Table 2
Fourteen children with GJB2 mutations who passed NHS.

Patient	Type of NHS performed	Age when hearing loss was first identified	Current level of hearing loss HL	GJB2 genotype
1	AOAE	1 year	Profound	c.235delC/c.235delC
2	AOAE	9 months	Profound	c.512ins4/p.R143W
3	AOAE	2 years	Moderate	c.235delC/p.Y136X-G45E
4	AOAE	1 year 6 months	Moderate	c.235delC/p.Y136X-G45E
5	AABR	1 year 7 months	Moderate	p.V37I/p.R143W
6	AOAE	3 years	Moderate	c.176-191del16/c.235delC
7	AABR	4 months	Severe	c.235delC/c.512ins4
8	AOAE	6 months	Severe	p.Y136X-G45E/p.V37I
9	AABR	1 year 6 months	Severe	c.235delC/c.235delC
10	AABR	1 year 6 months	Severe	c.235delC/p.Y136X-G45E
11	AABR	1 year 1 month	Severe	c.235delC/c.235delC
12	AABR	6 years	Mild	c.235delC/p.V37I
13	AOAE	2 years	Severe	c.235delC/p.Y136X-G45E
14	AOAE	7 months	Moderate	c.235delC/p.R143W

NHS: newborn hearing screening, AOAE: automated otoacoustic emissions, AABR: automated auditory brainstem response.

2004; Vohr et al., 1998; Xu et al., 2011). AOAE screening can result in false-negative findings in cases of auditory neuropathy (Maris et al., 2011), a hearing disorder characterized by normal outer hair cell function, as revealed by the presence of AOAE, and abnormal neural conduction of the auditory pathway, as revealed by the absence or severe abnormality of AABR. Although we have reported one auditory neuropathy case with *GJB2* alleles (Matsunaga et al., 2012), this type of case must be very rare. Meanwhile, AABR screens detect moderate or greater hearing losses (i.e. 40dBHL or greater) at high frequencies; therefore, mild HI or low-frequency HI cases can pass AABR (Deem et al., 2012). However, among our DFNB1 cases that passed NHS, all but one (No. 12) had worse than moderate HI including high frequency. This finding leads us to conclude that individuals with two pathogenic *GJB2* mutations sometimes fail to express the phenotype of hearing loss at birth but have a later onset in childhood. AABR has a higher passing rate as compared to AOAE (Abdul Wahid et al., 2012), and the use of a combination of AOAE and AABR screening testing ensures higher sensitivity than each alone (Xu et al., 2011), but it is currently unknown whether the combination screening can detect the non-penetrance of *GJB2* mutations.

Our results indicated that the genotypes of DFNB1 patients who passed NHS vary a great deal, suggesting that *GJB2* genotypes have little to do with non-penetrance at birth. However, cases No. 3 and No. 4 are sisters, sharing half of genes, and both presented hearing loss at almost the same timing, which leads to speculation of existence of genetic factors which delay the timing of *GJB2* mutations phenotype. From the standpoint of our results, conventional auditory screening programs have limitations in identifying delayed-onset hearing loss and its etiology. Recently, newborn genetic screening for common deafness-associated mutations has been proposed to compensate for the inherent limitations of conventional NHS by detecting subjects with mutations associated with mild-to-moderate, progressive, or late-onset HI (Schimmenti et al., 2011; Wu et al., 2011; Zhang et al., 2012).

5. Conclusion

Based on our results, prediction of hearing impairment phenotype based on *GJB2* genotype is possible in the Japanese population. We identified p.I128M and p.H73Y as novel pathological *GJB2* mutations. Increasing evidence indicates that not all infants with pathogenic *GJB2* mutations express hearing loss at birth. The extent to which this phenomenon occurs will only be evident with prospective studies using audiologic screening in parallel with molecular screening in all newborns, with longitudinal follow up of these infants. Until such data are available, it is important for all primary care providers to have a low threshold to re-evaluate an infant's hearing if parents raise a concern, irrespective of whether they have passed their NHS.

Conflict of interest

The authors declare that they have competing interests.

Acknowledgments

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Subgroups of enlarged vestibular aqueduct in relation with *SLC26A4* mutations and hearing loss

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None

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Abstract:

Objective:

To investigate possible association of hearing loss and *SLC26A4* mutations with the subgroups of enlarged vestibular aqueduct (EVA) morphology in Japanese subjects with hearing loss.

Study Design:

Retrospective multi-center study.

Methods:

Forty-seven subjects who had vestibular aqueduct with midpoint diameter greater than 1 mm by CT of the temporal bone were enrolled at multiple sites across Japan, and DNA samples and clinical data were collected. EVA morphology was classified into four subgroups by the pattern of enlargement: aperture, aperture & midpoint, midpoint, and borderline enlargement. Venous blood DNA samples were subjected to PCR-based direct sequencing of all exons and exon-intron boundaries of the *SLC26A4* gene.

