Table 2 Summary of the number of variants detected in each subject

Family	Subject	Number of genes analyzed	No.SNV/MNV*	No. non-synonymous SNV/MNV
1	III:3	61	414	84
	III:4	61	370	74
	IV:2	61	391	82
2	III:1	61	386	81
	III:2	61	422	87
	IV:1	61	435	82
	IV:3	61	400	84
3	II:1	61	383	82
	II:2	61	339	70
	III:1	61	350	74
	III:2	61	398	86
4	I:1	84	570	138
	I:2	84	569	126
	II:1	84	546	131
5	II:2	61	388	72
	II:4	61	374	87
	III:1	61	361	84
	III:2	61	396	85
6	I:1	61	429	96
	I:2	61	371	81
	II:1	61	378	86
	II:2	61	375	84
7	I:1	84	607	139
	I:2	84	554	126
	II:2	84	582	132
	II:1	84	539	117

*SNV, single nucleotide variant; MNV, multiple nucleotide variant.

present clinical features of the major and minor criteria other than hearing loss. Therefore, family 4 was considered to have non-syndromic hearing loss, DFNA23, based on the clinical information available at the time of this study.

In family 5 (Figure 1E), subjects III:1 and III:2 with hearing loss had compound heterozygous *MYO7A* mutations, c.6439-2A >G (intron 51) and c.6478T >G (p.W2160G). Subjects II:2 and II:4 with normal hearing had a heterozygous c.6439-2A >G mutation and a heterozygous p.W2160G mutation, respectively. *MYO7A* is responsible for DFNA11 (OMIM 601317) [37], DFNB2 (OMIM 600060) [38], and Usher syndrome 1B (OMIM 276900) [39]. Tryptophan 2160 in myosin 7A was found to be located in a carboxyl-terminal FERM domain in the myosin-tail (Figures 3A and Figure 3B); this domain reportedly associates with filamentous actin [40] and contributes to hair bundle formation. Molecular modeling predicted that the p.W2160G mutation would reduce hydrophobic interactions among residues in the center of the F3 subdomain of the FERM domain (Figures 3C and 3D). The p.W2160G mutation would destabilize the structure of the F3 domain and could result in disrupted protein interaction and stereocilial degeneration of the sensory hair cells [41,42].

In family 6 (Figure 1F), subjects II:1 and II:2 with hearing loss had a heterozygous *CDH23* mutation, c.719C>T (p.P240L), and a heterozygous *PCDH15* mutation, c.848G >A (p.R283H). Sanger sequencing revealed that the other subject with hearing loss (subject II:3) also had both heterozygous *CDH23* and *PCDH15* mutations. A p.P240L mutation in*CDH23* has been reported to be pathogenic [43]. Subject I:1 with normal hearing had a heterozygous mutation in *CDH23* (p.P240L), and subject I:2 with normal hearing had a heterozygous mutation in *PCDH15* (p.R283H). *CDH23* is responsible for both DFNB12 (OMIM 601386) and Usher syndrome 1D (OMIM 601067) [44], whereas *PCDH15* is responsible for both DFNB23 (OMIM 609533) and Usher syndrome 1F (OMIM 602083) [45]. Double heterozygous mutations of *CDH23*

