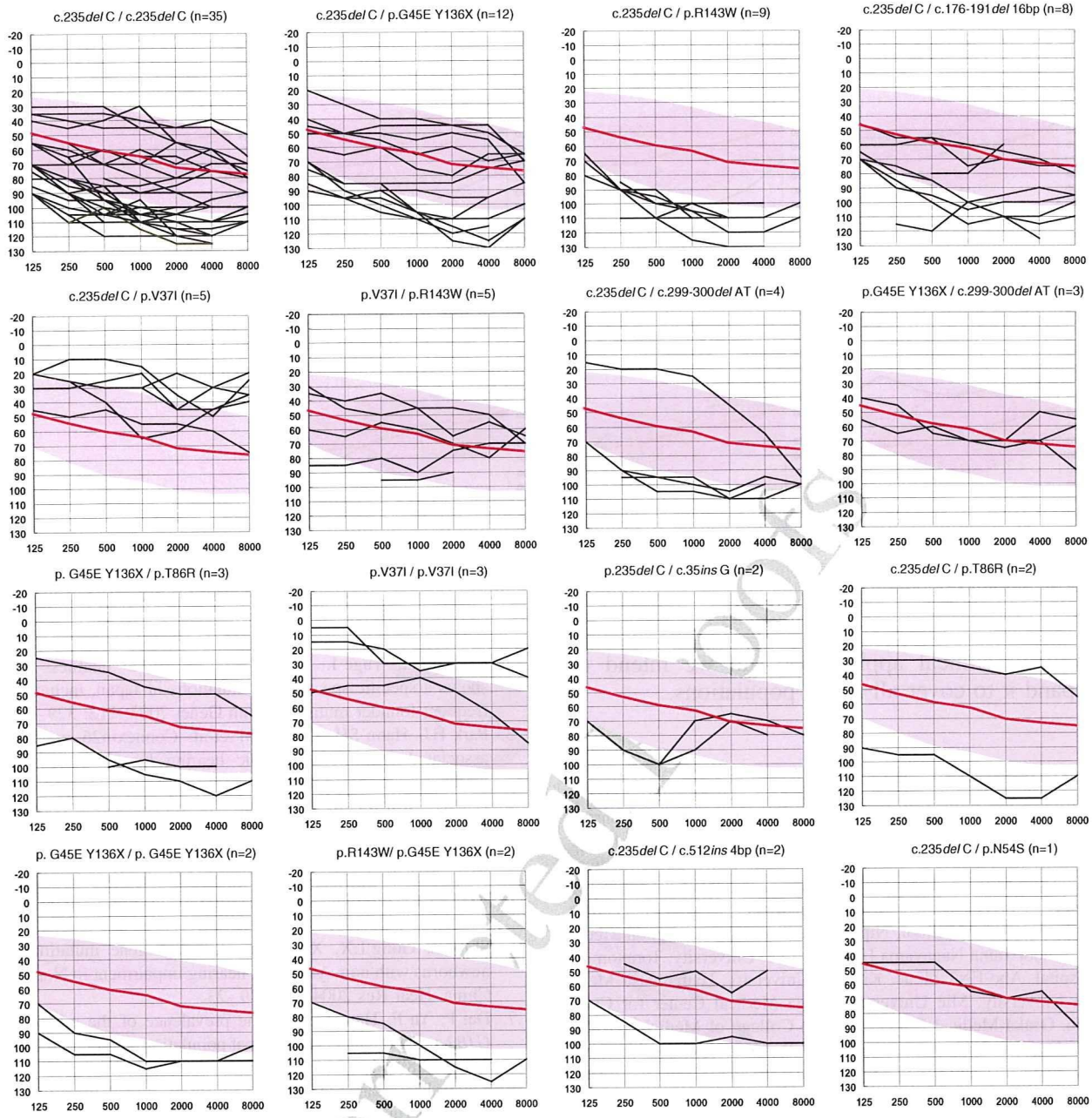

Fig. 2. Overlapping audiograms from the better ear for each genotype. The average audiogram from all subjects (1343 with bilateral sensorineural hearing loss) is indicated by a red line with standard deviation (shadow).

for the p.V37I allele, which is thought to be a milder phenotype), finding the hearing level of the latter to be significantly worse. Also, comparing only the milder p.V37I allele, the hearing level of p.V37I/p.R143W was worse than that of p.V37I/p.V37I and p.V37I/c.235*del*C. These results suggest that p.R143W leads to a worse phenotype than other *GJB2* mutations.

