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Clinical Case Reports

Case Report Open Access

A Novel Mutation of *MYO15A* Associated with Hearing Loss in a Japanese Family

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Abstract

Mutations in the *MYO15A* gene located on chromosome 17p11.2, are responsible for non-syndromic autosomal recessive profound hearing loss (DFNB3). Direct sequencing of 96 Japanese families with profound congenital hearing loss revealed one family with a novel homozygous mutation in *MYO15A*, a T to A transition at the nucleotide of 9413 (c.9413T>A) that encodes the MyTh4 domain of the protein (p. L3138Q). This is the first report of an East Asian hearing loss patient with a *MYO15A* mutation.

Keywords: DFNB3; MYO15A; Mutation; Hearing loss

Introduction

Hearing loss is one of the most common communication disorders in humans, affecting millions of individuals worldwide. To date, 95 loci for autosomal recessive sensorineural hearing loss (ARSNHL) have been reported and at 41 of these loci, the causative genes have been identified (Hereditary Hearing Loss Homepage: http://hereditaryhearingloss.org/). *MYO15A* is comprised of 66 exons distributed across 71 kbp of DNA on chromosome 17p11.2. The *MYO15A* mRNA transcript encodes a 3530 amino acid protein in its longest form. *MYO15A* has MyTh4 (Myosin-Tail like Homology region 4) domains, FERM (4.1 protein, Ezrin, Radixin, and Moesin) motifs, a SH3 (Src Homology 3) domain, and the PDZ domain.

In humans, 36 different *MYO15A* mutations have been reported and 35 of these cause congenital profound ARSNHL. The remaining *MYO15A* mutation was a heterozygous missense mutation detected in a Smith-Magenis syndrome patient who had moderate sensorineural hearing loss.

In this report, we describe the first identified novel missense *MYO15A* mutation in a Japanese ARSNHL patient together with a review of the previous literature. This mutation is located in a MyTh4 domain and is thought to disrupt normal *MYO15A* function, resulting in congenital hearing loss.

Subjects

DNA samples from 96 independent subjects who had profound congenital ARSNHL were collected from 33 ENT departments nationwide in Japan. All subjects gave prior written informed consent for participation in the project, which was approved by each hospital's ethical committee. Anamnestic and physical examinations were performed to exclude those with syndromic symptoms, outer and/or middle ear diseases, and environmental factors such as premature birth, or newborn meningitis. Controls were 192 Japanese healthy individuals with normal hearing confirmed by pure tone audiometry.

Mutation Analysis

All of the *MYO15A* exons were amplified using gene-specific primers described elsewhere [1]. PCR reactions were performed with 25 μl in 1.5 mM $MgCl_2$, 100 mM of each dNTP, 1U of Taq DNA polymerase, and 2 mM forward and reverse primers. After an initial denaturation at 95°C for 90 seconds, amplification was performed for 35 cycles of 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes. Then, a final extension was performed at 72°C for 5 minutes. Sequencing was performed with a BigDye™ v1.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Sequencing products were analyzed by an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Computer analysis to predict the effect of missense variants on *MYO15A* protein function was performed with Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), and Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/).

Results

Direct sequencing revealed a novel homozygous mutation of *MYO15A* at exon 57 (c.9413T>A) in one patient (Figure 1). This mutation (p. L3138Q) is located in the MyTh4 domain of the myosin 15a protein, and is predicted to be pathologic by prediction programs (Table 1). We also confirmed that the patient's father and mother had heterozygous mutations and that the mutation was absent in the controls. The patient had no mutations in *GJB2*, the gene most frequently involved with hearing impairment in Japanese, nor in mitochondrial 1555A>G.

In detail, the patient was a female with congenital severe to profound sensorineural hearing loss. At age one, her mother became aware of her hearing impairment because she did not speak. The patient visited the hospital for genetic testing the age of 17 (Figure 1). Computed Tomography examination indicated that she did not have any malformations, such as ossicular anomalies, cochlear hypoplasia, vestibular dilation or enlarged vestibular aqueduct. In addition, she had no history of vertigo. Her sister also had severe congenital hearing loss, but her parents, brother, and other relatives did not have hearing impairment (Figure 1). DNA samples were not obtained from her siblings.

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Exon	Domain	Nucleotide change	Amino acid change	Frequency	Control	Hereditary	Age of onset	Hearing level	Functional prediction	
									Poly Phen2 score	SIFT score
3	N-terminal extension	3658G>A	G1220R	1/96	0/192	Sporadic	Congenital	Severe	0	0.09
12	Motor	4322G>T	G1441V	1/96	0/192	Autosomal recessive	Congenital	Profound	0.785	0.01
30	MyTH4	6486delG	A2153fs	1/96	0/192	Sporadic	Congenital	Profound	-	-
57	MyTH4	9413T>A	L3138Q	1/96	0/192	Autosomal recessive	Congenital	Profound	0.791	0
65	-	10420A>G	S3474G	1/96	0/192	Sporadic	Congenital	Severe	0.427	-

Table 1: *MYO15A* variants found in this study.

