deteriorated and the patients over 100 dB was even worse. Since these patients hardly attained complete recovery after the conventional steroid therapy, spontaneous complete recovery could be considered to be quite rare. Therefore we consider they are suitable subjects to investigate the efficacy of treatment for ISSHL. In addition, limiting the degree of initial hearing loss could make the evaluation of treatment effect understandable. Any kind of measures such as degree of hearing recovery, final hearing levels, and a rate of patients with defined recovery or defined hearing level, were appropriately employed. On the other hand, when the entry criteria are limited to the patients with profound hearing loss, it would be difficult to collect enough number of patients within a limited period. However, the predicted sample size for treatment effect could be reduced because of the low recovery rate in such subjects. The ratio of the patients with over 100 dB who attained 40 dB or lower level at the final stage by steroid (+HBO) therapy was less than 0.05 (one for 30 in the past decade in our institution). If the new treatment is predicted to increase the ratio to 0.5, sample size needed was calculated at about 16 (two-sided significance level = 0.05, statistical power = 0.8). On the other hand the ratio of the patients with more than 15 dB recovery in overall patients including mild hearing loss by steroid therapy was about 0.7 [5], the calculated sample size was at about 63 to increase the ratio to 0.9.

Edaravone is recommended to start within 24 h after the onset of cerebral infarction. In the phase three RCT trial, a significant improvement in functional outcome was observed in the acute cerebral infarction patients who had been treated within 72 h from onset [13]. If ischemia is assumed as the pathogenesis in ISSHL, edaravone should be started at the early stage. Based on these reports, we set the entry criteria as within three days from the onset in this study. Although the differences of final hearing outcome due to time delay from first to third day were not observed in the edaravone group (data not shown), the patient criteria of earlier stage such as within 24 h from the onset might be more appropriate.

We used the half or same dosage (30 mg of 60 mg/day) of edaravone as cerebral infarction by intravenous administration. In dog, edaravone immediately distributed to cerebral spiral fluid (CSF) after intravenous infusion, the ratio of CSF/plasma concentrations of edaravone maintained around 60% [16]. Maetani et al. reported that edaravone had protective effect against ABR thresholds deterioration and inner hair cell death by intravenous administration (1 mg/kg) one hour after cochlea ischemia in Mongolian gerbils [15]. Although it is expected that intravenous infusion of edaravone also distribute to inner ear and rapidly scavenge free radical, the pharmacodynamics and pharmacokinetics of edaravone in inner ear of human has not been reported. Different administration dosage and methods; intratympanic infusion, might need to be investigated.

In the edaravone group, final hearing levels distributed wider than those in the control group. There was one case in the edaravone group whose hearing was at the level of out of scale in all frequencies at the initial visit and did not change at all after the treatment. We presumed that this particular case might have different pathogenesis from other patients. Although the patients with similar prognostic factors were selected as control, relatively small number of the patients in this study might be the factor not to be able to overcome the variety of pathogenesis in ISSHL.

In this study, final hearing levels between edaravone and control groups were almost same, an advantageous effect of adding edaravone, compared with the past treatment regimen including HBO, was not indicated. This result might suggest that edaravone could replace HBO in the treatment regimen for ISSHL. HBO holds some problems such as cost effectiveness and complications like aerotitis due to barotrauma in high frequency. In this study, edaravone did not produce any side effects. The treatment regimen including edaravone might be considered for ISSHL.

In the past two decades, we utilized HBO to treat the ISSHL patients with severe hearing loss. Therefore, we were not able to compare sufficiently the patients of this study with those treated without HBO. Previously we investigated the efficacy of HBO in ISSHL compared to steroid therapy; no significant difference was shown in overall cases. However, some possibility of superior effect of HBO was shown in the patients with over 90 dB of initial hearing loss; four of 47 patients in HBO but none of 12 patients in steroid showed complete recovery [17]. We speculate that HBO has no remarkable effect in ISSHL, we now present HBO as the one of the selective treatment measure for the patients with profound hearing loss who predicted poor prognosis. Although we consider that edaravone neither has remarkable effect compared with steroid therapy for ISSHL patients with profound hearing loss, it might have some possibility like HBO for improving the pathogenesis of ISSHL.

5. Conclusion

In this study, an advantageous effect of adding edaravone, compared with the past treatment regimen including HBO, was not indicated. Although this result might imply that edaravone could replace HBO in the treatment regimen for ISSHL, we consider that edaravone does not have remarkable effect compared with steroid therapy for ISSHL patients with profound hearing loss in the method of this study. Much effective treatment to such patients is greatly expected to be developed in the near future.

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ORIGINAL RESEARCH–OTOLOGY AND NEUROTOLOGY

Long-term prognosis of low-frequency hearing loss and predictive factors for the 10-year outcome

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ABSTRACT

OBJECTIVES: To determine the long-term prognosis of low-frequency hearing loss and predictive factors for the 10-year outcome of low-frequency hearing loss.
STUDY DESIGN: Case series with chart review.
SETTING: Tertiary referral center.
SUBJECTS AND METHODS: From 1979 to 1998, 466 consecutive patients with low-frequency hearing loss received initial treatment at the Hearing and Tinnitus Clinic of Keio University Hospital. Of the 49 eligible patients, pure-tone threshold data obtained over a period of 10 years after onset of low-frequency hearing loss were available for analysis. To determine the progression of hearing loss, we analyzed audiometric pattern changes. We also examined how the following factors affected 10-year prognosis: sex, age, side of hearing loss, accompanying dizziness, pre-therapeutic hearing thresholds at low frequencies, initial therapy results, and fluctuation of hearing during the first year after onset.
RESULTS: High- and pan-frequency hearing loss increased as time progressed. About half of the cases developed high- or pan-frequency hearing loss within 10 years of onset. Audiometric patterns measured at 10 years significantly correlated with those measured at one ($r = 0.57$), three ($r = 0.73$), and five years ($r = 0.85$). The 10-year prognosis significantly correlated with only two factors: initial therapy results ($r = 0.49$) and fluctuation of hearing during the first year ($r = 0.43$).
CONCLUSIONS: About half of the cases in our study developed high- or pan-frequency hearing loss within 10 years of onset of low-frequency hearing loss. The initial therapy results and fluctuation of hearing during the first year may indicate the long-term prognosis of patients presenting with low-frequency hearing loss.

In patients with sudden idiopathic sensorineural hearing loss, one study concluded that the prognosis of low-frequency hearing loss (LFHL) was better than that of high-frequency hearing loss (HFHL).[1] LFHL may have various causes. Some cases are believed to be due to cochlear hydrops and early-stage Ménière's disease.[2,3] Not all LFHL cases, however, are caused by cochlear hydrops.[4] Although the long-term hearing outcome of patients with Ménière's disease has been reported,[5,6] determination of the long-term prognosis of LFHL has received little attention.[7] One study reported that 39 patients with acute LFHL recovered almost completely.[8] Another study reported that the hearing of two LFHL patients progressed to profound hearing loss.[9] In treatment of a patient with LFHL, one of the main concerns is the long-term prognosis because it is not known whether recurrence or progression of hearing loss will occur, as is seen in Ménière's disease.

Therefore, we conducted a retrospective study on the long-term clinical course of LFHL to determine the prognosis of LFHL and identify predictive factors affecting long-term hearing outcome of patients with LFHL. We hypothesized that the prognosis of LFHL in some cases is poor. We also hypothesized that the 10-year outcome could be predicted by the initial clinical course.