The majority of our probands did not have tinnitus or vestibular dysfunction. Only 8% (5/65) of the patients with biallelic *GJB2* mutations had inner ear malformation, significantly lower than in the overall population with bilateral hearing loss, and in accordance with previous reports (14, 16, 17). Hearing loss patients with *GJB2* mutations also had a near absence of tinnitus, vestibular dysfunction and inner ear malformations.

In conclusion, our results describe the frequency of *GJB2* mutations and associated clinical features in a large Japanese cohort. Recently, based on our database of mutation spectrums found in Japanese, we have developed a genetic test for use in diagnostic screening for hearing loss based on the invader assay (18). This database will also

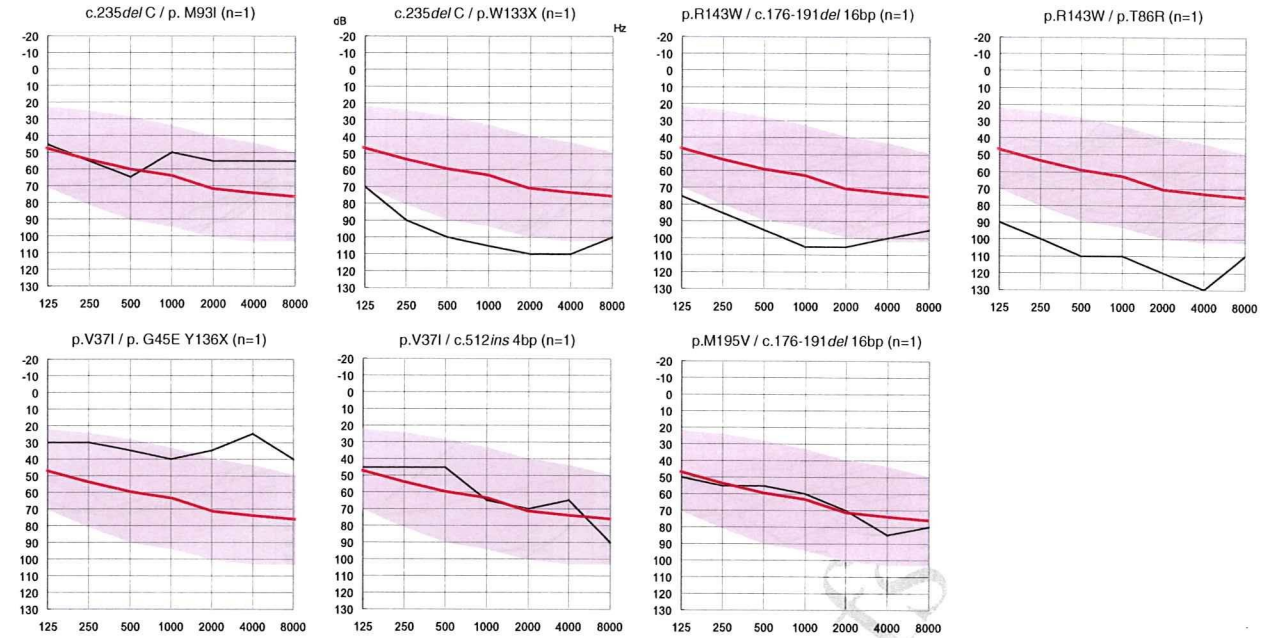

Fig. 2. Continued

facilitate clinical application, and we intend to expand it to cover all Asian populations.

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Conflict of interest

We, the authors, declare that there were no conflicts of interest in conjunction with this paper.