Exon	Domain	Nucleotide change	Amino acid change	Mutation type	Age of onset	Hearing level	Origin of family	References
Exon 2	N-terminal extension	373delCG	R125VfsX101	Frameshift	-	-	Ashkenazi Jewish	12
Exon 2	N-terminal extension	867C>G	Y289X	Nonsense	Congenital or prelingual	Moderate to severe	Turkey	11
Exon 2	N-terminal extension	1185dupC	E396fsX431	Frameshift	10-14 yrs	Moderate to severe	Pakistan	13
Exon 2	N-terminal extension	1387A>G	M463V	Missense	-	Severe to profound	Iran	14
Exon 2	N-terminal extension	3313G>T	E1105X	Nonsense	-	Profound	Pakistan	7, 13
Exon 2	N-terminal extension	3334delG	G1112fsX1124	Frameshift	-	Severe to profound	Pakistan	7, 13
Exon 3	Motor	4023C>T	Q1229X	Nonsense	Congenital	Profound	Pakistan	6
Intron 4	Motor	IVS4+1G>T	D1232fsX1241	Splice donor site	Congenital	Profound	Pakistan	6
Exon 5	Motor	3758C>T	T1253I	Missense	-	Severe to profound	India	7
Intron 5	Motor	IVS5+1G>A	T1253fsX1277	Splice donor site	-	Severe to profound	Pakistan	7
Exon 10	Motor	4176C>A	Y1392X	Nonsense	-	Severe to profound	Pakistan	7
Exon 10	Motor	4198G>A	V1400M	Missense	Congenital or prelingual	Severe to profound	Turkey	11
Exon 11	Motor	4240G>A	E1414K	Missense	-	-	Palestinian Arab	12
Exon 11	Motor	4273C>T	Q1425X	Nonsense	-	-	Turkey	15
Exon 12	Motor	4351G>A	D1451N	Missense	-	Severe to profound	India	7
Exon 12	Motor	4441T>C	S1481P	Missense	Congenital or prelingual	Severe to profound	Turkey	11,15
Exon 14	Motor	4652C>A	A1551D	Missense	-	-	Turkey	15
Exon 15	Motor	4669A>G	K1557E	Missense	-	Severe to profound	Pakistan	7
Exon 17	Motor	4904-4907delGAG	E1637del	Frameshift	-	Severe to profound	Iran	14
Exon 17	Motor	4998C>A	C1666X	Nonsense	-	Severe to profound	Tunisia	10
Exon 18	Motor	5117_5118GC>TT	L1706V	Missense	-	Severe to profound	Pakistan	7
Exon 19	Motor	5189T>C	G1730P	Missense	-	Severe to profound	Pakistan	7
Exon 20	Motor	5305A>G	T1769A	Missense	-	Severe to profound	Iran	14
Exon 22	Motor	5419-21delT	F1807L fsX6	Frameshift	-	Severe to profound	Iran	14
Exon 22	Motor	5492G>T	G1831V	Missense	-	Profound	Turkey	8
Exon 24	Motor	5810G>A	R1937H	Missense	-	Severe to profound	Iran	14
Exon 24	Motor	5807_5813delCCCGTGG	R1937TfsX10	Frameshift	Congenital or prelingual	Severe to profound	Turkey	11
Exon 26	IQ Motif	5925G>A	W1975X	Nonsense	-	Severe to profound	Iran	14
Exon 28	-	6061C>T	Q2021X	Nonsense	-	Severe to profound	Pakistan	7
Exon 29	MyTH4	6217C>T	P2073S	Missense	Congenital	Profound	Iran	1
Exon 30	MyTH4	6331A>T	N2111Y	Missense	Congenital	Profound	India	5
Exon 30	MyTH4	6337A>T	I2113F	Missense	Congenital	Profound	Indonesia	5
Exon 30	MyTH4	6371G>A	R2124Q	Missense	Congenital	Profound	Iran	1
Exon 31	MyTH4	6952C>T	T2205I	Missense	Congenital	Moderate	North America*	6
Exon 32	-	6731G>A	G2244E	Missense	-	Severe to profound	Pakistan	7
Exon 33	-	6796G>A	V2266M	Missense	-	Severe to profound	Pakistan, Turkey	7

Intron 37	-	IVS37 + 3G>C	-	Splice donor site	-	Severe to profound	Tunisia	10
Exon 41	-	7801A>T	K2601X	Nonsense	Congenital	Profound	India	5
Exon 44	FERM	8486G>T	Q2716H	Missense	Congenital	Profound	Pakistan	6
Exon 45	FERM	8158G>C	D2720H	Missense	-	Severe to profound	Pakistan	7
Exon 45	FERM	8183G>A	R2728H	Missense	-	-	Ashkenazi Jewish	12
Exon 48	FERM	8467G>A	D2823N	Missense	-	Severe to profound	Iran	14
Intron 50	-	IVS50-1G>C	-	Splice donor site	-	Profound	Turkey	8
Exon 51	SH3	8821_8822insTG	V2940fsX3034	Frameshift	-	Severe to profound	Pakistan	7
Intron 54	-	IVS54+1G>A	-	Splice donor site	-	Severe to profound	Tunisia	10
Exon 57	**MyTH4**	**9413T>A**	**L3138Q**	**Missense**	**Congenital or prelingual**	**Profound**	**Japan**	**This case**
Exon 57	MyTH4	9478C>T	L3160F	Missense	-	Severe to profound	Pakistan	7
Exon 62	FERM	9957_9960delTGAC	D3320fs	Frameshift	Frameshift	Severe to profound	Brazil**	9
Exon 62	FERM	9995_10002dupGCCG-GCCC	S3335AfsX121	Frameshift	Congenital or prelingual	Severe to profound	Turkey	11
Exon 65	-	10474C>T	Q3492X	Nonsense	-	Severe to profound	Pakistan	7
Exon 66	-	10573delA	S3525fs	Frameshift	Prelingual	Severe to profound	Brazil	9

*Mutation was found in a patient heterozygous at the DFNB3 locus with Smith-Magenis Syndrome.