Methods

We reviewed the medical records of 466 consecutive patients with LFHL. These patients started treatment at the Hearing and Tinnitus Clinic of the Keio University Hospital from 1979 to 1998. Acute LFHL is characterized by acute-onset sensorineural hearing loss at low frequencies (125, 250, and 500 Hz); a sum of hearing thresholds at low frequencies ≥ 70 dB; and a hearing threshold difference between both ears < 10 dB at high frequencies. The onset was sudden according to the patient's subjective symptoms, and days from the onset of LFHL to the first clinical visit were restricted to within 14 days. Patients with LFHL accompanied by vertigo attack were excluded from our study. For 61 of these patients, the pure-tone threshold data obtained over a period of ≥ 10 years after LFHL onset were available at the time of our review. Of the 61 patients, four patients developed definite Ménière's disease; these were excluded from our analysis. Eight patients were also excluded because they came to our hospital more than 14 days after LFHL onset, although subjectively the hearing loss

suddenly occurred. Patients with hearing loss related to a definite origin, such as perilymphatic fistula, were also excluded. The remaining 49 patients were included in our study; these presented with LFHL without vertigo. Retrocochlear lesions were ruled out in all 49 patients by MRI or auditory brain stem response (ABR).

Of the 49 patients, 19 were men and 30 were women. The mean (SD) age at the patients' first consultation was 46 (14) years (range 19-73 years). Hearing loss was left-sided in 31 patients, right-sided in 18 patients. The mean duration (SD) from the onset of hearing loss to the patients' last visit to our clinic was 12 (3) years (range 10-21 years). The mean hearing thresholds (SD) at each frequency at onset were 44 (13) dBHL at 125 Hz; 44 (16) dBHL at 250 Hz; 36 (16) dBHL at 500 Hz; 23 (14) dBHL at 1 kHz; 17 (11) dBHL at 2 kHz; 18 (12) dBHL at 4 kHz; and 25 (19) dBHL at 8 kHz.

We treated most of the LFHL patients with oral or intravenous corticosteroids (prednisolone, 30-60 mg per day as a starting dose); diuretics; and vasodilators. For patients who had psychological issues and resisted the medication therapy mentioned above, other treatment options included antidepressants and psychotherapy. Intratympanic steroids were not used for any of the patients in our study.[10]

We evaluated the LFHL patients' hearing thresholds one, three, five, 10, and 15 years after the onset of hearing loss and classified them into four categories on the basis of audiogram shape: normal hearing level (NHL), LFHL, HFHL, and pan-frequency hearing loss (PFHL). Spearman product moment correlation coefficients were used to evaluate the association between audiogram shapes at each time point. The audiogram shape for each type of hearing loss was defined as follows. In NHL, either hearing threshold was < 30 dBHL at all frequencies tested or the hearing threshold difference between both ears was < 10 dB at all frequencies tested. In LFHL, hearing loss was limited at low frequencies (125, 250, and 500 Hz), and hearing threshold difference between both ears was < 10 dB at high frequencies. In HFHL, hearing threshold difference between both ears was ≥ 10 dB at high frequencies. In PFHL, hearing loss was either ≥ 30 dB at all frequencies tested or ≥ 10 dB worse than the opposite ear at all frequencies tested. Patients with hearing loss at low and high frequencies but normal hearing at 1 kHz were classified as having HFHL.

Spearman product moment correlation coefficients were also used to determine the effect of the following factors on 10-year prognosis: sex; age; side of hearing loss; accompanying dizziness; pre-therapeutic hearing threshold at low frequencies (125, 250, and 500 Hz); result of the initial therapy; and fluctuation of hearing during the first year after onset. The results of the initial therapy were evaluated according to the following criteria:[11] complete recovery (hearing thresholds at low frequencies were less than 20 dB or the same level as the opposite ear); partial recovery (average of hearing thresholds at low frequencies improved more than 10 dB but was not at the same level as the opposite ear); unchanged (average hearing thresholds at low

frequencies improved < 10 dB); and progressive changes (hearing change other than the aforementioned three criteria). We considered a difference in the two most recent audiograms of > 15 dB at one frequency or > 10 dB at more than two frequencies indicative of hearing fluctuations.

All statistics were calculated with SPSS 15.0J for Windows (SPSS Inc., Chicago, IL); a P value < 0.05 was considered statistically significant.

This study was approved by the institutional review board at Keio University School of Medicine.

Results

The proportions of each type of audiogram measured at onset and at one, three, five, 10, and 15 years after onset are shown in Figure 1. At one year, 43 percent of the patients displayed NHL, whereas 57 percent of the patients displayed LFHL. The number of patients exhibiting either HFHL or PFHL increased as time progressed. Ten years after onset, 35 percent of the cases were classified as NHL, 24 percent as LFHL, 17 percent as HFHL, and 24 percent as PFHL. At 15 years, the proportion of cases displaying these four categories of audiogram shape was almost the same as that at 10 years.

The relationship between audiogram shape obtained at 10 years and that obtained at one, three, and five years is shown in Figure 2. Analyses of audiogram shape revealed that once hearing progressed to HFHL or PFHL, hearing never returned to NHL or LFHL. Spearman correlations between audiograms obtained at 10 years and those obtained at one, three, and five years were statistically significant; the coefficients were 0.57 at one year, 0.73 at three years, and 0.85 at five years ($P < 0.01$).

The 10-year prognosis significantly correlated with only two factors: initial therapy results ($r = 0.49$, $P < 0.01$) and hearing fluctuation during the first year ($r = 0.43$, $P < 0.01$). The other factors did not show significant correla-
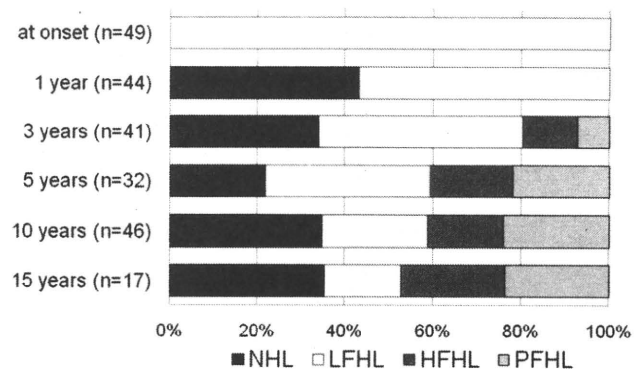
Figure 1 Proportions of four audiometric patterns at each time point after onset. Forty-nine cases were followed up for > 10 years. (The number of cases with available audiograms at each time point is different.) *HFHL*, high-frequency hearing loss; *LFHL*, low-frequency hearing loss; *NHL*, normal hearing level; *PFHL*, pan-frequency hearing loss.
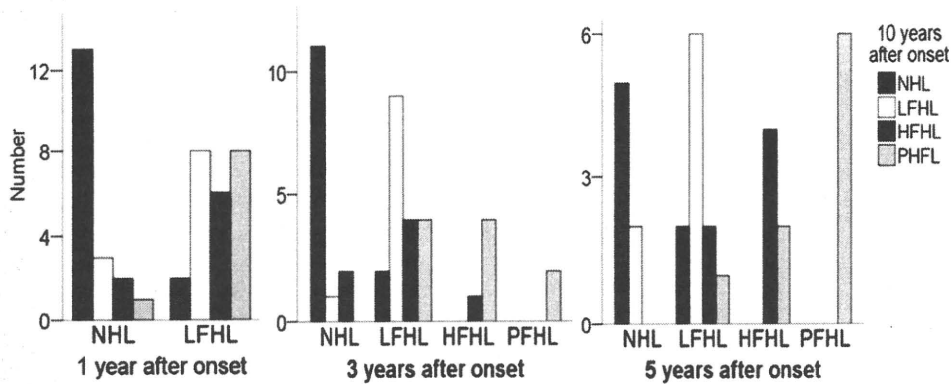
Figure 2   Relationship between audiometric patterns at 10 years and at one, three, and five years. *HFHL*, high-frequency hearing loss; *LFHL*, low-frequency hearing loss; *NHL*, normal hearing level; *PFHL*, pan-frequency hearing loss.

tions. Figure 3 shows the different 10-year outcomes in terms of the two significant factors. Twelve of 13 patients with good initial therapy results (complete recovery) had regained hearing at the 10-year point. All of these patients did not display hearing fluctuations during the first year after LFHL onset. By contrast, six of seven patients with poor initial therapy results (unchanged or progressive changes) experienced HFHL or PFHL by 10 years. All of these patients experienced hearing fluctuations during the first year after LFHL onset.