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Genetic background of candidates for EAS (electric-acoustic stimulation)

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Abstract

There is a certain number of patients with so-called ski-slope hearing loss, in which there is good hearing for lower frequencies in spite of little/no hearing in high frequencies. EAS (electric-acoustic stimulation) has recently been introduced for such patients with residual hearing at lower frequencies. Ski-slope hearing loss can have either a progressive nature or can be rather stable; therefore, decisions regarding timing of surgery are sometimes hampered. One advantage of genetic testing is that the possible prognosis for hearing, i.e. progressive or not, can be predicted for individual patients. The present study was performed to estimate the frequency of ski-slope hearing loss and investigate the genetic background of candidates for EAS. Using a 2587 subject DNA database of sensorineural hearing loss patients, 1) frequency of patients with ski-slope hearing loss, 2) their clinical features including inheritance mode, onset ages, and progression, and 3) involvement of four common genes with mutations in Japanese hearing loss patients, were evaluated. One hundred and fifty-one out of 2587 subjects fulfilled the audiological criteria for EAS. The frequency of patients possibly meeting the criteria for EAS was estimated to be 9.1% by restriction to probands only (139/1520). Various inheritance modes and onset ages were noted, with earlier onset in the patients with sporadic/recessive inheritance mode. Progressiveness was recognized in 56% of the patients. Genetic analysis identified mutations in 26.6% of the patients, including the mitochondrial 1555A>G mutation, and mutations in *SLC26A4, CDH23*, and *GJB2* genes, suggesting that at the least, these four genes may be involved in a certain group of patients, but also leaving possible genetic causes in the majority of the patients undetermined. As most of the patients showed a progressive nature in their hearing, genetic testing adds important additional information for candidates for EAS.

Key words: *ski-slope hearing loss, high frequency hearing loss, partial deafness, cochlear implantation*

Introduction

Cochlear implantation is currently the only available device for profound hearing loss patients and therefore has become a standard treatment choice worldwide. Although cochlear implantation has long been applied for patients with severe or profound hearing loss in all frequencies, recent advances in combined electric and acoustic stimulation (EAS) provide a chance of better speech perception for individuals with so-called ski-slope hearing loss. Selection criteria and decision making are sometimes difficult because of individual differences in progression, which is sometimes of a rather rapid progressive nature but other times rather stable. One advantage of genetic testing is that the possible prognosis for hearing, i.e. progressive or not, can be predicted for individual patients. Regarding genes responsible for hearing loss patients, to date, mutations in *GJB2* and *SLC26A4*, and the 1555A>G mutation in the mitochondrial 12S rRNA were found to be the major causes of hearing loss in Japanese patients (1). To date, no study has treated ski-slope hearing loss from an etiological viewpoint. The present study was performed to estimate the frequency of ski-slope hearing loss, audiological characteristics, and genetic background of candidates for EAS.

Subjects and methods

A 2587 subject DNA database of bilateral sensorineural hearing loss established by Shinshu University in collaboration with 33 ENT departments (mostly university hospitals) in Japan was used in this study. The database comprises 1520 unrelated Japanese probands (who had made their initial visit to a hospital) and their family members, with various inheritance modes and ages of onset. The composition of the 1520 probands was as follows: 355 subjects from

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autosomal dominant or mitochondrial families (two or more generations affected); 282 subjects from autosomal recessive families (parents with normal hearing and two or more affected siblings); and 738 subjects with sporadic deafness (also compatible with recessive inheritance or non-genetic hearing loss). All subjects gave prior informed consent for participation in the project and the ethics committee of each hospital approved the study.

Audiological selection criteria were based on the pure tone audiogram selection criteria as follows. Pure tone hearing levels were required to be 65dB HL for 125 Hz, 250 Hz and 500 Hz; 80dB HL for 2000 Hz; 85dB HL for 4000 Hz and 8000 Hz. Subjects with one of the above mentioned frequencies being out of the criteria limits by 10dB were included as potential candidates.

Mutation screening for *GJB2*, *SLC26A4*, and the 1555A>G mutation in the mitochondrial 12S rRNA, was performed in all of the patients as follows. Direct sequencing was used for *GJB2* (2), and restriction fragment length polymorphism (RFLP) was used for the 1555A>G mitochondrial mutation, as previously described (3). In patients with enlarged vestibular aqueduct, direct sequencing was used for *SLC26A4* because mutations in this gene have been restricted to the patients with this particular anomaly (4,5).