**Mutation was found in a heterozygous individual.

Table 2: DFNB3-causing *MYO15A* mutations.

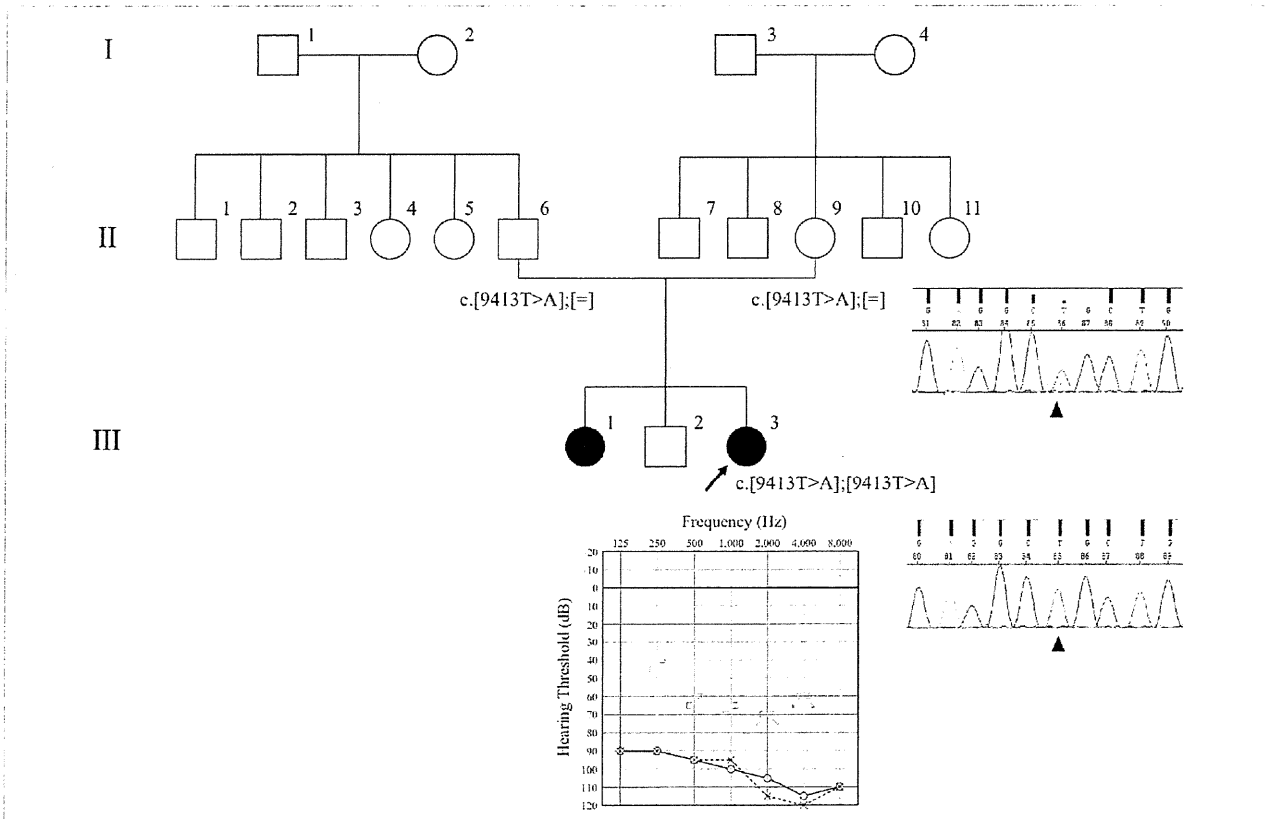

Figure 1: Family pedigree, audiogram of the patient, and lectropherogram analysis of MYO15A in the family. A novel homozygous mutation of MYO15A at exon 57 (c.9413T>A) is found in a patient with congenital severe to profound sensorineural hearing loss.

We also found other heterozygous variants: c.6824delG, p.G1441V, p.G1220R, and p.S3474G, each in a different independent patient, and none being found in the controls (Table 1).

Discussion

Myosin 15a protein is required for normal auditory function,

therefore *MYO15A* mutations cause ARSNHL. Mutations in this gene also cause the shaker 2(sh2) phenotype in mice. Sh2 mice are characterized by a vestibular defect and profound hearing loss [2,3] but such vestibular defects are not found in human carriers of *MYO15A* mutations. The stereocilia of hair cells of the sh2 mice are short and lack the characteristic staircase-like pattern [4].

In our patient, the novel *MYO15A* mutation located in the MyTH4 domain caused sensorineural hearing loss. In addition, this is the first *MYO15A* mutation found in an East Asian population. To date, 43 mutations in *MYO15A* were reported. Type of mutations, domains, and clinical features are summarized in Table 2 [1,5-15]. All *MYO15A* mutations previously reported were found in prelinguistic or congenital hearing loss patients, except for one Smith-Meganis syndrome patient [6]. Our patient had prelingual profound hearing loss, consistent with previous reports.

Of the 43 reported *MYO15A* mutations, six were missense mutations in the MyTH4 domains. Five of those six were found in homozygous state: p.N2111Y in Indians [5]; p.I2113F in Indonesians [5]; p.R2124Q and p.P2073S in Iranians [1]; and p. L3160F in a Pakistani family [7]. The sixth missense mutation was a heterozygous mutation, p. T2205I, in a North American family affected by Smith-Magenis syndrome [6] (Table 2).