Table 3 Summary of possible pathogenic mutations

Gene	Nucleotide change	Amino acid change	NCBI ID	dbSNP135	Allele frequency in 1000GENOME	Allele frequency in ESP6500	Allele frequency in Japanese control	PolyPhen-2 prediction (score)	PROVEAN prediction (score)	Pathogenicity	Family	Reference
ACTG1	c.802G>A	p.G268S	NM_001199954.1	None	-	0	0/192	Probably damaging (0.998	Deleterious (-4.504)	Possible	1	
POU4F3	c.1007delC	p.A336Vfs	NM_002700.2	None	-	0	0/192	-	-	Possible	2	
SLC26A5	c.390A>C	p.R130S	NM_198999.2	None	-	0	0/192	Benign (0.443)	Deleterious (-4.813)	Possible	3	
SLC26A5	c.209G>A	p.W70X	NM_198999.2	None	-	0	n.t*.	-	-	Possible	3	
SIX1	c.328C>T	p.R110W	NM_005982.3	rs80356459	No info	0	n.t.	Probably damaging (1.000)	Deleterious (-7.775)	Causative	4	35
MYO7A	c.6478T>G	p.W2160G	NM_000260.3	None	-	0	0/192	Probably damaging (1.000)	Deleterious (-12.649)	Possible	5	
MYO7A	c.6439-2A>G (intron 51)	Splice mutation	NM_000260.3	None	-	0	0/192		-	Possible	5	
CDH23	c.719C>T	p.P240L	NM_022124.5	rs121908354	1/2183	0	n.t.	Probably damaging (1.000)	Deleterious (-3.051)	Causative	6	43
PCDH15	c.848G>A	p.R283H	NM_001142763.1	None	-	1/13005	0/192	Probably damaging (0.998)	Neutral (-1.918)	Possible	6	
USH2A	c.12431delC	p.A4144GfsX23	NM_206933.2	None	-	0	0/190		-	Possible	7	

*n.t. = not tested

Table 4 Summary of variants with uncertain pathogenicity

Gene	Nucleotide change	Amino acid change	NCBI ID	dbSNP135	Allele frequency in 1000GENOME	Allele frequency in ESP6500	Allele frequency in Japanese control	PolyPhen-2 prediction (score)	PROVEAN prediction (score)	Pathogenicity	Family	Reference
DFNA5	c.781C>T	p.R261X	NM_004403.2	None	-	0	0/192	-	-	Uncertain	2	
USH2A	c.1346G>A	p.R449H	NM_206933.2	None	-	0	5/378	Benign (0.017)	Neutral (-0.880)	Uncertain	7	

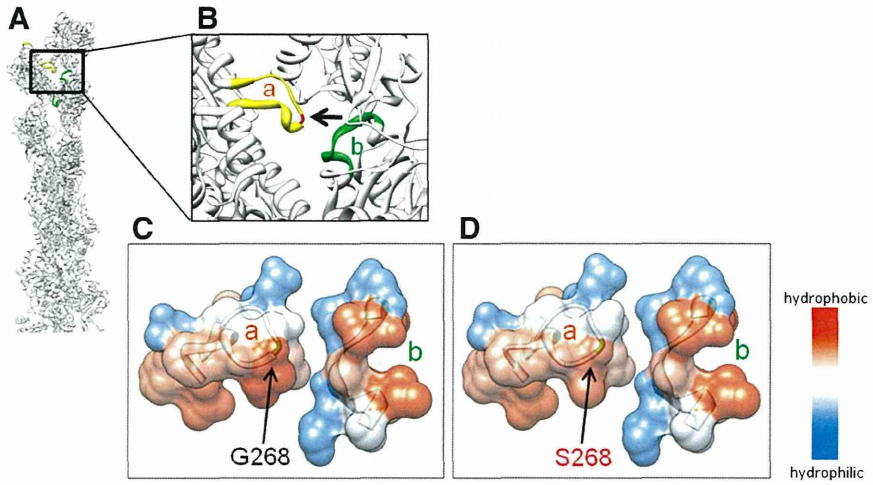

Figure 2 Molecular modeling of *ACTG* containing the p.G268S mutation. (A) Ribbon model of filamentous actin gamma 1. **(B)** Magnified ribbon model of filamentous actin gamma 1. Glycine residue 268 is shown in red and indicated by an arrow. Regions in yellow and green indicate the hydrophobic loop (262–274; a) and the corresponding interactive residues (281–289; b), respectively. **(C and D)** Vertical views of the regions a and b superimposed with predicted surface hydrophobicity in the wild type **(C)** and the p.G268S mutant **(D)**.