Discussion

The findings indicate that the hearing of some LFHL cases can progress to HFHL or PFHL, and that once LFHL

progresses to HFHL or PFHL, hearing never returns to NHL or LFHL. Our findings also indicate that the 10-year prognosis for those initially presenting with LFHL can be predicted by the clinical course during the first year after onset: initial therapy results and hearing level fluctuations during the first year.

Whether LFHL is an independent disease is controversial. LFHL is considered a subtype of idiopathic sudden hearing loss that has a better prognosis than other types of hearing loss.[1,12] Greater loss of hearing for low tones than for high tones accompanied by fluctuations in hearing has also been reported to be characteristic of endolymphatic hydrops.[13] Some electrocochleography studies showed that at least some LFHL cases are caused by endolymphatic hydrops;[2-4] however, only approximately 10 percent of
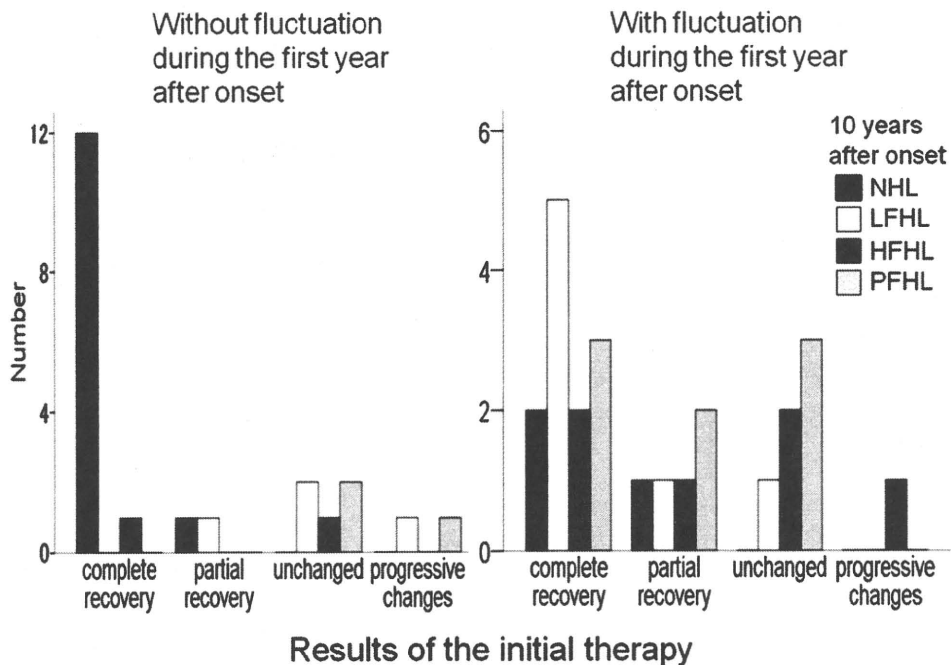

Figure 3   Ten-year outcomes according to initial therapy results and fluctuation of hearing during the first year after LFHL onset. *HFHL*, high-frequency hearing loss; *LFHL*, low-frequency hearing loss; *NHL*, normal hearing level; *PFHL*, pan-frequency hearing loss.

LFHL cases develop Ménière's disease.[3,4,7] The fact that only a few LFHL cases progress into Ménière's disease suggests that LFHL is not the same disease as Ménière's disease.[7]

An epidemiological study conducted in Japan revealed a high incidence of acute LFHL without vertigo, occurring at a rate of 42.8 to 65.7 per 100,000 persons,[11] indicating that otolaryngologists commonly encounter LFHL cases in their daily practice. Although some LFHL cases are known to fluctuate, recur, or progress, the > 10-year prognosis of LFHL has yet to be reported.[7,14] Moreover, the lack of long-term observations makes it difficult to predict the outcome of LFHL.[15]

In this study, we focused on audiometric pattern changes to describe the progression of LFHL. Strikingly, residual hearing loss after the initial therapy or fluctuations in hearing during the first year after LFHL onset may relate to the development of hearing loss at middle and high frequencies 10 years after onset. Our results indicate that otolaryngologists should pay particular attention to the following case scenarios: 1) cases that show complete recovery at first but display accompanying hearing fluctuations within one year of onset can progress to HFHL or PFHL (42%, 5 of our 12 cases); 2) cases with residual hearing loss after initial therapy (partial recovery, unchanged, or progressive changes) may progress to HFHL or PFHL (57%, 12 of 21 cases); 3) cases that respond poorly (unchanged or progressive changes) to initial therapy and that display fluctuations during the first year may experience HFHL or PFHL (86%, 6 of 7 cases).

Corticosteroids (prednisolone, 30-60 mg per day as a starting dose) were administered orally or intravenously to most of our patients as initial therapy. High-dose steroid or intratympanic steroid administration has been reported to be effective in some LFHL cases that responded poorly to initial treatments.[10,16] Thus, these two treatment options could mean a better outcome of long-term prognosis. A double-blind study showed that steroids are effective in the treatment of idiopathic sudden hearing loss (ISHL);[17] however, no treatment option has been proven to be effective for treating recurrent ISHL. Our results indicate that steroids may not be effective for treating HFHL or PFHL that progressed from LFHL, even though the hearing thresholds observed in HFHL or PFHL are within the "steroid-effective zone," that is, within moderate hearing loss, which is successfully treated with steroids.[17]

With regard to hearing loss progression at high frequencies, we should consider the effect of aging. To determine the effect of aging in our HFHL and PFHL cases, we calculated the annual rates of hearing changes at high frequencies (2, 4, and 8 kHz) for both ears during the first 10 years. The average rates of hearing change in affected ears were 3.9 dB at 2 kHz, 4.4 dB at 4 kHz, and 4.7 dB at 8 kHz. On the other hand, the average rates of hearing change in unaffected ears were 0.8 dB at 2 kHz, 0.8 dB at 4 kHz, and 2.1 dB at 8 kHz. This difference in hearing loss progression

between affected and unaffected ears suggests that the progression to HFHL and PFHL is not due to aging, but due to the effect of the disease. As this issue is beyond the scope of this paper, we will report the details of these differential hearing changes across both ears in another paper.