Results:

Four novel *SLC26A4* mutations were identified in the present study. *SLC26A4* mutations were detected in almost all subjects with aperture, aperture & midpoint, and midpoint enlargement. In contrast, 71% of subjects with borderline enlargement had no *SLC26A4* mutation. No significant difference was found in the distribution of truncating and non-truncating *SLC26A4* mutations between the EVA subgroups. In addition, no significant correlation was observed between the EVA subgroups and the hearing levels, incidence of hearing fluctuation, or progression of hearing loss.

Conclusion:

Subgroups of EVA morphology were significantly correlated with the presence or absence of *SLC26A4* mutation. In a subgroup analysis of subjects with *SLC26A4* mutations, however, differences in the EVA subgroups were not correlated with *SLC26A4* genotypes or characteristics of hearing loss.

Key words: Enlarged vestibular aqueduct, Pendred syndrome, DFNB4, SLC26A4, computed tomography, hearing loss

Level of Evidence: NA

INTRODUCTION

Enlarged vestibular aqueduct (EVA) is one of the most common inner ear deformities, often identified by CT in subjects with hearing loss.[1-5] The shape and size of the EVA differ between subjects. As such, a variety of radiographic criteria to define EVA have been published. Valvassori and Clemis[6] defined EVA as a vestibular aqueduct equal to or greater than 1.5 mm at the midpoint diameter. Jackler and De La Cruz[7] developed a criterion of a midpoint diameter greater than 2.0 mm, whereas Levenson and colleagues[8] proposed a cut-off of 2.0 mm at the external aperture diameter. Okumura et al.[9] suggested an external aperture diameter greater than 4.0 mm. Madden et al.[1] considered external aperture diameter greater than 2.0 mm and midpoint diameter greater than 1.5 mm as definitive, and midpoint diameter of 1.0- 1.5 mm as borderline enlargement. Vijayasekaran et al.[10] advocated the criteria of 0.9 mm at the midpoint diameter or 1.9 mm at the external aperture diameter.

Mutations in the *SLC26A4* gene have been identified as a major cause of vestibular aqueduct anomalies. *SLC26A4* mutations are known to cause Pendred syndrome (MIM #274600) and non-syndromic sensorineural deafness autosomal recessive type 4 (DFNB4, MIM #600791).[11-14] Some researchers have identified a correlation between *SLC26A4* mutations, EVA, and hearing loss, while others report no significant relationship amongst *SLC26A4* genotype and these phenotypes.[15] Previous studies have not evaluated the relationship between *SLC26A4* mutations and clinical features of hearing loss taking into consideration about morphologic variations of the EVA. We conducted a

multi-center study and differentiated subjects into subgroups according to vestibular aqueduct midpoint and external aperture diameters to examine a possible relationship between subgroups of EVA morphology, *SLC26A4* mutations, and hearing loss.

MATERIALS AND METHODS

We enrolled 47 bilateral EVA subjects with unilateral or bilateral sensorineural hearing loss of unknown causes (mean age 13.5 years, range 0 to 56 years; 33 children and 14 adults; 17 males and 30 females), and collected DNA samples and clinical data. Specifically, subjects whose bilateral vestibular aqueduct midpoint diameter was greater than or equal to 1 mm on temporal bone CT scans were included. The midpoint and external aperture diameters were measured perpendicular to the long axis of the vestibular aqueduct on the transverse plane, as shown in the upper right-hand inset in panel A of Fig. 1. Subjects were classified into the following four subgroups based on the morphologic characteristics of the vestibular aqueduct according to the criteria in Table I: aperture enlargement, aperture & midpoint enlargement, midpoint enlargement, and borderline enlargement.

For mutation analysis, genomic DNA was extracted from venous blood and subjected to PCR-based direct sequencing of the exons and exon-intron boundaries of the *SLC26A4* gene (GenBank NG_008489). For the purpose of this study, frameshift, splice site, and nonsense mutations were