For other minor responsible genes, frequencies are relatively small, and therefore one-by-one gene screening was performed in limited numbers of patients (64–319 patients depending on the gene (see reference (1)). For *CDH23*, 64 probands were analyzed using direct sequencing (6).

Results

One hundred and fifty-one (5.8%) out of the 2587 subjects registered in our database fulfilled the audiological criteria for EAS. The frequency of bilateral sensorineural hearing loss patients in the basic clinical population who may meet the criteria for EAS was estimated to be 9.1% by restriction to probands only (139/1520).

Regarding inheritance mode, 53% (74/139) of these patients had sporadic/recessive inheritance, 28% (39/139) dominant/mitochondrial inheritance, and in 19% (26/139) family history was unavailable (Table I).

Onset ages are shown in Table II. Onset ages were varied, and earlier onset ages were evident in the patients with sporadic/recessive inheritance mode.

Progressiveness was recognized in 56% (78/139) of the patients, regardless of inheritance mode (54% for sporadic/recessive inheritance, and 56% for dominant/mitochondrial) (Table III).

Table I. Inheritance mode of candidates for EAS ($n=139$).

Inheritance mode	Number (%)
Sporadic/recessive	74 (53%)
Dominant/mitochondrial	39 (28%)
Data unavailable	26 (19%)

Genetic analysis identified mutations in approximately 27% of the 145 patients, including the mitochondrial 1555A>G mutation ($n=18$, 12.9%), *SLC26A4* ($n=10$, 7.2%), *CDH23* ($n=6$, 4.3%) and *GJB2* mutations ($n=3$, 2.2%) (Table IV). Among the 2587 subjects, 178 were associated with the 1555>G mitochondrial mutation, 153 subjects harbored biallelic *GJB2* mutations, 61 subjects biallelic *SLC26A4* mutations, and eight biallelic *CDH23* mutations. Overlapped audiograms as well as average audiograms are shown in Figure 1A–D. Candidates rates (number of candidates/total patients with mutations) were high among the patients with the 1555A>G mitochondrial mutation (10.1%, 18/178), *SLC26A4* (16.4%, 10/61) and *CDH23* mutations (75%, 6/8) and low among the patients with *GJB2* mutations (2.0%, 3/153).

Discussion

There is a certain number of patients with residual hearing (sometimes normal or slightly elevated thresholds) at the lower frequencies, and profound deafness at the higher frequencies (the so-called ski-slope type hearing loss or partial deafness). Most of these patients do not show any abnormal pronunciation of consonants, indicating that they likely acquired progressive hearing loss at the higher frequencies. In spite of being hard of hearing due to the high-frequency involved hearing loss, they usually do not use hearing aids or use only standard hearing aids with limited efficiency. These cases also do not meet criteria for traditional cochlear implant.

Recent advances in surgical technique, and electrode design, and newly developed devices enable preservation of residual hearing (see reference 7, for review). The concept of EAS has expanded indications for cochlear implantation from profoundly deaf patients in all frequencies to patients with residual hearing at the lower frequencies. According to the present data based on a multicenter collaborative study, 9.1% of the patients who visited the academic referral center were estimated to fulfill the audiological criteria for EAS.

There has been no etiological study of ski-slope hearing loss, and although symmetrical audiograms strongly indicate the majority of cases are due to genetic causes, there have been few reports

Table II. Onset ages of the candidates for EAS (n=139).

	Number (%)					
Inheritance mode	-2 y.o	3-10	11-30	31-50	51-	Unknown
Sporadic/recessive	24 (32%)	12 (16%)	16 (22%)	7 (9%)	5 (7%)	10 (13%)
Dominant/mitochondrial	7 (18%)	12 (30%)	9 (23%)	6 (16%)	1 (2%)	4 (11%)

discussing the genetic background. According to Liu and Xu (1994) (8), non-syndromic hearing loss can be classified into several types on the basis of audiograms. In the autosomal dominant group there are three types of audiograms – sharply sloping, flat, and gently sloping; and two types in autosomal recessive – residual and sharply sloping. The present study is in agreement with their report where cases with a sharply sloping audiogram (which may correspond with ski-slope type) are either autosomal dominantly or autosomal recessively inherited. Dominant high-frequency sensorineural hearing loss can be classified into four types – steepest, less steep, gently sloping, and horizontal (9). Together with similarity of audiograms within the same family, Higashi hypothesized heterogeneity of dominant high-frequency sensorineural hearing loss, and actually the former two types may correspond with ski-slope hearing loss.