Furthermore, based on the prediction programs, two missense mutations, p. G1441V, p. L3138Q, are predicted to be pathologic variants (Table 1). However, except for p. L3138Q, all variants found in this study were identified as heterozygous and no associated mutation was found in the other allele.

The structure of the MyTH4 domain has not been fully characterized. In other myosins, it has been implicated in microtubule binding as well as actin binding to the plasma membrane. Some data suggest that the MyTH4/FERM domains are required for localization of Myosin15a to stereocilia tips. The co-localization of Myosin15a and whirlin proteins appears essential to form the complex at the stereocilia tips [16]. From our data combined with previous reports, the MyTH4 domain mutations interfere with the interaction between Myosin15a and whirlin, preventing the formation of the complex required for normal hearing [1]. *MYO15A* mutations have been found in each domain (Motor, MyTH4, N-terminal extension, FERM, and SH3) and caused similar clinical features including hearing level, implying the overall importance of *MYO15A* protein in cochlear function.

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References

1. Shearer AE, Hildebrand MS, Webster JA, Kahrizi K, Meyer NC, et al. (2009) Mutations in the first MyTH4 domain of MYO15A are a common cause of DFNB3 hearing loss. Laryngoscope 119: 727-733.
2. Probst FJ, Fridell RA, Raphael Y, Saunders TL, Wang A, et al. (1998) Correction of deafness in shaker-2 mice by an unconventional myosin in a BAC transgene. Science 280: 1444-1447.
3. Anderson DW, Probst FJ, Belyantseva IA, Fridell RA, Beyer L, et al. (2000) The motor and tail regions of myosin XV are critical for normal structure and function of auditory and vestibular hair cells. Hum Mol Genet 9: 1729-1738.
4. Belyantseva IA, Boger ET, Friedman TB (2003) Myosin XVa localizes to the tips of inner ear sensory cell stereocilia and is essential for staircase formation of the hair bundle. *Proc Natl Acad Sci U S A 100*: 13958-13963.
5. Wang A, Liang Y, Fridell RA, Probst FJ, Wilcox ER, et al. (1998) Association of unconventional myosin MYO15 mutations with human nonsyndromic deafness DFNB3. Science 280: 1447-1451.
6. Liburd N, Ghosh M, Riazuddin S, Naz S, Khan S, et al. (2001) Novel mutations of MYO15A associated with profound deafness in consanguineous families and moderately severe hearing loss in a patient with Smith-Magenis syndrome. Hum Genet 109: 535-541
7. Nal N, Ahmed ZM, Erkal E, Alper OM, Lüleci G, et al. (2007) Mutational spectrum of MYO15A: the large N-terminal extension of myosin XVA is required for hearing. Hum Mutat 28: 1014-1019.
8. Kalay E, Uzumcu A, Krieger E, Caylan R, Uyguner O, et al. (2007) MYO15A (DFNB3) mutations in Turkish hearing loss families and functional modeling of a novel motor domain mutation. Am J Med Genet A 143A: 2382-2389.
9. Lezirovitz K, Pardono E, de Mello Auricchio MT, de Carvalho E Silva FL, Lopes JJ, et al. (2008) Unexpected genetic heterogeneity in a large consanguineous Brazilian pedigree presenting deafness. Eur J Hum Genet 16: 89-96.
10. Belguith H, Alfa-Hmani M, Dhouib H, Said MB, Mosrati MA, et al. (2009) Screening of the DFNB3 locus: identification of three novel mutations of MYO15A associated with hearing loss and further suggestion for two distinctive genes on this locus. Genet Test Mol Biomarkers 13: 147-151.
11. Cengiz FB, Duman D, Sirmaci A, Tokgöz-Yilmaz S, Erbek S, et al. (2010) Recurrent and private MYO15A mutations are associated with deafness in the Turkish population. Genet Test Mol Biomarkers 14: 543-550.
12. Brownstein Z, Friedman LM, Shahin H, Oron-Karni V, Kol N, et al. (2011) Targeted genomic capture and massively parallel sequencing to identify genes for hereditary hearing loss in Middle Eastern families. Genome Biol 12: R89.
13. Bashir R, Fatima A, Naz S (2012) Prioritized sequencing of the second exon of MYO15A reveals a new mutation segregating in a Pakistani family with moderate to severe hearing loss. Eur J Med Genet 55: 99-102.
14. Fattahi Z, Shearer AE, Babanejad M, Bazazzadegan N, Almadani SN, et al. (2012) Screening for MYO15A gene mutations in autosomal recessive nonsyndromic, GJB2 negative Iranian deaf population. Am J Med Genet A 158A: 1857-1864.
15. Diaz-Horta O, Duman D, Foster J 2nd, Sirmaci A, Gonzalez M, et al. (2012) Whole-exome sequencing efficiently detects rare mutations in autosomal recessive nonsyndromic hearing loss. PLoS One 7: e50628.
16. Delprat B, Michel V, Goodyear R, Yamasaki Y, Michalski N, et al. (2005) Myosin XVa and whirlin, two deafness gene products required for hair bundle growth, are located at the stereocilia tips and interact directly. Hum Mol Genet 14: 401-410.