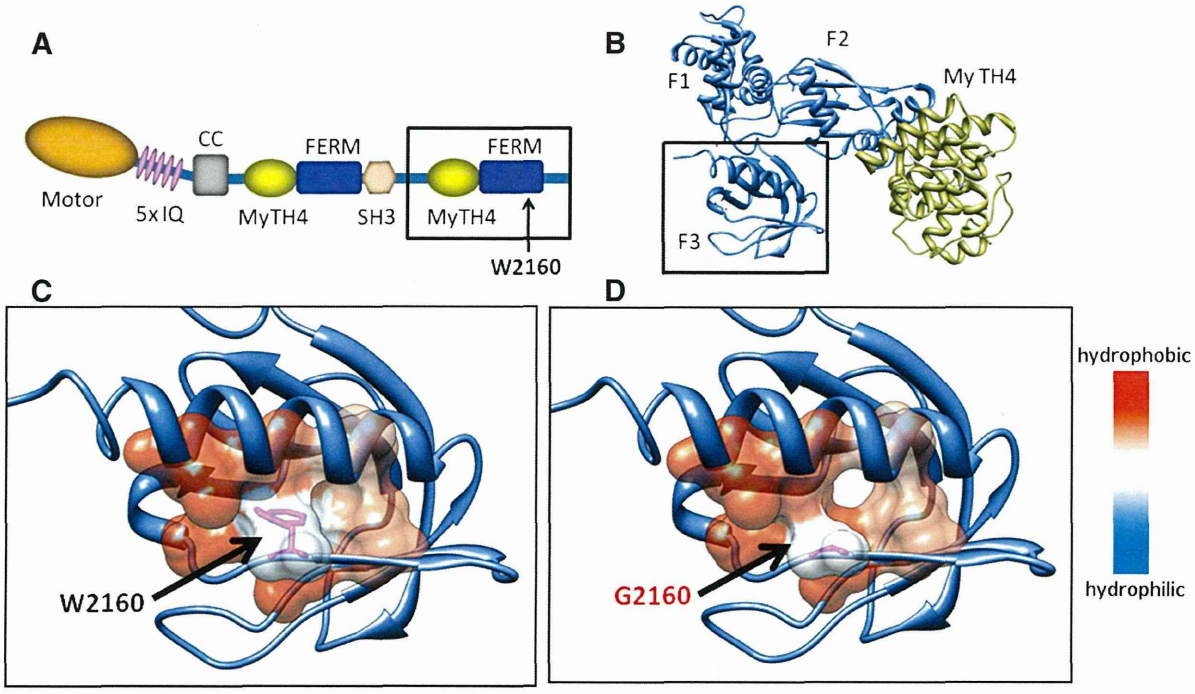

Figure 3 Molecular modeling of *MYO7A* containing the p.W2160G mutation. (A) Structural motif of myosin 7A. Tryptophan 2160 on the C-terminal 4.1 protein-ezrin-radixin-moesin (FERM) domain is indicated by an arrow. Motor, myosin motor domain; IQ, Isoleucine-glutamine calmodulin-binding motif; CC, coiled-coil domain; MyTH4, myosin tail homology 4 domain; SH3, Src homology 3 domain. **(B)** Ribbon model of the C-terminal FERM domain consisting of three subdomains (F1, F2, F3) and an MyTH4 domain. **(C, D)** Magnified ribbon model of the F3 subdomain superimposed with predicted surface hydrophobicity in the wild type **(C)** and the p.W2160G mutant **(D)**.

and *PCDH15* have been reported to be a digenic cause of hearing loss [46].

In family 7 (Figure 1G), subjects II:1 and II:2 with hearing loss did not have candidate mutations in the first 61 genes. Analysis of the additional 23 genes indicated a compound heterozygous *USH2A* variant or mutation, c.1346G >A(p.R449H) and c.12431delC (p.A4144GfsX23), in subjects with hearing loss, whereas subjects I:1 and II:2 with normal hearing had a heterozygous p.R449H variant and a heterozygous p.A4144GfsX23 mutation, respectively. *USH2A* is responsible for Usher syndrome 2A (OMIM 276901) [47]. Although *USH2A* with the p.R449H variant was not found on dbSNP135, 1000GENOME, or the Exome Variant Server, the allele frequency in Japanese control subjects with normal hearing was 1.3% (5/378).

In the remaining eight families, none of the detected variants co-segregated with hearing loss in the pedigrees (data not shown).