categorized as "truncating," and missense mutations as "non-truncating" mutations. Novel variants were defined as pathogenic if they (i) were non-synonymous, (ii) demonstrated low carrier rates (< 1%) in 96 normal control Japanese subjects, absence in database Exome Variant Server[16] and dbSNP,[17] and high amino acid conservation among various mammalian species, and (iii) were detected as heterozygous in association with the other allele with another heterozygous mutation already reported as pathogenic. Alteration of splice site was predicted by NNSPLICE.[18] Subjects with *SLC26A4* mutations were analyzed for the degree of hearing loss, fluctuations in hearing acuity, and progression of hearing loss to assess the relationship between these hearing parameters and EVA subgroups. Subjects underwent conditioned orientation reflex or conventional pure-tone audiometry, depending on their ages. Auditory steady state response measurements were utilized for five subjects who did not receive any of these audiometric tests. Hearing level was evaluated based on averages at 500, 1,000, 2,000, and 4,000 Hz (slight, 26–40 dB; moderate, 41–60 dB; severe, 61–80 dB; profound, ≥ 81 dB) according to the World Health Organization Grades of Hearing Impairment.[19] Subjects were considered to have fluctuating hearing loss if they had at least one bout of aggravation and recovery of hearing loss (at least 15 dB in one frequency). Subjects were considered to have progressive hearing loss if they showed aggravation of hearing loss by 10 dB or more at one or more frequencies within 10 years of interval. Statistical significance was assessed using the Fisher's exact test.

All procedures were approved by the Ethics Review Committee of National Hospital Organization

Tokyo Medical Center, Japan, and other participating institutions and were conducted only after written informed consent had been obtained from each subject or from the parents of the subjects.

RESULTS

Subgrouping of EVA and its association with SLC26A4 mutations

Fig. 1 shows typical CT findings in subjects with aperture enlargement (Fig. 1A), aperture & midpoint enlargement (Fig. 1B), midpoint enlargement (Fig. 1C), and borderline enlargement (Fig. 1D). Among 47 subjects, 21 (44%) were classified with aperture enlargement, 17 (36%) with borderline enlargement, 5 (11%) with aperture & midpoint enlargement, and 4 (9%) with midpoint enlargement (Fig. 2). All subjects had the same subgroup of enlargement bilaterally.

Genetic analysis of the 47 subjects showed that 34 (72%) had two *SLC26A4* mutation alleles (Table II), and the other 13 (28%) had no *SLC26A4* mutation alleles. None had a single *SLC26A4* mutation allele. The 34 subjects with two *SLC26A4* mutation alleles were diagnosed with Pendred syndrome or DFNB4. The majority of these subjects had aperture enlargement (n = 20, 59%), followed by aperture & midpoint enlargement (n = 5, 14%), borderline enlargement (n = 5, 14%), and midpoint enlargement (n = 4, 12%) (Fig. 2). On the other hand, most of the subjects without *SLC26A4* mutation alleles had borderline enlargement (n = 12, 91%), while the one remaining subject (8%) had aperture enlargement.

Frequency of subjects without *SLC26A4* mutation alleles in the borderline enlargement subgroup was significantly higher than those in the aperture enlargement and aperture & midpoint enlargement subgroups ($P < 0.0125$). It tended to be higher than that in the midpoint enlargement subgroup but it was not statistically significant ($P = 0.021$) probably due to the small number of subjects in the midpoint enlargement subgroup (n=4).

SLC26A4 mutations and genotypes in association with EVA morphology in subject with Pendred syndrome or DFNB4

The types and locations of all the *SLC26A4* mutations in 34 subjects with Pendred syndrome or DFNB4 were shown in Table II and Fig. 3. Five splice site mutations (c.601-1G>A (intron 5), c.919-2A>G (intron 7), c.1614+1G>A (intron 14), c.1708-32_1708-16del (intron 15), c.1707+5G>A (intron 15)), one non-sense mutation (p.L743X), two insertion/deletion mutations (p.S551Ffs13, Q705Wfs18), and 14 missense mutations (p.S28G, p.P76S, p.A372V, p.N392Y, p.R409H, p.T410M, p.T527P, p.I529S, p.Y556C, p.V659L, p.D669E, p.F692L, p.T721M, p.H723R) were detected. These included four novel mutations, p.S28G (c.82A>G), p.D669E (c.2007C>A), p.F692L (c.2074T>C), and c.1708-32_1708-16del (marked * in Table II) based on the criteria for novel mutations in the present study (described in methods). Electropherograms of the novel mutations and conservation of the amino acid residues among various species are shown in Fig. 3B and C. NNSPLICE predicted

c.1708-32_1708-16del to decrease probability of acceptor site at exon 16 from 0.49 (for normal allele) to 0.19 (for mutation allele), which is likely to cause aberrant splicing (Fig. 3C).

The list of subjects with two *SLC26A4* mutation alleles are shown in Table II. Analysis of genotypes of *SLC26A4* mutation alleles in these subjects showed that 20 (59%) had non-truncating/ non-truncating genotypes, 13 (38%) had non-truncating/ truncating genotypes, and 1 (3%) had truncating/ truncating genotypes (Fig. 4A). Comparison of the incidence of each genotype found no significant statistical difference between the subgroups of EVA morphology ($P = 1.000$).