In the present study, to understand the etiology of ski-slope hearing loss, genetic as well as clinical feature analyses were performed in the patients who fulfilled the audiological criteria. With regard to inheritance mode of these patients, 53% had sporadic/recessive inheritance, and 28% dominant/mitochondrial inheritance (Table I), indicating that various genes are involved in this category of hearing loss.

A high rate of patients with progressiveness was noted (56%) compared to overall (48%), and progressive nature was observed regardless of inheritance mode, indicating that progressiveness is one of the characteristic features of ski-slope hearing loss.

Onset ages were of great variation, also suggesting there are many responsible genes for this category of hearing loss. Earlier onset ages were noted in the patients with sporadic/recessive inheritance mode.

Ski-slope hearing loss may occur at various ages, and can have either a progressive nature or be rather stable; therefore, decisions regarding timing of surgery are sometimes hampered. There may be a great inter-individual variation regarding progressiveness, indicating that many different etiological differences may interact. Screening for commonly found responsible genes, proved at least four genes, including mitochondrial 12SrRNA, *SLC26A4*, *CDH23*, and *GJB2* are involved in this type of hearing loss, although candidate rates were different among the genes.

The 1555A>G mitochondrial mutation, which is known to result in high susceptibility to aminoglycoside antibiotics, has been identified as the most prevalent mitochondrial mutation (10). Hearing loss is usually high-frequency involved and progressive (3). Therefore, the present higher candidacy rate (10.1%) among the patients with this mutation, together with overlapped audiograms as well as average audiograms (Figure 1A), is consistent with the previously reported phenotype and there is a certain number of candidates for EAS in patients with this mutation.

The *SLC26A4* gene was initially identified as the gene responsible for Pendred syndrome, and is known to be involved in transportation of the chloride ion (11). The phenotype is known to range from Pendred syndrome to non-syndromic hearing loss associated with EVA (enlarged vestibular aqueduct) (12). Hearing is congenital/progressive, and usually high-frequency involved hearing loss (13). Patients acquire language but sometimes have incomplete pronunciation of consonants, indicating they may already have hearing loss at higher frequencies at the earlier (peri-lingual) ages. Overlapping audiogram (Figure 1B) suggested that some patients with this mutation are good candidates for EAS, but generally the slope is rather gentle. However, from the recent concept of preserving residual hearing it is still worth trying EAS for such patients with some (but not much) residual hearing at the lower frequencies.

CDH23 is known as the responsible gene for USH1D and DFNB12.

Table III. Progressiveness in the candidates for EAS (n=139).

	Number (%)		
Inheritance mode	Progressive	Non-progressive	Unknown
Overall	78 (56%)	44 (32%)	17 (12%)
Sporadic/recessive (n=74)	40 (54%)	24 (32%)	10 (14%)
Dominant/mitochondrial (n=39)	22 (56%)	10 (26%)	7 (18%)

Table IV. Responsible genes in the candidates for EAS (n=139).

Genes identified	Number (%)
Mitochondrial 1555A>G	18 (12.9%)
SLC26A4	10 (7.2%)
CDH23	6 (4.3%)
GJB2	3 (2.2%)