Discussion

In the present study we selected Japanese subjects that had hereditary hearing loss without *GJB2* mutations, mitochondrial mutations, enlarged vestibular aqueduct or auditory neuropathy-associated *OTOF* mutations, and we aimed to detect the spectrum of rare deafness genes in these patients. Targeted NGS for 84 deafness genes resulted in identification of candidate genes in 7 of 15 families and revealed the diverse spectrum of rare deafness genes in Japanese subjects with nonsyndromic hearing loss for the first time. This is the first report of mutations in *ACTG1*, *POU4F3*, and *SLC26A5* in Japanese families with hearing loss. Families 5, 6, and 7 appeared to have candidate mutations or variants in *MYO7A*, *CDH23*, *PCDH15*, and *USH2A*, all of which are associated with Usher syndrome [39,44,45,47]. Our results are in contrast to an NGS study of a different ethnic group [48], which showed *TMC1* mutations to be the prevalent candidate cause of hearing loss.

For the eight families without candidate genes, hearing loss could be attributable to mutations in non-captured regions including regulatory domains of the 84 genes, other unidentified deafness genes, unknown multigenic causes, copy number variations, or chromosomal structural change.

Double heterozygous mutations

In family 5, double heterozygous mutations of *CDH23* and *PCDH15* were detected as a candidate cause. This combination of double heterozygous mutations has been reported [46]. Cadherin 23 and protocadherin 15 consist of the upper and lower part of tip link, respectively, which is critical for proper function of mechanotransduction channels on the stereocilia of the sensory hair cells [49]. In addition, P240 of CDH23 is on the extracellular cadherin 1 domain, and R283 of PCDH15 is on the extracellular cadherin 2 domain, which are considered to interact with each other for tip-link bound [49], raising the possibility that the double heterozygous mutations could lead to a destabilized tip-link.

Additional findings of double heterozygous mutations associated with hereditary hearing loss have been reported for *KCNJ10* and *SLC26A4* [50] and for *FOXI1* and *SLC26A4* [51], and some mutated genes may have a modifying effect [52]. Although most NGS pipelines, including ours, focus on identifying monogenic causes of disease, development of a detection strategy for digenic and oligogenic causes of disease should be considered in the future.

Discrimination of mutations from variants

The key challenge for the diagnostic application of NGS is to distinguish causal alleles from the numerous nonpathogenic variants present in each individual. In the present study, for example, the high allele frequency of *USH2A* with the p.R449H variant in Japanese control subjects implied that pathogenicity of this variant was unlikely. Ethnic diversity of genetic variance has been reported in deafness genes such as *OTOF* [12] and *CDH23* [43,53], and integration of a database of genetic variants with allele frequencies in a specific ethnic group would increase the certainty of the causative nature of genetic mutations by filtering out variants that occur with high frequency. This would facilitate targeted NGS analysis for genetic diagnosis of hearing loss.

Additional files

Additional file 1: The 84 genes that were targeted for next-generation sequencing.

Additional file 2: Clinical features of family members.

Additional file 3: Audiograms of subjects with hearing loss in the seven families in which candidate genes were detected. Figure legend: Hearing level as a function of frequency in subject IV:2 from family 1 (A), subject III:3 from family 1 (B), subject IV:3 from family 2 (C), subject III:1 from family 2 (D), subject III:2 from family 2 (E), subject III:1 from family 3 (F), subject II:1 from family 4 (G), subject III:1 from family 5 (H), subject II:2 from family 6 (I), subject II:3 from family 6 (J), and subject II:2 from family 7 (K). Open circles with solid lines represent air conduction thresholds of the right ear; crosses with dotted lines represent air conduction thresholds of the left ear; [symbols represent bone conduction thresholds of the right ear;] symbols represent bone conduction thresholds of the left ear; arrows pointing to the bottom left represent scale-out hearing level of the right ear; arrows pointing to the bottom right represent scale-out hearing level of the left ear.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
HM and NS carried out capturing and sequencing the DNA samples, interpreted the data, and drafted the manuscript. CT carried out capturing and sequencing the DNA samples. AS and JK worked on DNA sequencing and interpreting the data. KN carried out molecular modeling of gene

products. KKosaki and TM designed the study and interpreted the data. NM, KKaga, and TM contributed to accumulation and interpretation of clinical data. TM finalized the manuscript. All authors read and approved the final manuscript.