Characteristics of hearing loss in association with EVA morphology in subjects with Pendred syndrome or DFNB4

The hearing levels, incidence of hearing fluctuation, and progression of hearing loss in subjects with two *SLC26A4* mutation alleles are shown in Table II. Relation between the hearing level and EVA morphology was examined in the ears of 34 subjects (68 ears) (Fig. 4B). Thirty-four ears (50%) had profound hearing loss in total. No significant differences in the hearing levels were detected between the subgroups of EVA morphology ($P = 0.462$). In order to exclude the effect of aging in this analysis, we also stratified the subjects into two groups (age 0-9 and 10y or older) and conducted the same analysis. These analyses also demonstrated the same results, indicating that the difference in ages among subgroups did not affect distribution of subjects among different hearing levels (data not

shown). Next, the relation between hearing fluctuation and EVA morphology was investigated in 28 subjects for whom relevant audiometric data were available (Fig. 4C). Hearing fluctuations were detected in 15 subjects (54%) in total, and no significant differences were noted in the incidence of hearing fluctuations between the subgroups of EVA morphology (P = 0.209). Lastly, the relation between progression of hearing loss and EVA morphology was analyzed in 29 subjects for whom relevant clinical data were available (Fig. 4D). Twenty subjects (69%) had progressive hearing loss in total, and the results showed no significant differences in the incidence of progressive hearing loss between the subgroups of EVA morphology (P = 0.207).

DISCUSSION

Although a variety of EVA criteria using the midpoint and aperture diameters of the vestibular aqueduct have been proposed to date,[1,6-10] our study is the first attempt to divide EVA into subgroups based on the shape and size of the vestibular aqueduct, and the first to investigate the possible relationship of these subgroups with genotypes and audiometric findings. *SLC26A4* mutations were detected in 72% of the Japanese subjects with bilateral EVA. Among these *SLC26A4* mutations, four mutations were novel. The discovery of these novel mutations would expand the *SLC26A4* mutation spectrum, thereby contributing to a more accurate gene-based diagnosis of hearing loss with EVA.

Nearly all subjects with aperture, aperture & midpoint, and midpoint enlargement presented *SLC26A4* mutations, suggesting that subjects with these EVA subgroups are most likely to be diagnosed with Pendred syndrome or DFNB4. On the other hand, only approximately 30% of subjects with borderline enlargement had *SLC26A4* mutation, which suggests that majority of subjects with this EVA subgroups have a pathological mechanism other than Pendred syndrome or DFNB4.

None of the 47 EVA subjects enrolled in the present study had only a single *SLC26A4* mutation allele. This finding is a striking contrast with previous research reporting single *SLC26A4* mutation alleles in approximately one-third of Caucasian subjects with EVA.[3,4,20-22] This discrepancy might be associated with Japanese subjects who were reported to have distinct spectrum of *SLC26A4* mutations from Caucasian subjects.[22] One possible explanation is that the development of EVA in the Caucasian population may more frequently involve mutations in the introns or promoter regions of the *SLC26A4* than that in the Japanese population. Another possibility is that Caucasian population may have higher mutation frequencies in genes causing digenic hearing loss in association with heterozygous *SLC26A4* mutations (e.g., *KCNJ10* and *FOXI1*) than Japanese population.[23-25] The other possible explanation for the discrepancy is that the present study registered only subjects with bilateral EVA, whereas previous studies included those with unilateral hearing loss or unilateral EVA. This implicates the hypothesis that biallelic mutations of *SLC26A4* are more strongly associated with bilateral EVA.

Our analysis of subjects with *SLC26A4* mutations revealed no significant difference in the

proportion of truncating and non-truncating *SLC26A4* mutations between subgroups of EVA morphology. This suggests that, in addition to malfunction of the *SLC26A4* protein, environmental factors or genes other than *SLC26A4* may contribute to variations in vestibular aqueduct morphology.

Some researchers argue that there is no significant relationship between the degree of the EVA and the severity and progression of hearing loss and hearing fluctuations, while others propose that there is a significant relationship.[26] In the present study, no significant differences were detected in the level, fluctuation, and progression of hearing loss between the subgroups of EVA morphology, indicating that characteristics of hearing loss cannot be predicted based on the EVA morphology in subjects with Pendred syndrome or DFNB4.

CONCLUSION

Almost all the subjects with aperture, aperture & midpoint, and midpoint enlargement of EVA had two *SLC26A4* mutation alleles, whereas more than two thirds of subjects with borderline enlargement of EVA had no *SLC26A4* mutation alleles. Analysis of subjects with two *SLC26A4* mutation alleles revealed no significant correlation between the morphologic subgroups of EVA and *SLC26A4* genotypes or characteristics of hearing loss, suggesting that the subgroups of EVA morphology may be associated with factors other than genotypes of *SLC26A4* mutations and that the subgroups of EVA morphology are not a predictive factor for characteristics of hearing loss.

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