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ORIGINAL ARTICLE

Mutation spectrum and genotype–phenotype correlation of hearing loss patients caused by *SLC26A4* mutations in the Japanese: a large cohort study

Maiko Miyagawa[1], Shin-ya Nishio[1], Shin-ichi Usami[1] and The Deafness Gene Study Consortium[2]

Mutations in *SLC26A4* cause a broad phenotypic spectrum, from typical Pendred syndrome to nonsyndromic hearing loss associated with enlarged vestibular aqueduct. Identification of these mutations is important for accurate diagnosis, proper medical management and appropriate genetic counseling and requires updated information regarding spectrum, clinical characteristics and genotype–phenotype correlations, based on a large cohort. In 100 patients with bilateral enlarged vestibular aqueduct among 1511 Japanese hearing loss probands registered in our gene bank, goiter data were available for 79, of whom 15 had Pendred syndrome and 64 had nonsyndromic hearing loss. We clarified the mutation spectrum for the *SLC26A4* mutations and also summarized hearing levels, progression, fluctuation and existence of genotype–phenotype correlation. *SLC26A4* mutations were identified in 82 of the 100 patients (82.0%). Of the Pendred syndrome patients, 93% (14/15) were carriers, as were 77% (49/64) of the nonsyndromic hearing loss patients. Clinical characteristics of patients with *SLC26A4* mutations were congenital, fluctuating and progressive hearing loss usually associated with vertigo and/or goiter. We found no genotype–phenotype correlations, indicating that, unlike in the case of *GJB2* mutations, the phenotype cannot be predicted from the genotype. Our mutation analysis confirmed the importance of mutations in the *SLC26A4* gene among hearing loss patients with enlarged vestibular aqueduct and revealed the mutation spectrum, essential information when performing genetic testing.
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Keywords: congenital hearing loss; DFNB4; enlarged vestibular aqueduct; goiter; Pendred syndrome; *SLC26A4*

INTRODUCTION

Based on our genetic screening, *SLC26A4* is the second most common responsible gene in Japanese deafness patients.[1] Mutations in the *SLC26A4* gene are known to be responsible for a broad phenotypic spectrum, from typical Pendred syndrome to nonsyndromic hearing loss with enlarged vestibular aqueduct (EVA). The prevalent association of *SLC26A4* mutations in these patients (90% in Pendred syndrome and 78.1% in nonsyndromic hearing loss associated with EVA) indicates the importance of this gene in the pathophysiology of this category of hearing impairment.[2] More than 160 mutations have been found in *SLC26A4* (Pendred/BOR Homepage, http://www.healthcare.uiowa.edu/labs/pendredandbor/), and different mutational spectrums among different ethnic groups have been reported.[2] The identification of *SLC26A4* mutations enables more appropriate genetic counseling and proper medical management for these patients. For such clinical application, updated information regarding mutation spectrum, clinical characteristics and genotype–phenotype correlations based on a large cohort is needed. In addition to our previous reports,[1–7] the present study was performed using a large cohort of patients to collect updated data and summarize these data to enable more precise decision making by ear, nose and throat clinicians.

MATERIALS AND METHODS

Subjects

Data on 1511 independent probands and 1545 family members were collected from 33 ear, nose and throat departments nationwide in Japan and registered in our gene bank. All subjects or next of kin, caretakers or guardians on behalf of the minors/children gave prior written informed consent for participation in the project, and the Shinshu University Ethical Committee as well as the respective Ethical Committees of the other participating institutions of the Deafness Gene Study Consortium (Hokkaido University, Hirosaki University, Iwate Medical University, Tohoku University, Yamagata University, Fukushima Medical University, Jichi Medical University, Gunma University, Nihon University, Nippon Medical School, Nippon Medical School Tama Nagayama

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Hospital, Jikei University, Toranomon Hospital, Kitasato University, Hamamatsu Medical University, Mie University, Shiga Medical Center for Children, Osaka Medical College, Hyogo College of Medicine, Kobe City Medical Center General Hospital, Wakayama Medical University, Okayama University, Yamaguchi University, Ehime University, Kyushu University, Fukuoka University, Nagasaki University, Kanda ENT Clinic, Miyazaki Medical College, Kagoshima University and Ryukyus University) approved the study.

Computerized tomography scan was used to diagnose EVA (according to the criteria of EVA: a diameter of >1.5 mm at the midpoint between the common crus and the external aperture), and they were clinically well characterized by repeated auditory examinations.

The 100 subjects (51 males and 49 females) from among the 1511 probands who met the criteria of bilateral EVA and who ranged in age from 0 to 59 years with a mean age of 13.9 years at the time of examination were enrolled in the current study. Fifteen subjects had Pendred syndrome and 64 had nonsyndromic hearing loss.

The controls were 192 unrelated Japanese healthy individuals with normal hearing evaluated by auditory testing.

Mutation analysis

To identify *SLC26A4* mutations, a DNA fragment containing all the exons of *SLC26A4*, including flanking intronic sequences, was sequenced as described elsewhere.[4] New variants were tested in 192 unrelated normal hearing controls.

Possible pathologic mutations were defined as (1) mutations found to be homozygotes or compound heterozygotes (and determined by segregation study); (2) variants that were not found, or were very few, in the 192 control subjects; and (3) amino acids that were well conserved among various species.

Clinical evaluations

Hearing levels were determined by pure-tone audiometry in adults. For the young patients, conditioned orientation response audiometry or auditory steady-state response was used. Clinical data, including hearing loss progression, fluctuation, episodes of tinnitus and vestibular dysfunction (vertigo, dizziness) and goiter, were collected by anamnestic evaluation. For genotype–phenotype correlation analysis, one-way analysis of variance (Tukey's honest significant difference (HSD) test), Kruskal–Wallis test and multivariate statistics (multiple regression analysis and logistic regression analysis) were used.