One limitation in our study is the high rate of dropouts. Only 13 percent (61 of 466) of the patients had pure-tone threshold data spanning over a period of ≥ 10 years after LFHL onset. Another 11 percent (49 of 466) of the patients visited our hospital for five or more years. However, the remaining 76 percent (356 of 466) of the patients stopped visiting our hospital within a few years, occasionally even within a few months, after LFHL onset. We attempted to contact some of these LFHL patients by letter; however, only about 30 percent of them replied (unpublished data). Thus, LFHL patients need to be followed through a prospective study with fewer dropouts. In the present study, for many of our patients, we did not conduct tests to determine the etiology of LFHL. However, 15 patients did undergo a glycerol test, which was positive in only three patients. The results of the glycerol tests did not correlate with the prognosis. Electrocochleography was not performed on any of the patients.[18] Although electrocochleography has been shown to effectively detect endolymphatic hydrops in acute LFHL patients,[2] it is not useful for predicting the prognosis of LFHL.[3] Additionally, otoacoustic emission testing is not a suitable prognostic test for LFHL.[19] On the basis of our current findings, we propose that the clinical course is important for predicting the prognosis of LFHL.

Conclusion

The hearing of about half of the LFHL patients in our study progressed to HFHL or PFHL within 10 years after LFHL onset. Our data indicate that the long-term prognosis could be predicted by initial therapy results and the occurrence of hearing fluctuations during the first year after onset.

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Disclosures

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Short Report

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

Tsukada K, Nishio S, Usami S, and the Deafness Gene Study Consortium. A large cohort study of *GJB2* mutations in Japanese hearing loss patients. Clin Genet 2010: 78: 464–470. © John Wiley & Sons A/S, 2010

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 by 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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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.T123N (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 who were diagnosed at an earlier age: 25.7% (108/420) in those diagnosed at age 0–3, 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 probands diagnosed at an earlier age (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

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

Amino acid change	Nucleotide change	Patients					Controls				Evolutionary conservation	Reference
		Allele (n = 2686)	Allele frequency (%)	Homozygous (n)	Compound heterozygous (n)	Heterozygous (n)	Alleles (n = 504)	Allele frequency (%)	Controls (n = 252)	Carrier rate (%)		
—	c.235 delC	142	5.29	34	45	28	2	0.40	2	0.80	NA	Fuse et al. (19)
p.V37I	c.109G>A	47	1.75	3	11	30	3	0.60	3	1.20	Yes	Abe et al. (1)
p.G45E[c]	c.134G>A											
p.Y136X[c]	c.408C>A	34	1.27	1	22	10					Yes	Fuse et al. (19)
p.R143W	c.427C>T	18	0.67	0	16	2					Yes	Brobby et al. (20)
—	c.176_191 del16bp	15	0.56	0	10	5					NA	Abe et al. (1)
—	c.299-300 delAT	11	0.41	0	8	3					NA	Abe et al. (1)
p.T86R	c.257C>A	8	0.30	0	5	3					Yes	Ohtsuka et al. (2)
—	c.512insAACG	3	0.11	0	3	0					NA	Hismi et al. (21)
—	c.35insG	2	0.07	0	2	0					NA	Estivill et al. (22)
p.I71T[b]	c.212T>C	2	0.07	0	0	2					Yes	Ohtsuka et al. (2)
p.T8M	c.23C>T	1	0.04	0	0	1					Yes	Kenna et al. (23)
p.I33N[b]	c.98T>A	1	0.04	0	0	1					Yes	This study
p.A49V[b]	c.146C>T	1	0.04	0	0	1					Yes	Ohtsuka et al. (2)
p.N54S	c.161A>G	1	0.04	0	1	0					Yes	This study
p.Y68C[a]	c.203A>G	1	0.04	0	0	1					No	This study
p.M93I	c.276G>A	1	0.04	0	1	0					Yes	Wu et al. (24)
p.K112M[b]	c.335A>T	1	0.04	0	0	1					Yes	This study
—	c.376-377 delAA[e]	1	0.04	0	0	1					NA	This study
p.W133X	c.398G>A	1	0.04	0	1	0					NA	Primignani et al.[f]
p.K168R[b]	c.503A>G	1	0.04	0	0	0					Yes	This study
p.M195V	c.583A>G	1	0.04	0	1	0					Yes	This study
—	c.605ins46bp	1	0.04	0	0	1					NA	Yuge et al. (25)
p.F191L	c.571T>C	0	0	0	0	0	1	0.20	1	0.40	yes	Feng et al. (26)
p.R127H	c.380G>A	0	0	0	0	0	1	0.20	1	0.40	yes	Seeman et al.[f]
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. (19)
p.T123N[d]	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]Variant probably representing polymorphism because no evolutionary conservation was observed.

[b]Variants with unproven pathogenic nature.

[c]p.G45E and p.Y136X(c.134G>E) mutations are on the same parental allele.

[d]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.

[e]c.376-377 delAA 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.

[f]Ballana E, Ventayol M, Rabionet R et al. Connexins and deafness Homepage. World wide web URL: http://www.crg.es/deafness.

Table 2. The frequency of GJB2 mutations and diagnostic age

	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
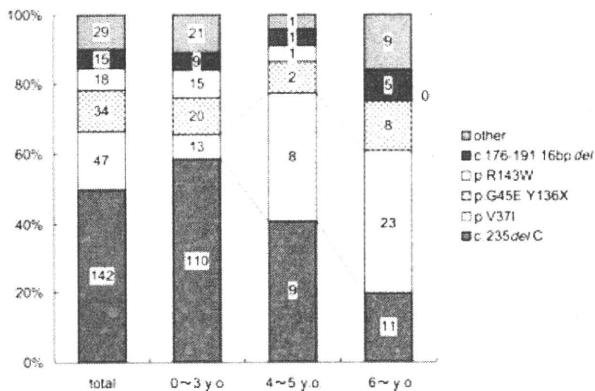
Fig. 1. Frequency of mutant GJB2 alleles in different diagnostic age groups. c.235*del*C was mainly found in the group diagnosed at up to 3 years, where it was significantly higher than in age 6 and over (p < 0.01; χ² test). On the contrary, p.V37I was mainly found in the diagnostic age groups of 4–5, 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; χ² 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; χ² 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; χ² 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 those diagnosed at age 0–3 (for practicality categorized as congenital hearing loss). 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 patients diagnosed at age 0–3.

