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Vestibular Functions of Hereditary Hearing Loss Patients with *GJB2* Mutations

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Key Words
GJB2 gene · Caloric test · Cervical vestibular evoked myogenic potential

Abstract
Objectives: Mutations in the *GJB2* gene have been of particular interest as it is the most common causative gene for congenital deafness in all populations. Detailed audiological features, including genotype-phenotype correlations, have been well documented. However, in spite of abundant gene as well as protein expression in the vestibular end organs, neither vestibular symptoms nor vestibular functions have yet been elucidated. In the present study, vestibular functions were evaluated in patients diagnosed with *GJB2*-related deafness. ***Subjects and Methods:*** Vestibular functions were evaluated by caloric test and cervical vestibular evoked myogenic potential (cVEMP) testing in 24 patients with biallelic *GJB2* mutations. ***Results and Discussion:*** Twenty-one of 23 patients (91.3%) had normal caloric responses and significantly lower cVEMP amplitudes than the control subjects. In the patients who were able to undergo vestibular testing, the mostly normal reactions to caloric testing indicated that the lateral semicircular canal was intact. However, the majority of *GJB2* patients showed low cVEMP reactions, indicating a saccular defect.

Introduction

Hereditary hearing loss affects about 1 in 1,000 infants in developed countries, and genetic causes account for at least 50% of all childhood hearing loss [Morton, 1991]. Nowadays, it is known that mutations in various kinds of genes cause hearing loss.

Mutations in the *GJB2* gene are the most common genetic cause of both congenital and hereditary hearing loss worldwide. A series of previous studies have demonstrated that about 15–25% of patients with congenital hearing loss have *GJB2* mutations [Azaiez et al., 2004; Dai et al., 2009; Tsukada et al., 2010; Chan and Chang, 2014]. To date, more than 100 *GJB2* variations have been reported (see the connexin-deafness homepage: http://www.davinc.crg.es/deafness), and hearing loss ranges from mild to profound according to differences in the genotype; therefore, genotype-phenotype correlations are well documented, and this type of hearing loss is thought to be nonprogressive. Detailed audiological features, including genotype-phenotype correlations and progression, in patients with these mutations have been well studied [Snoeckx et al., 2005; Tsukada et al., 2010; Minami et al., 2013; Chan and Chang, 2014].

However, there have been only a few reports on vestibular function despite abundant *GJB2* gene and protein expression in the vestibular end organs [Kikuchi et

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al., 1994; Forge et al., 2003]. Here we summarize the results of our comprehensive study on vestibular function in patients with hearing loss caused by *GJB2* mutations.

Subjects and Methods

Twenty-four patients (7 males and 17 females) with biallelic *GJB2* mutations underwent cervical vestibular evoked myogenic potential (cVEMP) and/or caloric testing in this study. Caloric testing was performed for 23 subjects (mean age 16.3 years, range 5–60), and cVEMP was assessed for 22 subjects. As the cVEMP results were affected by cochlear implantation, we excluded cochlear-implanted ears from the final analysis, resulting in the cVEMP results for 36 ears of 21 subjects (mean age 15.0 years, range 5–41) being included.

As a control, vestibular testing was performed for 70 unaffected ears with normal hearing in patients with unilateral hearing loss caused by mumps, unilateral cochlear nerve deficiency or sudden deafness. Caloric testing was performed in 64 of these 70 subjects, with the data for 43 of the subjects (mean age 16.8 years, range 5–62) used as age-matched controls. cVEMP testing was also performed in 69 of the 70 subjects, with the data for 42 of the subjects (mean age 14.6 years, range 5–42) used as age-matched controls. Informed written consent was obtained from all subjects, control subjects and, in the case of minors, the next of kin, caretakers or guardians prior to participation in the study. The study and consent procedure were approved by the Ethical Committee of Shinshu University.

Mutation Analysis

To identify *GJB2* mutations, a DNA fragment containing the entire coding region was amplified using the primer pair Cx48U/Cx1040L, as described elsewhere [Abe et al., 2000]. Polymerase chain reaction (PCR) products were sequenced and analyzed with an ABI sequencer 3130XL (Applied Biosystems, Life Technologies, Carlsbad, Calif., USA).

Audiological Evaluation

The hearing level was determined by pure-tone audiometry. The average threshold in the conversation frequencies (0.5, 1.0, 2.0 and 4.0 kHz) was calculated for the better ear, and the severity of hearing loss was categorized as mild (20–40 dB), moderate (41–70 dB), severe (71–95 dB) or profound (>95 dB) (see GENDEAF recommendations: http://hereditaryhearingloss.org).