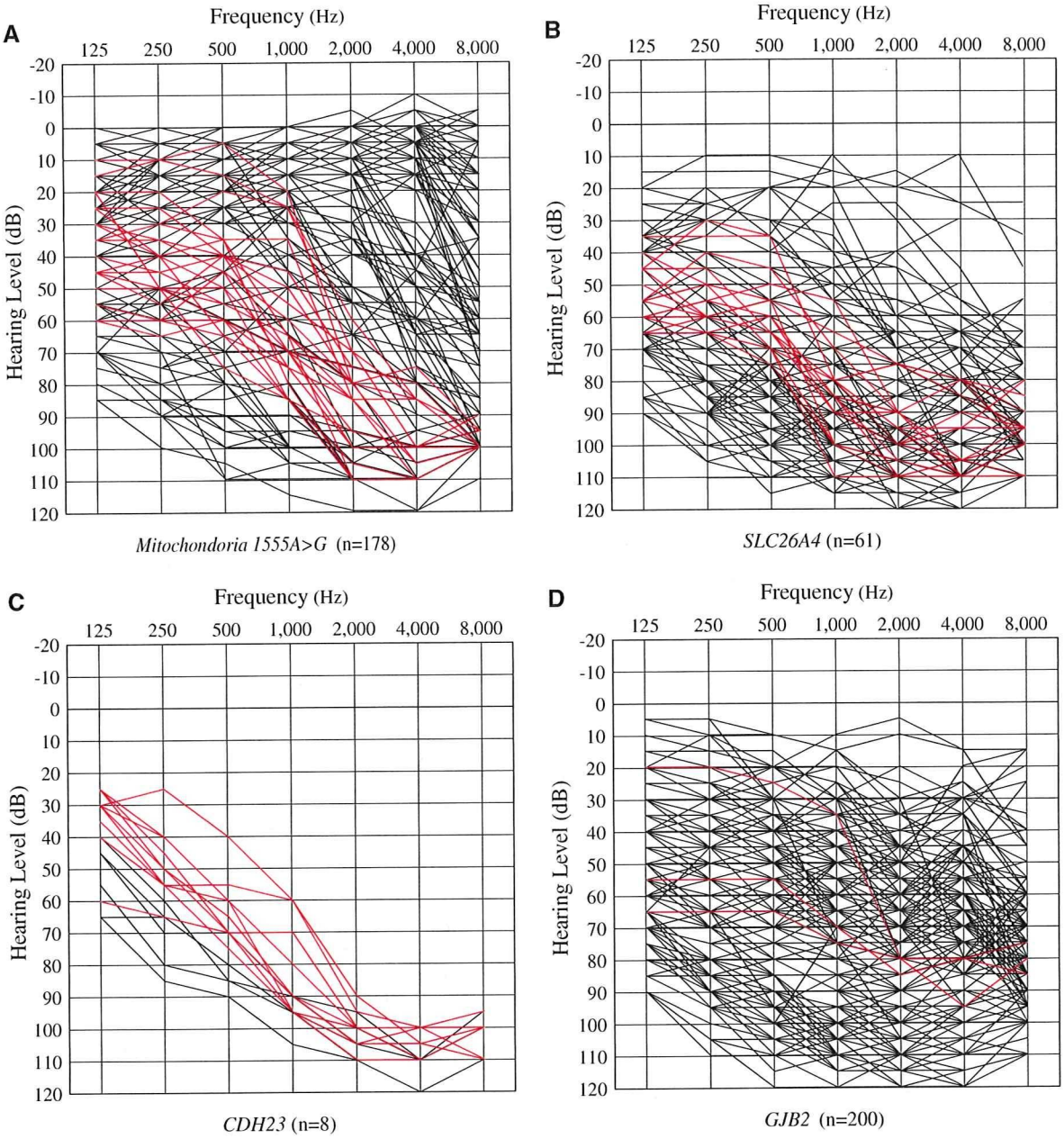

Figure 1. Overlapping audiogram of the patients with mutations. Candidates for EAS are indicated with red lines (A, mitochondrial 1555A>G; B, *SLC26A4;* C, *CDH23;* D, *GJB2*).

Encoded protein cadherin 23 is important for maintaining tip links (14). Patients with this mutation have high-frequency involved progressive hearing loss (6), suggesting that there is a significant number of EAS candidates. Although only a limited number of patients ($n=64$) with *CDH23* mutations were analyzed in this study, overlapping audiograms also indicated that they are good candidates for EAS (Figure 1C).

GJB2 is known to be the most prevalent gene responsible for congenital hearing loss worldwide (see reference 15, for review). Encoded protein, Connexin 26, is known to participate in potassium ion recycling in the inner ear. Currently, more than 100 different *GJB2* mutations are associated with recessive forms of non-syndromic hearing loss (see reference 15, for review). Overlapping audiograms of the 153 patients with bi-allelic *GJB2* mutations showed rather flat or gently sloping audiograms (Figure 1D). As hearing loss is usually reported to be non-progressive, there may be only a small number of the patients with *GJB2* mutations who are indicative for EAS. Only 2.0% of the patients with *GJB2* mutations in this study fit the criteria for EAS.