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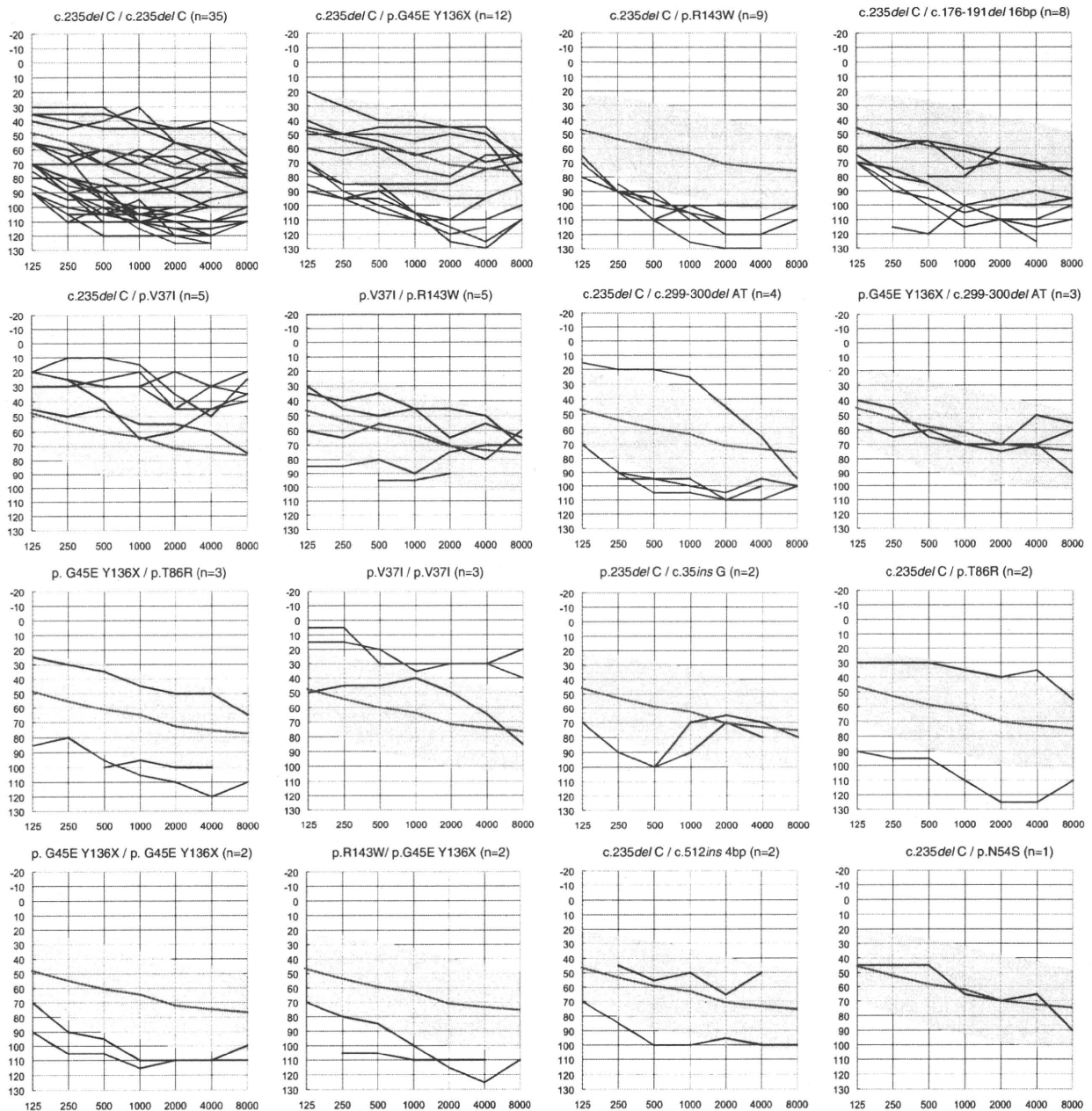
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Fig. 2. Overlapping audiograms from the better ear for each genotype. The average audiogram from all subjects (1343 with bilateral sensorineural hearing loss) is indicated by a red line with standard deviation (shadow).

We compared homozygous for c.235*del*C with compound heterozygous with p.R143W (except 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
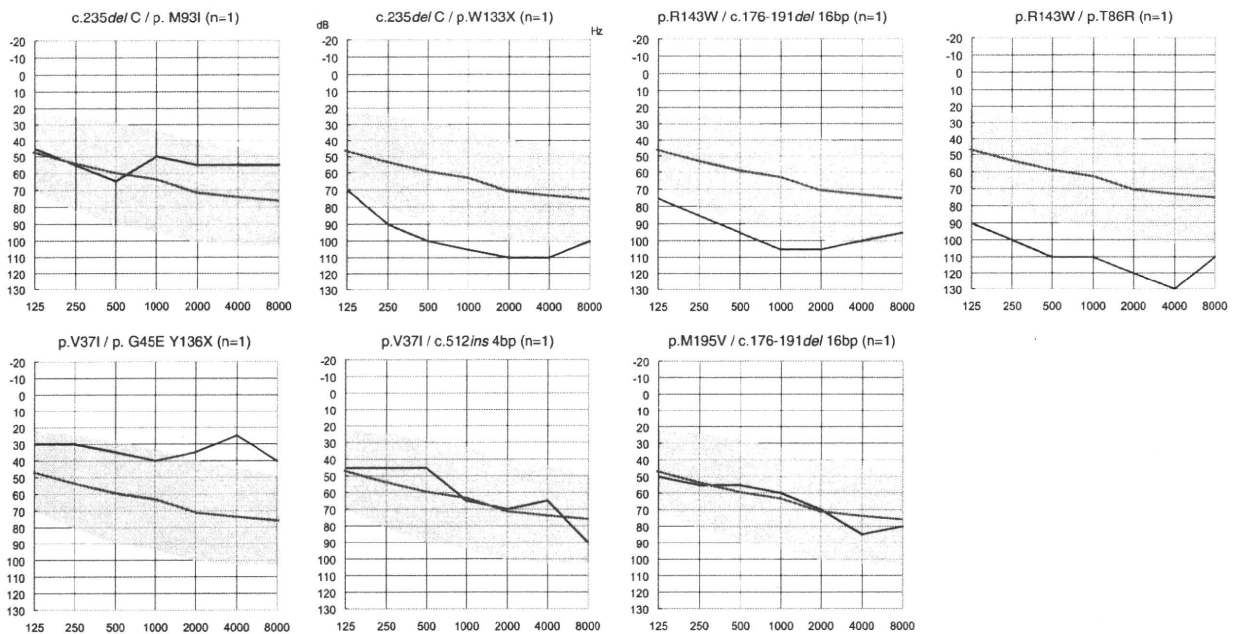
Fig. 2. Continued

diagnostic screening for hearing loss based on the invader assay (18). This database will also facilitate clinical application, and we intend to expand it to cover all Asian populations.

Acknowledgements

Conflict of interest

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

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SHORT COMMUNICATION

Hair roots as an mRNA source for mutation analysis of Usher syndrome-causing genes

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mRNA is an important tool to study the effects of particular mutations on the mode of splicing and transcripts. However, it is often difficult to isolate mRNA because the organ or tissue in which the gene is expressed cannot be sampled. We previously identified two probable splicing mutations (c.6485+5G > A and c.8559-2A > G) during the mutation analysis of USH2A in Japanese Usher syndrome (USH) type 2 patients, but we could not observe their effects on splicing because the gene is expressed in only a few tissues/organs, and is not expressed in peripheral lymphocytes. In this study, we used hair roots as a source of mRNA of USH-causing genes, and successfully detected the expression of seven, except USH1C and CLRN1, of the nine USH-causing genes. We used RNA extracted from the hair roots of a patient who has both c.6485+5G > A and c.8559-2A > G mutations in USH2A in a compound heterozygous state to observe the effects of these mutations on transcripts. Reverse-transcription PCR analysis revealed that c.6485+5G > A and c.8559-2A > G inactivated splice donor and splice acceptor sites, respectively, and caused skipping of exons. Thus, RNA extracted from hair roots is a potential powerful and convenient tool for the mutation analysis of USH-causing genes.
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INTRODUCTION

To perform mutation analysis in the study of hereditary diseases, we generally used genomic DNA for the detection of mutations in exons and adjacent intronic regions of the gene of interest. Further, mRNA, if available, can also be used for the detection of mutations and for determining their effects on transcripts. However, except in the cases when the gene of interest is expressed in blood cells, it is difficult to isolate mRNA because the organ or tissue in which the gene of interest is expressed cannot be sampled.

We recently performed mutation analysis of USH2A gene for Japanese patients of Usher syndrome (USH) type 2, and identified 14 mutations, including 11 novel ones.[1] Of these mutations, two were splicing mutations, c.6485+5G > A and c.8559-2A > G in introns 33 and 42, respectively. We determined the pathogenicity of these mutations using supportive data, but could not examine their effect on pre-mRNA splicing because of the difficulty in obtaining USH2A mRNA. The expression of USH2A mRNA is restricted to a few tissues, including the retina and the cochlear, and is absent in peripheral lymphocytes.[2] Similarly, peripheral lymphocytes do not express mRNA of any other USH-causing genes, except DFNB31.[3]

Here, we attempted to use hair roots as a source of USH-causing gene mRNA. We successfully detected the mRNA expression of most USH-causing genes and analyzed the effect of the above-mentioned USH2A mutations on pre-mRNA splicing. This is the first report on the mRNA expression of USH-causing genes in hair roots.