Vestibular Tests

The patients were examined by caloric testing and cVEMP testing to obtain data on semicircular canal function and otolithic function, respectively.

In the cVEMP testing, electromyography (EMG) was performed using a pair of surface electrodes mounted on the upper half and sternal head of the sternocleidomastoid muscle, respectively. The electrographic signal was recorded using a Neuropack evoked potential recorder (Nihon Kohden Co. Ltd., Tokyo, Japan). Clicks lasting for 0.1 ms at 105 dB nHL were presented through a headphone. The stimulation rate was 5 Hz, the bandpass filter intensity was 20–2,000 Hz, and analysis time was 50 ms. The responses to 200 stimuli were averaged twice. As the P13–N23 amplitude of the cVEMP based on the unrectified EMG activity is correlated with sternocleidomastoid muscle activity during the test [Colebatch et al., 1994], we measured sternocleidomastoid muscle activity using the background-integrated EMG response; i.e., the area under the averaged rectified EMG curve from –20 to 0 ms before sound stimulation. The correction of the P13–N23 amplitude was calculated as follows [Shojaku et al., 2001]:

corrected amplitude = P13–N23 amplitude of the averaged unrectified EMG (μV)/the average background-integrated EMG of 20 ms (μV).

In the caloric testing, the maximum slow phase velocity (SPV) was measured by cold water irrigation (20°C, 5 ml, 20 s). We defined an SPV value below 10°/s as representing areflexia and a value between 10 and 20°/s as hyporeflexia.

Statistical Analysis

For all analyses, SPSS for Windows software (IBM Co., Chicago, Ill., USA) was used, and Mann-Whitney U tests were applied when comparing differences between patients with *GJB2* mutations and the normal controls. Correlations between the severity of hearing and vestibular functions were calculated using Pearson's correlation coefficient.

Results

The *GJB2* mutations and vestibular and audiological results for all patients are summarized in table 1.

Semicircular Canal Function

Only 2 out of the 23 patients (8.7%) who underwent caloric testing showed an areflexic (G10) or hypoflexic (G3) response in the unilateral ear.

Figure 1 shows a comparison between caloric test results for the patients with *GJB2* mutations and normal controls. There was no statistically significant difference in the median SPV value between patients with *GJB2* mutations and the control subjects (31.9 vs. 40.8°/s; $p = 0.121$, Mann-Whitney U test).

Saccular Function

We performed cVEMP testing on 36 ears of 21 patients with *GJB2* mutations (mean age 15.0 years, range 5–41) included in this study. Figure 2 shows a comparison between the corrected amplitude of cVEMP for the patients with *GJB2* mutations and age-matched normal controls. The median corrected amplitude was 0.570 in the patients with *GJB2* mutations, which was statistically lower ($p < 0.001$, Mann-Whitney U test) than that of the normal controls (n = 42; 1.41). Seventeen out of 21 patients with *GJB2* mutations (80.1%), or 22 out of 36 ears (61.1%), showed a value lower than 0.64, which was the cutoff for amplitude in the cVEMP results for the lowest 5% of the normal control subjects.

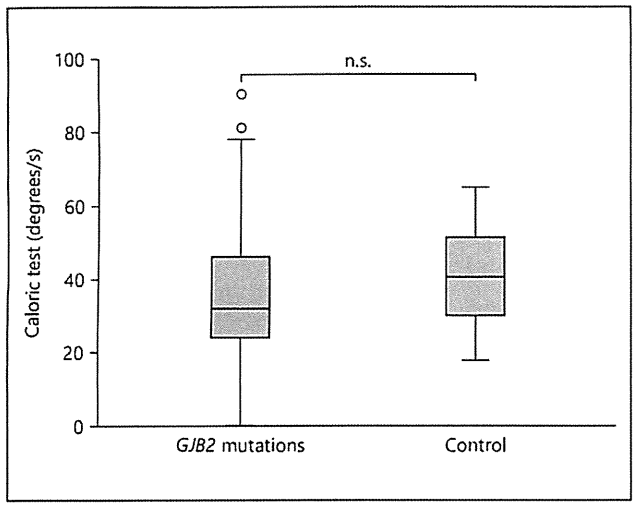

Fig. 1. The mean SPV in the patients with biallelic *GJB2* mutations and the control group. n.s. = No statistically significant difference (Mann-Whitney U test).