The present study clearly revealed some responsible genes for ski-slope hearing loss, and genetic testing is potentially useful for estimating progressiveness and decision making for EAS in the future.

However, at the same time, in the majority of patients the cause is still unknown, and screening for various genes should be continued to understand the etiology of this type of hearing loss. In the literature, there have been many responsible genes described as having high-frequency involved hearing loss (16).

In the present study, progression is based on the anamnestic evaluation; therefore, actual speed of progression should be refined in future studies.

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Short Report

Factors that affect hearing level in individuals with the mitochondrial 1555A>G mutation

Lu SY, Nishio S, Tsukada K, Oguchi T, Kobayashi K, Abe S, Usami S. Factors that affect hearing level in individuals with the mitochondrial 1555A>G mutation.
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The mitochondrial 1555A>G mutation is one of the most common mutations responsible for hearing loss in Asians. Although the association with aminoglycoside exposure is well known, there is great variation in the severity of hearing loss. We analyzed hearing levels in 221 Japanese individuals with this mutation and attempted to identify relevant covariants including (i) age, (ii) aminoglycoside exposure, (iii) heteroplasmy ratio, and (iv) other gene mutations. At every age, average hearing levels were worse than those in normal subjects, suggesting that mitochondrial function itself may affect the severity of hearing loss. Although the hearing loss in individuals with the 1555A>G mutation progressed with age, the rate did not differ from that of the normal subjects. Those who had reported aminoglycoside exposure had moderate-to-severe hearing impairment regardless of age, confirming that such exposure is the most important environmental variable. We also confirmed the presence of heteroplasmy, which is known to modify the expression of other mitochondrial diseases, but found no evidence for a significant correlation with hearing impairment. A high prevalence of *GJB2* heterozygous mutations was noted, indicating that these mutations may exhibit epistatic interaction with the 1555A>G mutation.

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Key words: 12S rRNA – 1555A>G mutation – aminoglycosides – *GJB2* – hearing loss – mitochondria

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The 1555A>G mutation in the mitochondrial 12S rRNA gene (1) is the commonest mitochondrial mutation associated with hearing loss. Generally associated with aminoglycoside exposure (2, 3), there are also well-documented patients without a history of exposure (4–6). Systematic screening of Japanese hearing loss patients revealed that approximately 3–5% of these subjects had the 1555A>G mitochondrial mutation, and in those patients who had reported aminoglycoside exposure, the mutation was found in 33% (1, 7). This mutation has been found not only in patients with late-onset hearing loss but also in those with congenital/early-onset sensorineural hearing loss (8). The mitochondrial 1555A>G mutation has been considered to be transmitted in the homoplasmic state, but there have been recent reports of patients with heteroplasmy (8, 9). In an effort to prevent severe deafness, we distribute a drug use warning card advising avoidance of aminoglycosides to 1555A>G mutation family members who are not yet affected (10). The hearing impairment associated with aminoglycoside exposure is usually a bilateral, progressive, high-frequency sensorineural loss. Although it is clear that the patients who report a history of aminoglycoside exposure have a more severe hearing impairment, the severity of deafness is variable (4, 6), suggesting the contribution of additional factors. Age-related expression/progression of hearing loss is one possible factor (4, 5). The existence of modifier genes has also been postulated (11–14), although no candidate genes have been identified. Finally, it was also recently reported that heteroplasmy ratios of the mitochondrial 1555A>G mutation appear to be associated with phenotype variability (9). In order to clarify the possible involvement of these factors in the severity of hearing loss, we investigated the effect of (i) age, (ii) aminoglycoside exposure, (iii) heteroplasmy ratio, and (iv) other gene mutations in 221 individuals with the 1555A>G mutation.