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Methods paper

GJB2-associated hearing loss undetected by hearing screening of newborns

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ABSTRACT

The hearing loss caused by *GJB2* mutations is usually congenital in onset, moderate to profound in degree, and non-progressive. The objective of this study was to study genotype/phenotype correlations and to document 14 children with biallelic *GJB2* mutations who passed newborn hearing screening (NHS). Genetic testing for *GJB2* mutations by direct sequencing was performed on 924 individuals (810 families) with hearing loss, and 204 patients (175 families) were found to carry biallelic *GJB2* mutations. NHS results were obtained through medical records. A total of 18 pathological mutations were identified, which were subclassified as eight inactivating and 10 non-inactivating mutations. p.I128M and p.H73Y were identified as novel missense *GJB2* mutations. Of the 14 children with biallelic *GJB2* mutations who passed NHS, eight were compound heterozygotes and 3 were homozygous for the c.235delC mutation in *GJB2*, and the other three combinations of non-c.235delC mutations identified were p.Y136X-p.G45E/p.V37I heterozygous, c.512ins4/p.R143W heterozygous, and p.V37I/p.R143W heterozygous. These 14 cases demonstrate that the current NHS does not identify all infants with biallelic *GJB2* mutations. They suggest that the frequency of non-penetrance at birth is approximately 6.9% or higher in DFNB1 patients and provide further evidence that *GJB2* hearing loss may not always be congenital in onset.

1. Introduction

More than half the incidence of congenital hearing loss is due to hereditary factors. Mutations in the *GJB2* gene encoding connexin 26 (Cx26) are the most common cause of nonsyndromic autosomal recessive sensorineural hearing loss (DFNB1) in many populations (Morton and Nance, 2006). To date, more than 150 mutations, polymorphisms, and unclassified variants have been described in the *GJB2* gene (https://research.cchmc.org/LOVD2/home.php). The mutation spectrum and prevalence of mutations vary significantly across different ethnic groups (Smith and Van Camp, 1993).

DFNB1-causing allele variants of *GJB2* alter the function of the encoded protein, Cx26, in the inner ear, and they may also alter trafficking. Cx26 aggregates in groups of six around a central 2–3 nm pore to form a doughnut-shaped structure called a connexon (Maeda et al., 2009). Connexons from contiguous cells covalently bond to form intercellular channels. Aggregations of connexons are called plaques and are the constituents of gap junctions. The gap junction system may be involved in potassium circulation, allowing ions that enter hair cells during mechanosensory transduction to be recycled to the stria vascularis (Zhao et al., 2006). Hearing loss caused by *GJB2* mutations is usually congenital in onset, moderate to profound in degree, and non-progressive (Smith and Van Camp, 1993). However, it is reported

Abbreviations: AABR, automated auditory brainstem response; ABR, auditory brainstem-evoked response; AOAE, automated otoacoustic emissions; Cx26, connexin 26; HI, hearing impairment; I/I, biallelic inactivating; I/NI, compound heterozygous inactivating/non-inactivating; NHS, newborn hearing screening; NI/NI, biallelic non-inactivating; PolyPhen-2, polymorphism phenotyping v2.

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that some cases of *GJB2*-related sensorineural hearing loss have delayed disease onset (Norris et al., 2006; Pagarkar et al., 2006).