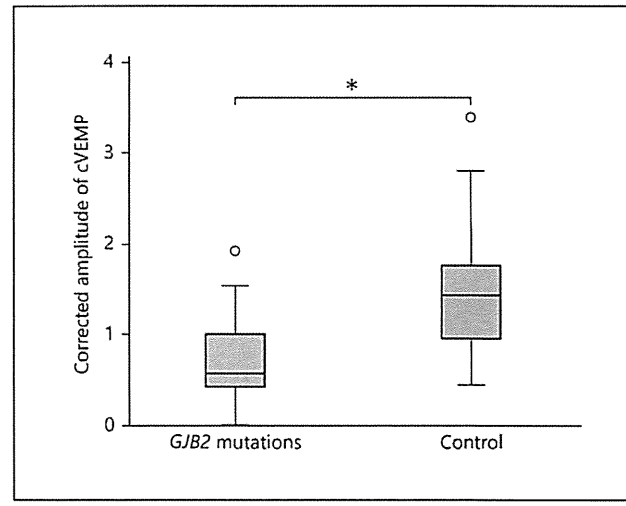

Fig. 2. The mean cVEMP corrected amplitude of the patients with biallelic *GJB2* mutations and the control group. * $p < 0.001$, Mann-Whitney U test.

Table 1. Results of audiological and vestibular testing of patients with biallelic *GJB2* mutations

Patient No.	Mutation	Age, years	Sex	Vertigo	Hearing levels	Caloric test, degrees/s		cVEMP	
						R	L	R	L
G1	c.176–191 16bp *del*/c.235*del*C	8	M	–	profound	47.9	61.92[1]	0.42	0[1]
G2	c.235*del*C/p.M93I	10	F	–	moderate	38.23	46.38	0.46	1.54
G3	c.176–191 16bp *del*/c.235*del*C	12	F	–	profound	9.34	24.23	0.42	0.48
G4	c.235*del*C/c.235*del*C	7	M	–	profound	22.89	41.24[1]	0	1.860[1]
G5	c.235*del*C/p.R143W	20	M	–	profound	22.18	25.82	0.4	0.86
G6	c.235*del*C/p.G45E Y136X	25	F	–	severe	20.83	70.87	1.28	0.52
G7	c.235*del*C/p.G45E Y136X	22	F	–	profound	34.64[1]	24.87	0.200[1]	1
G8	p.V37I/p.V37I	9	M	–	moderate	43.69	48.54	1	0.5
G9	c.235*del*C/c.235*del*C	6	M	–	profound	59.27[1]	88.8	0.860[1]	1.4
G10	p.G45E Y136X/c.299–300*del*AT	6	F	–	severe	0	33.18	1.22	1.92
G11	c.176–191*del* 16bp/p.R143W	17	F	–	profound	90.71	25.48	0.6	0.38
G12	c.235*del*C/c.235*del*C	6	M	–	profound	34.33	39.21	1.26	0
G13	c.235*del*C/c.235*del*C	29	F	–	moderate	n.a.	n.a.	0.6	0.96
G14	p.G45E Y136X/c.176–191*del* 16bp	60	F	–	profound	45.92	50.15[1]	n.a.	n.a.[1]
G15	c.235*del*C/c.235*del*C	16	F	–	profound	30.6	23.17	n.a.	n.a.
G16	c.235*del*C/p.R143W	10	F	–	profound	27.27	39.33[1]	0.86	0[1]
G17	c.235*del*C/p.R143W	12	F	–	profound	20.21[1]	20.54[1]	0[1]	0[1]
G18	c.235*del*C/p.G45E Y136X	13	F	–	moderate	68.89	81.2	0.4	0
G19	c.235*del*C/c.235*del*C	25	F	–	moderate	21.22	25.91	0.54	0.72
G20	c.235*del*C/c.235*del*C	28	F	–	profound	22.93	28.95	0.48	0.46
G21	c.235*del*C/p.T86R	5	M	–	profound	57.72[1]	64.81	0[1]	0
G22	c.235*del*C/p.G45E Y136X	9	F	–	severe	31.93	35.85	0.46	1.04
G23	c.235*del*C/p.G45E Y136X	7	F	–	severe	27.43	24.26	1.02	0.64
G24	c.235*del*C/c.299–300*del*AT	41	F	–	profound	27.17	24.33	0.26	0.64

– = No symptom; n.a. = Not available.
[1] After cochlear implantation.