Materials and methods

Subjects

The subjects in this study were 221 Japanese individuals from 67 families with the 1555A>G mutation, ranging in age from 2 months to 87 years. The number of affected members in individual families ranged from 1 to 24 with an approximate average of 3.3. The control group used to determine *GJB2* allele frequency was composed of 252 independent Japanese subjects with normal hearing.

Methods

Audiological analysis
Hearing level was classified using a pure-tone average over 500, 1000, 2000, and 4000 Hz in the better hearing ear. The hearing tests were performed at ages 4–87 years.

Mutation analysis
We screened for the 1555A>G mitochondrial DNA (mtDNA) mutation as described previously (4). In brief, total DNA including genome DNA and mtDNA was extracted from the blood, and the mitochondrial nucleotides 1252 through 1726 were amplified by polymerase chain reaction (PCR). To identify the *Alw*26I site, digestion was performed with a restriction enzyme (*Alw*26I). An ABI sequencer 3100XL (Perkin Elmer Co., Ltd, Waltham, MA) was used to confirm the 1555A>G mutation by direct sequencing.

To identify *GJB2* mutations, a DNA fragment containing the entire coding region was amplified using the primer pair Cx48U/Cx1040L (15). PCR products were sequenced and analyzed with an ABI sequencer 3100XL (Perkin Elmer Co., Ltd). [See Abe et al. (15) for details of the sequencing analysis methods.]

Heteroplasmy ratio of the 1555A>G mitochondrial mutation
The Hitachi FMBIO II image scanning machine (Hitachi Co., Ltd, Minatoku, Tokyo, Japan), a fluorescence imaging system, was used to quantify the heteroplasmy ratio by detection of fluorescently labeled and digested PCR products as described below. A 459 bp DNA fragment was amplified with Ex *Taq* DNA polymerase (Takara Bio Inc., Ohtsushi, Shiga, Japan) using 200 ng of DNA from the subject as a template. Primer sequences were as follows: upper primer, 5′- GCCTATATACCGCCATCTTC -3′; lower primer, 5′- TCTGGTAGTAAGGTGGAGTG -3′. The upper primer was fluorescently labeled at 5′ with rhodamine. PCR conditions were 95°C for 6 min, followed by 27 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 50 s and 72°C for 7 min. The PCR products were digested with restriction endonuclease *Alw*26I (Fermentas; 2.5 units, 37°C for 8–16 h). The subsequent PCR products were digested at 37°C for 8–16 h with 2.5 units of *Alw*26I (Fermentas). Two fluorescent products, wild type (300 bp) and/or mutant (459 bp), were detected because the 1555A>G mutation destroys the restriction site for *Alw*26I. The fluorescent intensity of the mutant bands in quantification experiments from two independent PCR amplifications was used to estimate the proportion of mutant copies in heteroplasmic subjects. We subcloned the insert including the 1555 position into the pDrive cloning vector using a QIAGEN PCR cloning kit (10) (QIAGEN, Hilden, Germany) as an appropriate standard of mutant heteroplasmy. The standard mixtures containing different amounts of wild-type and mutant synthesized oligonucleotides were used with analytical runs to quantify heteroplasmy of mtDNAs.

Statistical analyses

Student's *t*-test was used to compare average hearing levels of subjects with and without *GJB2* mutations and with and without aminoglycoside exposure.

Results

The hearing loss of individuals with the 1555A>G mutation progressed with age; however, the rate of progression did not differ from that found in the normal population (Fig. 1a). The aminoglycoside exposure group had moderate-to-severe hearing impairment regardless of age (Fig. 1b). The existence of heteroplasmy was confirmed in 10 individuals from eight families; however, no apparent correlation was found between heteroplasmy ratio and hearing loss severity (Fig. 1c). There was a high prevalence of *GJB2* heterozygous mutations in individuals bearing the 1555A>G mitochondrial mutation (Table 1), and their hearing levels tended to be worse (without *GJB2* mutation, 35.4 dB; with *GJB2* mutation, 42.0 dB), but the difference was not statistically significant (Fig. 1d). All the *GJB2* mutations found were in heterozygous state, and no subjects were associated with biallelic mutations. There was no correlation between mutation genotype and hearing level.

Discussion

The average hearing level in people with the 1555A>G mutation was worse than that in normal populations at any age (Fig. 1a). This