To identify prelingual bilateral severe to profound hearing loss, newborn hearing screening (NHS) was implemented from the year 2000 in some areas of Japan; it has now been extended to 60% of all newborns in the country (Adachi et al., 2010; Kasai et al., 2012). Two types of physiological tests are approved for the first phase of newborn audiologic screening: automated otoacoustic emissions (AOAE) and automated auditory brainstem response audiometry (AABR). Both methods have advantages and disadvantages, but they are equally efficacious (Chiong et al., 2007). An important limitation of existing NHS programs has been the fact that not all forms of prelingual hearing loss are expressed at birth. Conditions such as congenital cytomegalovirus infection (Stehel et al., 2008), enlargement of the vestibular aqueduct (Dewan et al., 2009), and the m.1555A>G in the 12S rRNA gene (Chen et al., 2011) can all lead to prelingual hearing loss that is not present at birth and affected persons can pass NHS. Even in the case of DFNB1, some c.35delG mutations and p.V37I mutations have been previously reported to cause hearing loss that was not detected through NHS (Chan et al., 2010; Norris et al., 2006). In this study, we present our experience with *GJB2*-associated hearing loss, describing 14 children who initially passed their NHS but were subsequently identified with hearing loss.

2. Materials and methods

This study was approved by the ethical committee of the National Tokyo Medical Center and by the ethical committees of all research institutes collaborating in this study. Informed consent was obtained by each research center from every participant or from the parents of minors. From November, 2002, to August, 2012, 924 individuals (810 families) with hearing loss underwent genetic testing by direct sequencing for *GJB2* mutations. They were collected from all over Japan as part of a multicenter study of *GJB2*. The entire coding regions of *GJB2* were amplified using the primer pair 5′-CTAGTGATTCCTGTGTTGTGTG-3′/5′-TTTGTGTTGGGAAATGCTTGCGA-3′. Two hundred and four patients (175 families) were found to carry pathological biallelic *GJB2* mutations. Screenings of alleles in a normal-hearing population were performed for novel mutations.

Genetic and audiological data were described according to the recommendations of the Genetic Deafness (GEN-DEAF) study group on genotype/phenotype correlations (Mazzoli et al., 2003). According to these guidelines, the groups were categorized as follows: mild hearing impairment (HI) (20–40 dB), moderate HI (41–70 dB), severe HI (71–95 dB), and profound HI (>95 dB). Audiometric data were obtained by various techniques. In 94 cases, hearing levels were obtained via pure tone audiometry, with measurements collected at frequencies ranging from 0.125 kHz to 8 kHz. For 89 young children, conditioned orientation reflex or play audiometry was used to measure hearing thresholds. Testing also included auditory brainstem-evoked response (ABR) or auditory steady-state response in 18 infants. A four-frequency average at 0.5, 1, 2, and 4 kHz from the better-hearing ear was used to compare subgroups of patients. Three patients were excluded from the genotype/phenotype analysis because of insufficient audiometric data. NHS results were obtained by medical records. NHS was performed once by either AOAE or AABR. Eight of these patients initially had their hearing screened by AOAE, six were screened by AABR.

3. Results

3.1. GJB2 mutation spectrum and degree of hearing impairment (HI) in the three classes of genotypes

A total of 18 mutations were identified and then subclassified, eight as inactivating, such as c.235delC, and 10 as non-inactivating mutations, such as p.V37I (Table 1). The most common mutation was c.235delC. We defined three genotype classes: biallelic inactivating (I/I; 141 patients [69.1%]), biallelic non-inactivating (NI/NI; 20 patients [9.8%]), and compound heterozygous inactivating/non-inactivating (I/NI; 43 patients [21.1%]). In the I/I class, 37.4% of patients had profound HI, 30.2% had severe HI, and 30.9% had moderate HI. In the I/NI class, 34.9% of patients had profound HI, 16.3% had severe HI, and 32.6% had moderate HI. More than three-quarters (80%) of patients in the NI/NI class had only a moderate or mild degree of HI, and only one patient (5%) in this class had profound HI (Fig. 1).

p.I128M and p.H73Y were identified as novel missense *GJB2* variants, in a heterozygote with accompanying known pathogenic alleles. These mutations were predicted as likely to be damaging variants according to polymorphism phenotyping v2 (PolyPhen-2). PolyPhen-2 is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations (Adzhubei et al., 2010). Screening of 364 alleles in a normal-hearing population did not identify the p.I128M mutation, and one of 376 alleles in a normal-hearing population screening was identified as p.H73Y mutation. Both amino acids isoleucine-128 and histidine-73 lie in a region of the Cx26 protein that is highly conserved in vertebrates (Fig. 2).