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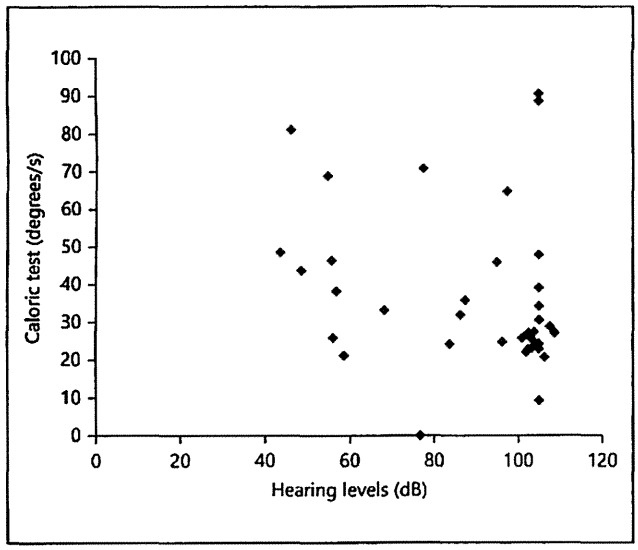

Fig. 3. The relationship between hearing level and caloric test results in patients with biallelic *GJB2* mutations.

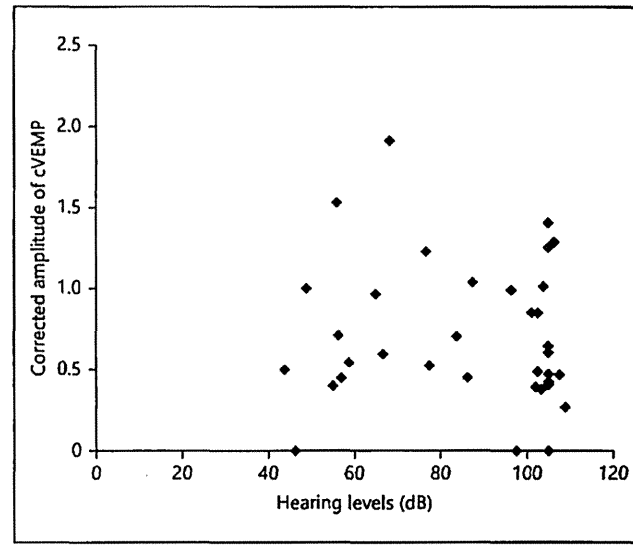

Fig. 4. The relationship between hearing level and cVEMP results in patients with biallelic *GJB2* mutations.

Relationship between Hearing Level and Vestibular Function

With regard to the correlation between hearing level and vestibular functions, no statistically significant correlations were found between the severity of hearing level and SPV (R = –0.2; fig. 3) or corrected amplitude of cVEMP results (R = –0.1; fig. 4).

Discussion

GJB2 encodes the connexin 26 (Cx26) protein, a member of the connexin family and related gap junction proteins. Connexins oligomerize to form hexameric hemichannels called connexons, which are present in the plasma membrane, where they can bind with connexons from adjacent cells to form functional gap junctions [Bruzzone et al., 1996]. The expression of *GJB2* has been documented in a variety of cells and tissues. In the cochlea, Cx26-containing gap junctions are proposed to maintain cochlear homeostasis and the circulation of metabolites, such as inositol 1,4, 5-trisphosphate, which is critical for cellular function and the survival of cochlea-supporting cells [Kikuchi et al., 1995; Martinez et al., 2009; Xu and Nicholson, 2013].

Previous immunocytochemical reports have shown the abundant expression of Cx26 in the supporting cells and fibrocytes of the rat and human vestibular end organs [Kikuchi et al., 1994; Forge et al., 2003], indicating that Cx26 functions in the vestibular end organs as well as in the cochlea. In spite of the abundant *GJB2* gene and protein expression in the vestibular end organs, there have been only a few reports on the vestibular function of patients with hearing loss caused by *GJB2* mutations, and the reports to date included only a small number of subjects or subjects with only heterozygous *GJB2* mutations [Todt et al., 2005; Kasai et al., 2010]. In this study, 21 out of 23 patients (91.3%) had normal caloric responses. On the other hand, the corrected amplitude of cVEMP for *GJB2* mutation patients was significantly lower than that for the normal control subjects, and 17 out of 21 patients (80.1%) showed a value that was lower than the lowest 5% of the normal control subjects. In the patients who were able to undergo vestibular testing, the mostly normal reactions to caloric testing indicated that the lateral semicircular canal was intact. However, the majority of *GJB2* mutation patients showed lower cVEMP reactions, revealing a saccular defect. In support of our results, previous reports also showed pathological cVEMP results in 3 of 5 (60%) patients [Kasai et al., 2010] and 5 of 7 (71.4%) patients [Todt et al., 2005] with *GJB2* mutations, and cochleosaccular degeneration in human temporal bones with compound heterozygous c.35*del*G mutations [Jun et al., 2000].