RESULTS

SLC26A4 mutation spectrum

There were a total of 39 *SLC26A4* mutations found in the probands with bilateral EVA (Table 1). These mutations were either homozygous, compound heterozygous or heterozygous with no other mutations being detectable. There were two nonsense mutations (p.S610X, p.L727X), three deletion frameshift mutations (c.322delC, c.917delT, c.1219delCT) and three insertion frameshift mutations (c.139insC, c.1652insT, c.2111ins GCTGG). Seven splice site mutations were found (c.416-1G>A, c.600+1G>T, c.601-1G>A, c.919-2A>G, c.1001+1G>A, c.1002-9A>G and c.1707+5G>A).

There were 24 missense mutations (p.P76S, p.T94I, p.P123S, p.M147V, p.P297Q, p.K369E, p.A372V, p.N392Y, p.G396E, p.T410M, p.A434T, p.G439R, p.S448L, p.T527P, p.I529S, p.S532I, p.C565Y, p.R581S, p.S657N, p.V659L, p.S666F, p.T721M, p.H723R and p.H723Y). To evaluate the evolutionary conservation of the amino acids affected by these missense mutations, we made an alignment of the *SLC26A4* amino acid sequence of four mammalian species: human, rat, cow and mouse. On the basis of this alignment, all missense mutations had changed evolutionary conserved amino acids. Of these mutations, nine variants had not been reported. We checked the 192 control subjects with normal hearing, but with the exception of p.H723R in 1 case, no mutations were detected.

Sequencing identified mutations in 82 of the 100 patients (82.0%). Mutations were detected in 93% of those with Pendred syndrome (14/15) and 77% (49/64) of those with nonsyndromic hearing loss. Of these, 15/100 (15.0%) were homozygous, 51/100 (51.0%) were compound heterozygous and 16/100 (16.0%) were heterozygous (Table 2).

The most frequent mutation was p.H723R that accounted for 36.0%, and the second was c.919-2A>G found in 7.0%, followed by c.1707+5G>A (4.0%). Frequency of the other 36 mutations was very low (0.5–2.0%).

Clinical findings

Table 2 shows the clinical details for the 100 subjects.

The subjects had an average hearing level of 80.9 dB (7.5–112.5 dB), with hearing loss that was mild in 5, moderate in 22, severe in 37, profound in 19 and unknown in 12. Regarding onset age of hearing loss, 45 patients were congenital, 18 were prelingual (1–3 years old), 20 were from 4 to 14 years and 17 were unknown. These results clearly indicated that early onset is dominant in patients with EVA. Also, 70 patients (70%) showed progressive hearing loss and 56 patients (56.0%) felt fluctuation of hearing. With regard to the 79 patients for whom data on vertigo were available, 41 patients complained of vertigo and 38 did not. Of the 79 patients for whom data on goiter were available, 15 had goiter and 64 did not, with an onset age from 12 to 33 years. As to family history, all families were recessive inheritance or sporadic cases.

Genotype–phenotype correlations for diagnostic age, fluctuation, vertigo, tinnitus and goiter are summarized in Figure 1.

We defined nonsense or frameshift mutations as truncating (T) and missense mutations as nontruncating (NT) and classified the genotypes as truncating/truncating (T/T), truncating/nontruncating (T/NT) or nontruncating/nontruncating (NT/NT). Significant differences were not found between the groups in any of the clinical features (Tukey's HSD test was used for diagnostic age and Kruskal–Wallis test was used for fluctuation, vertigo, tinnitus and goiter, all tests indicated $P>0.05$; Figure 1). Figure 2 shows the relationship between hearing loss severity and the mutation (T or NT) that also showed no significant differences (Tukey's HSD test, $P>0.05$). We also performed multivariate statistics (multiple regression analysis and logistic regression analysis) and we found that only the age of the patients correlated with the hearing loss severity while the genotype of *SLC26A4* mutations did not significantly affect the hearing loss severity ($P>0.05$).

DISCUSSION

The present large cohort study revealed a high prevalence (82%; 82/100) of *SLC26A4* mutations in sensorineural hearing loss patients with EVA in Japanese. The frequency (8.7%) is the second most common next to *GJB2* that is found in 16.2% of overall and 25.6% of congenital hearing loss patients.[1]

Our mutation analysis results confirmed the previous reports that indicated the importance of this gene among hearing loss patients with EVA. This study also added novel mutations and summarized updated data for the precise molecular diagnosis.

First, the high prevalence (82%) of *SLC26A4* mutations in EVA patients is compatible with the high prevalence of *SLC26A4* mutations reported in eastern Asians; that is, 97.9% in Chinese,[8] and 92% in Koreans.[9] These frequencies are higher than those reported in Caucasoid populations (20% in Americans,[10] 40.0% in French[11] and 28.4% in Spanish[12]). It is still an open question whether other genes are involved in the EVA patients without *SLC26A4* mutations.