Table 1
GJB2 mutation spectrum.

Mutation and genotype		No. of subjects	
Inactivating/inactivating (I/I)			
Homozygous	c.235delC/c.235delC	65	(31.9%)
	p.Y136X-G45E/p.Y136X-G45E	9	(4.4%)
	c.35delG/c.35delG	1	(0.5%)
	c.176-191del16/c.176-191del16	1	(0.5%)
Compound heterozygous	c.235delC/p.Y136X-G45E	28	(13.7%)
	c.235delC/c.176-191del16	13	(6.4%)
	c.235delC/c.299-300delAT	11	(5.4%)
	c.235delC/c.512ins4	3	(1.5%)
	c.235delC/c.605ins46	1	(0.5%)
	c.235delC/p.W134X	1	(0.5%)
	c.235delC/c.35delG	1	(0.5%)
	c.176-191del16/p.Y136X-G45E	3	(1.5%)
	c.512ins4/p.Y136X-G45E	1	(0.5%)
	c.299-300delAT/p.Y136X-G45E	1	(0.5%)
	c.299-300delAT/p.W134X	2	(1.0%)
Total I/I		141	(69.1%)
Non-inactivating/non-inactivating (NI/NI)			
Homozygous	p.V37I/p.V37I	4	(2.0%)
	p.R143W/p.R143W	1	(0.5%)
Compound heterozygous	p.V37I/p.R143W	8	(3.9%)
	p.V37I/p.V84M	1	(0.5%)
	p.V37I/p.V84L	1	(0.5%)
	p.V37I/p.T86R	2	(1.0%)
	p.R143W/p.M195V	1	(0.5%)
	p.R143W/p.H73Y	1	(0.5%)
	p.T86R/p.T26A	1	(0.5%)
Total NI/NI		20	(9.8%)
Inactivating/non-inactivating (I/NI)			
Compound heterozygous	c.235delC/p.T86R	9	(4.4%)
	c.235delC/p.R143W	12	(5.9%)
	c.235delC/p.V37I	6	(2.9%)
	c.235delC/p.R32H	1	(0.5%)
	c.235delC/p.I128M	1	(0.5%)
	p.Y136X-G45E/p.V37I	4	(2.0%)
	p.Y136X-G45E/p.T86R	1	(0.5%)
	p.Y136X-G45E/p.R143W	1	(0.5%)
	c.299-300delAT/p.V37I	3	(1.5%)
	c.299-300delAT/p.R143W	1	(0.5%)
	c.512ins4/p.R143W	2	(1.0%)
	c.512ins4/p.V37I	1	(0.5%)
	c.176-191del16/p.R143W	1	(0.5%)
Total I/NI		43	(21.1%)

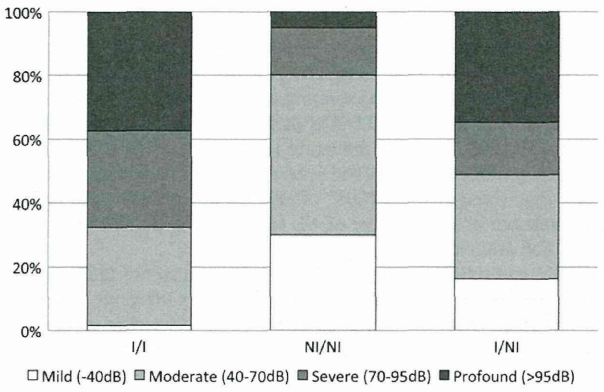

Fig. 1. Degree of hearing impairment in the three classes of genotypes. Of the 204 persons studied, 141 (69.1%) segregated two inactivating mutations that represented 15 different genotypes. Twenty of the 204 persons studied (9.8%) segregated two non-inactivating mutations that represented 9 different genotypes. Of the 204 individuals studied, 43 (21.1%) segregated one inactivating and one non-inactivating mutation that represented 13 different genotypes.