MATERIALS AND METHODS

Collection of hair roots

At least 30 hair root samples were collected from the scalp of normal Japanese individuals and a USH type 2 patient. The patient had c.6485+5G > A and c.8559-2A > G mutations in USH2A in a compound heterozygous state (see the patient C152 in a previous report[1]). The institutional review board of Hamamatsu University School of Medicine approved this study, and written informed consent was obtained from all participants before enrollment.

Reverse-transcription PCR of USH-causing genes

Total RNA was extracted from the hair roots using the SV Total RNA Isolation System (Promega, Madison, WI, USA). Next, 2 μg of total RNA was reverse transcribed with oligo(dT) primers by using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) for all nine known USH-causing genes was amplified using specially designed PCR primers (Table 1). The PCR mixtures (total volume, 20 μl) contained 2 μg cDNA, 1.0 M betaine (Wako, Osaka, Japan), 1.5 mM MgSO4, 0.3 μM each primer and 0.4 U KOD Plus DNA polymerase (Toyobo, Osaka, Japan). The amplification conditions were as follows: denaturation at 94 °C for

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Table 1 Nucleotide primers designed for PCR and sequencing of Usher syndrome-causing genes

Template	Primer sequences (5' to 3')	Exon	Annealing temperature (°C)[a]	Product length (bp)
MYO7A cDNA	F: TGAGATTGGGGCAGGAGTTCGACG	2	68	428
	R: GATGATGCAGCACTGGTCTCGGCT	4		
USH1C cDNA	F: AGTGGCCCGAGAATTCCGGCATAA	1	64	359
	R: CTGCCTGACCGCCTTTGATGAGGT	4		
CDH23 cDNA	F: GGTCGGCTTTGCCCTTCCACTCTT	11	64	449
	R: GTCCCGTGTCCTTGTCCAGCGAGA	14		
PCDH15 cDNA	F: TGCCAAACACTCGTGATTGCCGTC	8	64	330
	R: GACCGGCAAAGGCAGGAAGAGGAT	11		
USH1G cDNA	F: CCCACTCTCTGGGCTGCCTACCAT	1	68	443
	R: GTGAGGCTGGAGAAGCTGAGGGTGT	2		
USH2A cDNA	F: TAACTGCTTGCACTTTGGCTGGCT	31	64	613
	R: GTTAGGGCCTCACTGGCCTCACTC	35		
USH2A cDNA	F: GTGGTGACAGTGCTGGAACCCGAT	41	64	563
	R: ACAGTCACTTCTCGGCTCGGTGTAA	44		
GPR98 cDNA	F: ACTCACCTTTTGGCTTGGTGGGCT	53	64	533
	R: AAAGCTTCCAGCCAGCCGGACTAC	56		
DFNB31 cDNA	F: CTGCGCGTCAACGACAAATCCCTG	1	64	371
	R: CCTGGGTCCACGCCAGTGATGTAA	3		
CLRN1 cDNA	F: GCAATCCCAGTGAGCATCCACGTC	2	64	368
	R: GGGAACTGAAATCCAGCAAGTCGT	3		

Abbreviations: F, forward; R, reverse.
[a]The amplification conditions were as follows: denaturation at 94 °C for 2 min; followed by 40 cycles of treatment at 98 °C for 10 s, 64 or 68 °C for 30 s (see this column), and 68 °C for 1 min; and final extension at 68 °C for 5 min.
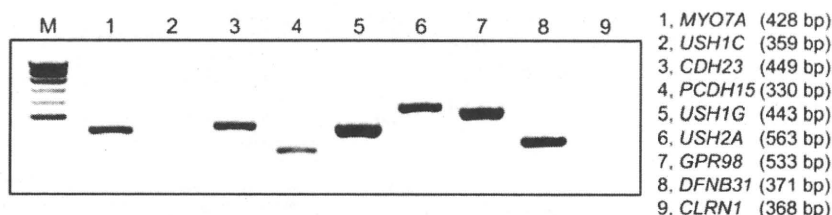
Figure 1 RT-PCR analysis of USH-causing genes. mRNA expression of all USH-causing genes, except USH1C and CLRN1, was detected in normal control hair roots. PCR was performed using 2 μg cDNA (total volume, 20 μl) with 40 cycles.

2 min; followed by 40 cycles of treatment at 98 °C for 10 s, 64 or 68 °C for 30 s (described in Table 1) and 68 °C for 2 min; and final extension at 68 °C for 5 min.

RESULTS

Detection of mRNA of USH-causing genes in hair roots

Total RNA was prepared from the scalp hair root samples obtained from normal individuals. Reverse-transcription PCR (RT-PCR) analysis revealed the mRNA expression of all USH-causing genes, except USH1C and CLRN1, in hair roots (Figure 1).

Detection of the splicing abnormality caused by USH2A splicing mutations

We next attempted to detect the splicing abnormality caused by the compound heterozygous mutations c.6485+5G>A and c.8559-2A>G in USH2A. Total RNA was prepared from the hair root samples obtained from the patient, and RT-PCR was performed using primers to amplify the cDNA between exons 31 and 35. Agarose gel electrophoresis of the RT-PCR products revealed two bands—a larger band corresponding to the normal sequence and a smaller band corresponding to the mutant sequence (Figure 2a). Sequence analysis of the mutants revealed that c.6485+5G>A causes skipping of exon 33 (160 bp) and presumably creates a premature stop codon in exon 34

through a frameshift. Similarly, RT-PCR performed using primers to amplify the cDNA between exons 41 and 44 revealed that c.8559-2A>G causes skipping of exon 43 (123 bp) (Figure 2b) and presumably induces a 41-amino-acid deletion. These results revealed that c.6485+5G>A and c.8559-2A>G inactivated splice donor and splice acceptor sites, respectively, and this finding confirmed the pathogenicity of these mutations.

DISCUSSION

RT-PCR analysis revealed the mRNA expression of seven of the nine USH-causing genes in hair roots. It has been reported that the mRNA of one USH-causing gene, MYO7A (causes USH type 1B), can be detected in the nasal epithelium;[4] however, obtaining MYO7A mRNA would necessitate invasive and painful tissue sampling methods. In contrast, collecting hair roots from the scalp is not an invasive procedure. Further, analysis of total RNA obtained from the hair roots of the patient with USH type 2 revealed that the two intronic mutations c.6485+5G>A and c.8559-2A>G inactivated a splice donor and splice acceptor sites, respectively, and both these mutations resulted in exon skipping. This is the first report to describe the RT-PCR analysis of USH2A mutations and show that the mutations close to the splice donor/acceptor sites cause splicing errors.
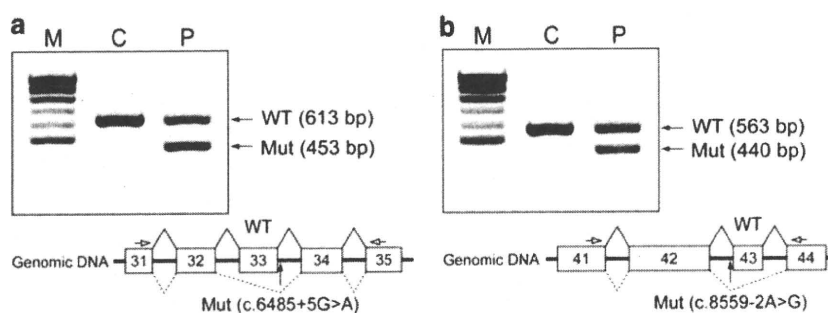
Figure 2 (**a**) Products of RT-PCR performed using primers to amplify *USH2A* cDNA between exons 31 and 35. The c.6485+5G>A mutation caused skipping of exon 33 (160 bp) and was presumed to create a premature stop codon in exon 34 through a frameshift. (**b**) Products of RT-PCR performed using primers to amplify *USH2A* cDNA between exons 41 and 44. The c.8559-2A>G mutation caused skipping of exon 43 (123 bp) and was presumed to create a 41-amino-acid deletion. Boxes with a number represent the exons. The solid and dotted lines that connect exons show the manner of splicing in the wild type and mutant, respectively. The distance between exons does not indicate the actual intron sizes. The open arrows indicate the PCR primers, and the closed arrows indicate mutations in introns. M, molecular marker (100 bp ladder); C, control; P, patient; WT, wild type; Mut, mutant.