Table 1 Possible pathogenic variants found in enlarged vestibular aqueduct (EVA) subjects ($n = 100$)

			Frequency (n = 100)				
Nucleotide change	*Amino acid change*	*Exon*	*Homozygote*	*Compound heterozygote*	*Heterozygote*	*Allele frequency (in 200 alleles)*	*References*
c. 139insC		1		1		0.50	This study
c. 266C>T	p. P76S	2		1		0.50	Suzuki *et al.*[5,6]
c. 281C>T	p. T94I	3		1		0.50	Wang *et al.*[7,8]
c. 322delC		4		1		0.50	Tsukamoto *et al.*[2,4]
c. 367C>T	p. P123S	4		1		0.50	Tsukamoto *et al.*[2,4]
c. 416-1G>A		Intron 4		2		1.00	Tsukamoto *et al.*[2,4]
c. 439A>G	p. M147V	5		2		1.00	Tsukamoto *et al.*[2,4]
c. 600+1G>T		Intron 5		1		0.50	This study
c. 601-1G>A		Intron 5		1		0.50	Tsukamoto *et al.*[2,4]
c. 890C>A	p. P297Q	7		1		0.50	This study
c. 917delT		7			1	0.50	Tsukamoto *et al.*[2,4]
c. 919-2A>G		Intron 7	1	11	1	7.00	Coucke *et al.*[21]
c. 1001+1G>A		Intron 8		2		1.00	Coyle *et al.*[22]
c. 1002-9A>G[a]		Intron 8		1		0.50	This study
c. 1105A>G	p. K369E	9		1		0.50	Usami *et al.*[2,3]
c. 1115C>T	p. A372V	9		1		0.50	Usami *et al.*[2,3]
c. 1174A>T	p. N392Y	10		3		1.50	Park *et al.*[14,16]
c. 1187G>A	p. G396E	10		1		0.50	This study
c. 1219delCT		10		1		0.50	This study
c. 1229C>T	p. T410M	10	1	1		1.50	Coyle *et al.*[22]
c. 1300G>A	p. A434T	11			1	0.50	This study
c. 1315G>A	p. G439R	11		1		0.50	Suzuki *et al.*[5,6]
c. 1343C>T	p. S448L	11		1		0.50	Wang *et al.*[7,8]
c. 1579A>G	p. T527P	14		2		1.00	Suzuki *et al.*[5,6]
c. 1586T>G	p. I529S	14		1		0.50	Wang *et al.*[7,8]
c. 1595G>T	p. S532I	14		2		1.00	Usami *et al.*[3,17]
c. 1652insT		15		3	1	2.00	Tsukamoto *et al.*[2,4]
c. 1694G>A	p. C565Y	15		1		0.50	Tsukamoto *et al.*[2,4]
c. 1707+5G>A		Intron 15	1	6		4.00	Park *et al.*[8,9]
c. 1743G>C	p. R581S	16		2		1.00	Iwasaki *et al.*[5,18]
c. 1829C>A	p. S610X	17		1		0.50	Tsukamoto *et al.*[2,4]
c. 1970G>A	p. S657N	17		1		0.50	Tsukamoto *et al.*[2,4]
c. 1975G>C	p. V659L	17		3		1.50	Wang *et al.*[7,8]
c. 1997C>T	p. S666F	17		1		0.50	Tsukamoto *et al.*[2,4]
c. 2111ins GCTGG		19		1	1	1.00	Usami *et al.*[2,3]
c. 2162C>T	p. T721M	19		1	1	1.00	Usami *et al.*[2,3]
c. 2168A>G	p. H723R	19	11	40	10	36.00	Usami *et al.*[2,3]
c. 2168C>T	p. H723Y	19	1			1.00	This study
c. 2180T>A	p. L727X	19		1		0.50	This study

[a]c. 1002-9A>G, uncertain pathogenicity.

Mutations in *FOXI1*,[13] a modulatory gene of *SLC26A4*, were not found in our series of patients (data not shown). As seen in previous mutation screening reports, we encountered a significant number of heterozygous cases without a second mutation even after direct sequencing of the coding region of the gene. It is highly likely that there is one more occult mutation somewhere because patients with heterozygous mutation are associated with EVA.

Second, it is evident that the mutation spectrum found in the Japanese population is quite different from that in Caucasoid populations, but similar to the mutation spectrum reported in the Asian populations, especially Koreans.[8–12,14] There are two frequent mutations in east Asians, namely p.H723R and c.919-2A>G. p.H723R is most prevalent in the Japanese and Korean populations,[8] whereas c.919-2A>G is most common in the Chinese.[7]

The existence of a genotype–phenotype correlation is still controversial.[6,12,15] Mutations in *SLC26A4* can cause a broad phenotypic spectrum, from typical Pendred syndrome to nonsyndromic hearing loss associated with EVA. In the present study, various features of the phenotype were compared with the genotypes. We defined nonsense or frame shift mutations as truncating (T) and missense mutations as non-truncating (NT) and classified the genotypes as truncating/truncating (T/T), truncating/non-truncating (T/NT), or non-truncating/non-truncating (NT/NT). However, statistical differences were not found between the groups in any of the clinical features (χ^2 tests, $P > 0.05$; Figure 1).

Concerning the relationship between the severity of hearing loss and individual *SLC26A4* mutations, several functional studies have demonstrated the property of transporter function.[16–18] Furthermore,