3.2. Fourteen children with GJB2 mutations who passed NHS

We retrospectively identified 14 children with mutations in the *GJB2* gene, each of whom passed the hearing screening performed at birth but was later found to have hearing loss (Table 2). The incidence rate was 6.9% among all the DFNB1 patients. Furthermore, to restrict the analysis to those patients born after 2000, when NHS began in Japan, 8.9% of the *GJB2* hereditary hearing loss patients passed NHS. The age at which hearing loss was confirmed ranged from 4 month to 6 years old. A patient with mild hearing loss (No. 12) was the latest identified. Of these 14 children, three were homozygous for the c.235delC mutation, and eight were compound heterozygous for c.235delC (4 c.235delC/p.Y136X-p.G45E, 1 c.235delC/c.512ins4, 1 c.235delC/c.176-191del16, 1 c.235delC/p.V37I and 1 c.235delC/p.R143W). The other three combinations of non-c.235delC mutations identified were p.Y136X-p.G45E/p.V37I heterozygous, c.512ins4/p.R143W heterozygous, and p.V37I/p.R143W heterozygous. p.Y136X-p.G45E and c.512ins4 are truncating mutations, and both p.V37I and p.R143W are nontruncating mutations.

4. Discussion

4.1. Two novel missense GJB2 pathological mutations, p.I128M and p.H73Y, have now been identified

The spectrum of pathologic *GJB2* allelic variants diverges substantially among populations, as reflected by specific ethnic biases for common mutations. The c.35delG allele is common among Caucasians (Estivill et al., 1998); c.167delT is most common in the Ashkenazi Jewish population (Morell et al., 1998); and c.235delC is most common in the Japanese population (Ohtsuka et al., 2003). We identified p.I128M and p.H73Y as novel pathological mutations. The p.H73Y *GJB2* mutation shows that hydrophilic His73 (hydropathy index −3.2) is replaced by less hydrophilic tyrosine (hydropathy index −1.3). In the p.I128M *GJB2* mutation, isoleucine (hydropathy index 4.5) is replaced by less hydrophobic methionine (hydropathy index 1.9).

4.2. Inactivating mutations of GJB2 associated with a greater degree of HI than non-inactivating mutations

This finding is consistent with a previous report on a large collection of patients from 16 countries (Europe, Israel, the USA, and Australia) (Snoeckx et al., 2005) and other multicenter studies from North America (Putcha et al., 2007) and Japan (Oguchi et al., 2005). According to most previous reports, heterozygotes with I/NI mutations demonstrated less severe HI than homozygotes with inactivating mutations (Chan et al., 2010; Kenna et al., 2010; Snoeckx et al., 2005). However, our results demonstrated that I/NI mutations were as severe as I/I mutations. Although the mechanism underlying the varying phenotype in patients with *GJB2* mutations is still unclear, mutation-specific genotype differences, as well as differences in other genetic and environmental modifiers, are likely to explain the variability in the severity of hearing loss.

4.3. Children with GJB2 mutations are not always identified by traditional screening methods during the newborn period

The introduction of NHS has radically altered the average age at which hearing loss is first recognized and has revealed that some cases of profound prelingual hearing loss pass newborn screening. Our results indicate that the frequency with which *GJB2* hereditary hearing loss patients passed NHS is at least 6.9%. Because it is unlikely that we identified all the cases that passed NHS, we estimate that the incidence of non-penetrance at birth is even higher. Considering only those patients born after 2000, when NHS began in Japan, 8.9% of *GJB2* hereditary hearing loss patients passed NHS.

Penetrance of hearing loss in pathological biallelic *GJB2* mutations was reported almost complete (Kenneson et al., 2002). The non-penetrance of *GJB2* mutations at birth in this present study is unexplainable with the comorbidity of causes for progression of hearing loss or postnatally acquired hearing loss. Two possibilities may explain why the 14 children with *GJB2* mutations passed NHS: a false-negative result in the screening and late-onset hearing loss. Although there has been no controlled long-term study specifically evaluating the actual false-negative rate in NHS, in some groups the false-negative rate appears to be very low (Hall et al.,

Fig. 2. Multiple alignments of Cx26 orthologs. Arrows indicate affected amino acids.

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.

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

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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.