Generally, mRNA is very useful for mutation analysis, especially in the case of coding-sequence mutations in large multi-exon genes, splicing mutations and regulatory-region mutations that affect the expression levels. Of these, the use of mRNA to determine the effect of a mutation on splicing as we revealed in this report is the most important advantage because we still cannot accurately predict splicing changes from DNA sequence alterations, especially if the alterations occur at a distance from splicing donor/acceptor sites[5] or within exonic splicing enhancers.[6]

Thus, mRNA extracted from hair roots is a potentially powerful and convenient tool for mutation analysis in USH-causing genes. Further, it is also reasonable to hypothesize that the mRNA of genes that cause deafness can be detected in hair roots, and this may facilitate easier and more accurate mutation analysis.

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Original Paper

Audiology Neurotology

Specific Localization of Five Phosphatidylcholine Species in the Cochlea by Mass Microscopy

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Key Words

Abstract

Phosphatidylcholine (PC), a phospholipid, is a basic structural component of cell membranes. PC species exhibit various binding patterns with fatty acids; however, the distributions of PC species have not been studied in the cochlea. In recent years, imaging mass spectrometry has been used as a biomolecular visualization technique in medical and biological sciences. We recently developed a 'mass microscope' consisting of a mass spectrometry imager with high spatial resolution equipped with an atmospheric-pressure matrix-assisted laser desorption/ionization and quadrupole ion trap time-of-flight analyzer. In this study, we applied the mass microscope to analyze cochlear tissue sections. The imager allowed visualization of the localization of PC species in each region of the cochlea. The structures of the PC species were determined using tandem mass spectrometry. PC(16:0/18:1) was highly localized in the organ of Corti and the stria vascularis. PC(16:0/18:2) was mainly observed in the spiral ligament. PC(16:0/16:1) was found primarily in the organ of Corti. These distributional differences may be associated with the cellular architecture of these cochlear regions.

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Introduction

The cochlea is essential for auditory transduction. Each of its highly organized structures has a different role in this process. For example, the organ of Corti, which lies on the basilar membrane, is responsible for mechanoelectrical transduction. The stria vascularis, on the lateral wall of the cochlear duct, supplies the endolymph with ions and energy. The fine architecture of the cochlea has been studied using various scientific methods, including anatomical, electrophysiological, molecular-biological, and other approaches [Wangemann and Schacht, 1996; Ashmore and Gale, 2000].

Over the past 2 decades, rapid progress in research on hereditary hearing loss has identified numerous constituent molecules in the cochlea, including ion channels, transporters, cell junctions, and the Usher interactome [Adato et al., 2005; Wangemann, 2006; Lang et al., 2007]. The authors have also localized an endocytic receptor and a component of the extracellular matrix in the cochlea [Mizuta et al., 1999; Hosokawa et al., 2009]. All of these molecules are proteins.

In addition to proteins, lipids are basic structural components of biological membranes, and their roles in the cochlea have also been investigated. Lipids play a significant role as a phosphoinositide second-messenger system in the cochlea [Ogawa and Schacht, 1994]. Their localiza-

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tion has been studied using radioactive probes in cultured cochlear tissue [Niedzielski and Schacht, 1991]. Phosphatidylcholine (PC) species are one class of phospholipids, and their activity is related to the activity of several anti-oxidant enzymes, such as glutathione reductase and superoxide dismutase, in the brain [Benzi et al., 1989]. Oxidative stress has been strongly associated with aging. In the cochlea, PC species have been shown to influence age-related hearing loss [Seidman et al., 2002]. However, PC species exhibit various binding patterns with fatty acids [Kuksis and Marai, 1967], and their distribution and function based on these various binding patterns remain elusive.

We have developed an imaging mass spectrometry (IMS) approach that uses a matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight (MALDI-QIT-TOF) mass spectrometer [Shimma et al., 2008; Sugiura and Setou, 2010]. This method can be used to analyze the distribution and structure of various biomolecules, including PC species, in tissue sections as mass images [Kimura et al., 2009]. Differing distributions of PC species have recently been reported for the brain [Mikawa et al., 2009; Sugiura et al., 2009] and the eye [Hayasaka et al., 2008] in mice. We recently developed a new mass spectrometry (MS) imager, called a 'mass microscope' [Harada et al., 2009], to obtain higher spatial resolution than a conventional imager can provide. In this study, we used this mass microscope to define the distributions of PC species in unfixed sections of the guinea pig cochlea.

Materials and Methods

Tissue Preparation
Three-week-old male Hartley guinea pigs (SLC, Hamamatsu, Japan) with normal Preyer's reflex were anesthetized with pentobarbital (50 mg/kg body weight, i.p.) and decapitated in accordance with an animal study protocol approved by the Hamamatsu University School of Medicine Animal Care and Use Committee. The temporal bones were isolated, and the cochleae were dissected. The cochleae were immediately frozen in liquid-nitrogen-cooled isopentane without fixation. The frozen cochleae were then immersed in a 2% sodium carboxymethylcellulose solution for embedding and stored at -80°C. Before sectioning, the cochleae were kept for 20 min at -20°C. Consecutive 15-μm sections of the frozen cochleae were prepared using a cryostat (Cryocut CM 1950; Leica Microsystems, Wetzlar, Germany). The sections were mounted on glass slides coated with indium tin oxide (Bruker Daltonics, Bremen, Germany).

Mass Microscope for IMS
A mass microscope (Shimadzu, Kyoto, Japan) is a high-resolution (spatial resolution, 10 μm) IMS instrument with MALDI-QIT-TOF combined with an optical microscope under atmospheric pressure [Harada et al., 2009]. The MALDI chamber contains a charge-coupled device (CCD) camera (Olympus, Tokyo, Japan).

A matrix of 2,5-dihydroxybenzoic acid (DHB; Bruker Daltonics) was prepared at a concentration of 50 mg/ml DHB in 70% methanol (Kanto, Tokyo, Japan) and 0.1% trifluoroacetic acid (Kanto). DHB matrix solution (1 ml) was sprayed uniformly for 30 min over the section with a 0.2-mm nozzle caliber airbrush (Procon Boy FWA Platinum; Mr. Hobby, Tokyo, Japan). During spraying, the distance between the nozzle and the tissue surface was maintained at 15 cm. After the DHB matrix was allowed to dry, the glass slide coated with indium tin oxide was placed on the mass microscope target plate. All analyses were performed in the positive-ion mode. The experimental parameters for the imaging were: scan pitch, 10 μm; pixels, 221 × 250; number of laser irradiations per pixel, 200, and laser pulse energy, 0.13 μJ. The mass microscope was operated in the range of m/z 400–1000 to detect the ion signals of the PC species.