There are a number of hypotheses regarding intact semicircular function and decreased saccular function. A previous report showed that the survival of vestibular hair

cells was observed in the utricle and crista ampullaris of Cx30−/− mice, although Cx30 is required for the survival of saccular hair cells [Qu et al., 2007]. Colocalization of Cx26 and Cx30 [Forge et al., 2003] in the vestibular organ suggested that they are coassembled from Cx26 and Cx30, so Cx26 may not be required for the survival of the utricle and ampullae in the same way. In addition to Cx26 and Cx30, RT-PCR and cDNA microarray analyses have shown that many other connexin genes, namely Cx29, Cx30 and Cx43, are expressed in the inner ear [Ahmad et al., 2003]. The vestibular organs express other connexins as well as Cx26 and Cx30, and it may be that channels containing these connexins can compensate for functional deficiencies caused by *GJB2* mutations in the semicircular canal.

Mutations in several different genes whose products appear to be involved in the maintenance of cochlear homeostasis cause nonsyndromic deafness and the death of outer hair cells [Steel and Kros, 2001], and both the targeted ablation of the mouse *cx26* gene from the organ of Corti and vestibular supporting cells [Cohen-Salmon et al., 2002] and the expression of a dominant negative *cx26* mutation [Kudo et al., 2003] also lead to the death of outer hair cells with no obvious effect on the vestibular organ. Therefore, the impairment of intercellular communication may have more generalized detrimental effects on cochlear homeostasis to which the organ of Corti is particularly sensitive, whereas the vestibular organs may be less dependent on efficient cell-to-cell communication. Both mice with a *cx30* deletion and mature mice with targeted ablation of *cx26* demonstrate hearing loss and do not generate endocochlear potentials [Cohen-Salmon et al., 2002; Teubner et al., 2003]; therefore, the disruption of intercellular communication and cochlear cell death may impair the ability to generate or maintain endocochlear potentials. The endocochlear potential is essential for auditory function in mammals, but there is no equivalent in the vestibular system [Wangemann, 1995]. It is well established that the utricle, ampullae and common crus of the semicircular canals all contain vestibular dark cell epithelia that secrete K^+ and distribute Cx26 [Masuda et al., 2001], while the saccule is devoid of vestibular dark cells [Kimura, 1969; Burnham and Stirling, 1984; Marcus and Shen, 1994; Marcus and Shipley, 1994]. Based on these and other observations [Sellick and Johnstone, 1972], it was proposed that the saccular endolymph originates from the cochlea by longitudinal flow or diffusion and is not produced in this organ. Changes in the endolymph in the cochlea might have a direct influence on the saccular endolymph and induce degeneration and hypofunction of the saccule.

It is well known that a number of genotype-phenotype correlations are associated with hearing impairment [Snoeckx et al., 2005; Tsukada et al., 2010; Chan and Chang, 2014]. In this study, we evaluated the correlation between hearing level and vestibular function. There were no significant correlations between hearing level and SPV ($R = -0.2$) or corrected amplitude of cVEMP results ($R = -0.1$). As most of the patients in this study had a c.235*del*C mutation that is thought to be associated with a severer phenotype, we could not find any genotype-phenotype correlations among the vestibular functions. More genotypes should be evaluated in future to clarify the existence of any correlations.

In this study, no patients complained of vertigo or dizziness. Our previous study also showed only 4% (3/75) of patients with *GJB2* mutations had episodes of vertigo, dizziness or faintness [Tsukada et al., 2010]. Although associated symptoms of chronic saccular dysfunction are unknown, hearing loss in these patients is known to be congenital. Therefore, it is conceivable that vestibular compensation may occur in the central nervous system in spite of the presence of a saccular defect.

A previous report also described the results of utricular function using subjective haptic vertica [Todt et al., 2005]. Unfortunately, we could not investigate utricular function in this study, but we plan to undertake further work on utricular functions, such as ocular VEMP, in the near future.

Conclusion

The high incidence of saccular defects in patients with *GJB2* mutations is indicative of the genetic background. Although hearing loss occurs in these patients, saccular dysfunctions are well compensated and may not decisively influence the quality of life.

This result will also facilitate the clinical application of genetic counseling for these patients and their families.

Acknowledgments

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