Identification of the PC Species
The same section was used for raster scanning and tandem MS (MS/MS) analyses. The ion signals produced by raster scanning were identified as PC species using MS/MS analyses. MS/MS analyses of the cochlear sections were performed at a laser pulse energy of 0.05–0.13 μJ to determine the optimal energy. In the product ion spectrum, when the product ions corresponded to a specific neutral group loss, such as the choline head group including trimethylamine [(CH_3)$_3$N] alone or with the cyclophosphane ring [(CH_2)$_2$PO$_4$H], and fatty acids from a PC [Hsu and Turk, 2003], the m/z value of the PC precursor ion was input into the metabolite MS search (http://www.hmdb.ca/labm/jsp/mlims/MSDbParent.jsp). This website provides information on the numbers of carbons and unsaturated bonds in each fatty acid of a PC species [Hayasaka et al., 2009]. In this study, to determine the detailed compositions of the fatty acids, the neutral loss of at least one fatty acid from the PC in the MS/MS analysis was required. The distribution of biomolecules that were identified by MS/MS analysis was visualized as mass images using BioMap software (http://www.maldi-msi.org). This software was provided at no cost by Novartis (Basel, Switzerland).

Statistical Analyses
Representative areas 1600 μm² (40 × 40 μm) in size were selected in each region of the cochlea. Within these areas, the ion intensity of each m/z value corresponding to the identified PC species was averaged. The statistical significance of differences in the averaged ion intensity of each region among the 5 cochlear sections was determined by one-way analysis of variance followed by the Scheffé test. Statistical analyses were performed using StatView 5.0 software (SAS Institute, Tokyo, Japan).

Results

Cochlear Tissue Sections
Before using the mass microscope, we established that fine sections could be obtained from frozen cochlear tissue. Sections stained with hematoxylin and eosin are

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Fig. 1. Optical image of a section of a guinea pig cochlea stained with hematoxylin and eosin. The entire cochlea from the basal turn to the apex is shown (**a**). Scale bar = 500 μm. The area surrounded by the green square is enlarged and shown in **b**. This section shows the spiral ganglion (SG), the neurons in the osseous spiral lamina (n), the spiral limbus (L), the organ of Corti (Co), the stria vascularis (StV), and the spiral ligament (SL). These images were created from a number of smaller rectangular images. Scale bar = 100 μm.
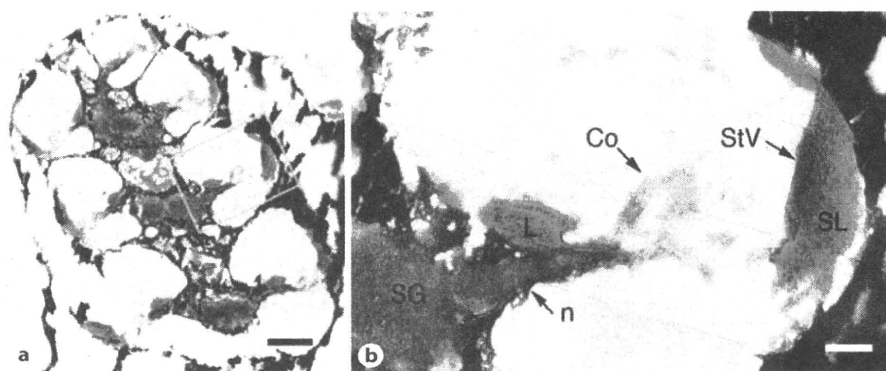
shown in figure 1a. In the enlarged image, the spiral ganglion, the neurons in the osseous spiral lamina, the spiral limbus, the organ of Corti, the stria vascularis, and the spiral ligament are identified from the basal turn to the apex (fig. 1). The structure of the organ of Corti in these sections was less well defined than in the aldehyde-fixed and decalcified sections. However, we could identify cells of the organ of Corti between the spiral limbus and the lateral wall of the cochlear duct. We did not assess Reissner's membrane because it was not sufficiently stable in the unfixed frozen cochlear sections.

Mass Spectra for Each Region of the Cochlea
Figure 2a is a photomicrograph of an unfixed frozen cochlear section under the mass microscope. This tissue section was adjacent to the section shown in figure 1a. Each region of the cochlea is also identified in this section. We used the mass microscope to analyze the area enclosed by the green square in figure 2a. Mass spectra were obtained for each region of the cochlea outlined in white (fig. 2b–f). Ion peaks at m/z 754.53, 772.52, 780.54, 782.55, 796.51, 798.54, and 810.57 were identified as precursor ions for MS/MS analyses.

Identification of the Biomolecules
We performed MS/MS analyses to identify the structures of the biomolecules associated with the precursor ions shown in figure 2b–f. Representative results of MS/MS analysis are shown in figure 3. This product ion spectrum from m/z 782.55 is characteristic of [PC(16:0/18:1) + Na]$^+$, according to a previous report [Hsu and Turk, 2003]. In the MS/MS analysis for m/z 782.55, fragment peaks at m/z 723.50 and 599.48 were detected. PC species with cation adducts often exhibit these two fragment peaks, corresponding to neutral losses of 59 Da [trimethylamine, $(CH_3)_3N$] and 183 Da [choline head group,

$(CH_3)_3N(CH_2)_2PO_4H$] from a precursor peak, in biological tissues [Sugiura and Setou, 2009]. The type of cation adducted to the biomolecule is generally assumed to be a sodium or potassium ion in biological tissues. The difference of 21.95 Da between m/z 577.53 and 599.48 suggested that a sodium ion was replaced with a proton adducted to the fragmented PC. Two minor peaks, m/z 441.25 and 467.23, in the product ion spectrum indicated fatty acids associated with the PC species [Hayasaka et al., 2009]. These peaks were consistent with the neutral losses of fatty acids 18:1 (282 Da) and 16:0 (256 Da) from m/z 723.50. Therefore, we identified the biomolecule with m/z 782.55 as [PC(16:0/18:1) + Na]$^+$.

In the same manner, we analyzed six additional precursor ions, and the results are summarized in table 1. The peaks at m/z 754.53, 772.52, 780.54, 796.51, 798.54, and 810.57 were identified as [PC(16:0/16:1) + Na]$^+$, [PC(16:0/16:0) + K]$^+$, [PC(16:0/18:2) + Na]$^+$, [PC(16:0/18:2) + K]$^+$, [PC(16:0/18:1) + K]$^+$, and [PC(18:0/18:1) + Na]$^+$, respectively. The metabolite MS search was helpful in the identification of m/z 754.53 and 810.57. The difference between the peaks at m/z 780.54 and 796.51 was determined to be due to the adduction of either a sodium or potassium ion to PC(16:0/18:2). The peaks at m/z 782.55 and 798.54 were likewise due to differing ions adducted to PC(16:0/18:1). Thus, we identified five different PC species in the cochlear section (table 1).

Distributions of the PC Species in the Mass Images
Finally, we reconstructed mass images for cochlear sections from the peaks identified above using BioMap software. The signal intensities in the mass spectra depended on the region of the cochlea being studied (fig. 2). The cochlear nerve region is visible in the representative mass image of figure 4, because this section is closer to the modiolus than the section shown in figure 1a. The