Table 2 Phenotypes and genotypes of affected EVA subjects

ID	*Age*	*Mutation allele 1/allele 2*	*Age of awareness*	*Progression*	*Fluctuation*	*Tinnitus*	*Vertigo*	*Goiter*	*Threshold (Rt) (dB)*[a]	*Threshold (Lt) (dB)*[a]	*Hearing level in the low frequencies*[b]
77	12	p.[917delT];[=]	12	+	+	+	+	−	58.75	45	49.375
237	7	p.[T721M];[H723R]	0	+	−	−	+	−	112.5	68.75	83.75
334	23	p.[A372V];[H723R]	0	NA	NA	+	NA	NA	96.25	83.75	81.9
695	4	p.[K369E];[H723R]	0	+	−	NA	NA	−	100	90	89.4
752	18	p.[1652insT];[=]	1	−	−	+	+	+	98.75	102.5	96.3
1045	25	p.[H723R];[H723R]	0	+	NA	−	+	+	78.75	90	85.6
1306	3	p.[919-2A>G];[H723R]	0	NA	NA	NA	NA	NA	NA	NA	NA
1365	20	p.[T721M];[=]	2	NA	NA	NA	NA	NA	96.25	105	96.9
1379	10	p.[1001+1G>A];[H723R]	0	+	+	−	−	NA	66.25	46.25	57.5
1432	6	p.[H723R];[=]	0	+	−	−	−	NA	102.5	105	100.0
1625	16	p.[919-2A>G];[H723R]	0	+	+	NA	+	NA	100	95	88.1
1795	NA	p.[H723R];[=]	NA	NA	N/A	NA	NA	NA	NA	NA	NA
1820	12	p.[H723R];[H723R]	5	+	+	−	−	NA	72.5	73.75	61.3
1957	7	p.[S666F];[H723R]	3	+	+	NA	NA	−	95	101.25	93.8
1961	12	p.[C565Y];[H723R]	0	+	N/A	NA	NA	NA	108.75	110	103.8
2010	12	p.[416-1G>A];[H723R]	9	+	+	−	−	+	80	91.25	81.3
2202	4	p.[P297Q];[T527P]	3	+	−	−	−	−	77.5	76.25	73.8
2331	31	p.[H723R];[H723R]	0	+	+	+	+	+	90	100	87.5
2449	1	p.[139insC];[322delC]	0	NA	NA	−	+	−	100	85	92.5
2462	52	p.[M147V];[H723R]	2	+	+	−	−	−	98.75	95	88.1
2498	0	p.[919-2A>G];[1001+1G>A]	0	+	+	NA	−	−	86.25	86.25	83.8
2538	10	p.[H723R];[H723R]	3	+	+	−	−	+	81.25	55	66.9
2621	3	p.[R581S];[H723R]	0	+	+	−	−	−	91.25	91.25	90.0
2695	13	p.[T527P];[H723R]	2	+	+	+	+	−	62.5	61.25	63.1
2728	3	p.[919-2A>G];[H723R]	1	+	+	−	−	−	97.5	97.5	93.8
2798	15	p.[H723R];[H723R]	4	+	+	NA	+	+	52.5	96.25	66.3
2804	2	p.[1707+5G>A];[H723R]	0	+	+	−	−	−	78.75	78.75	82.5
3072	44	p.[G439R];[H723R]	6	+	+	+	+	−	110	108.75	105.0
3074	21	p.[H723R];[=]	2	+	+	+	+	+	105	106.25	99.4
3298	6	p.[919-2A>G];[H723R]	0	+	+	+	+	−	73.75	110	86.9
3301	4	p.[416-1G>A];[H723R]	0	+	+	+	+	−	65	72.5	68.1
3442	6	p.[919-2A>G];[H723R]	NA	+	NA	+	+	−	81.25	50	60.0
3450	14	p.[H723R];[H723R]	0	+	+	+	+	−	110	73.75	87.5
3561	6	p.[H723Y];[H723Y]	4	NA	NA	NA	NA	NA	83.75	65	71.3
3994	59	p.[601-1G>A];[H723R]	10	+	+	+	+	+	96.0	94	91.3
3996	8	p.[H723R];[1652insT]	0	+	−	+	−	−	100	110	98.1
3999	8	p.[H723R];[1652insT]	0	+	+	−	+	−	30	50	40.0
4050	5	p.[M147V];[H723R]	1	+	+	+	+	−	107.5	85	93.8
4097	3	p.[N392Y];[1002-9A>G]	0	−	−	−	−	−	106.25	85	93.1
4098	26	p.[N392Y];[919-2A>G]	2	−	+	+	+	−	110	37.5	71.3
4102	5	p.[N392Y];[H723R]	0	+	+	+	+	−	95	78.75	83.1
4131	10	p.[H723R];[=]	8	+	+	−	−	−	81.25	60	70.6
4144	21	p.[H723R];[H723R]	4	+	NA	+	+	−	93.75	105	95.6
4232	15	p.[V659L];[H723R]	NA	−	+	+	+	−	60	92.5	69.4
4299	4	p.[S532I];[2111ins GCTGG]	3	−	+	−	+	−	17.5	70	42.5
4305	14	p.[A434T];[=]	0	+	−	+	−	−	110	110	105.0
4320	10	p.[G396E];[S532I]	NA	+	+	+	−	−	72.5	80	72.5
4338	6	p.[R581S];[H723R]	0	+	+	+	+	−	78.75	52.5	64.4
4380	10	p.[1707+5G>A];[H723R]	2	+	+	−	−	−	96.25	81.25	84.4
4386	21	p.[H723R];[H723R]	NA	+	+	+	+	+	77.5	93.75	85.0
4398	4	p.[1652insT];[H723R]	2	+	+	+	+	−	70	97.5	86.9
4434	8	p.[T410M];[1707+5G>A]	1	+	+	−	+	−	92.5	100	91.3
4469	11	p.[H723R];[=]	0	+	NA	−	−	−	20	21.25	16.9
4485	40	p.[H723R];[=]	10	+	+	+	+	−	56.25	65	58.8

Table 2 (Continued)

ID	Age	Mutation allele 1/allele 2	Age of awareness	Progression	Fluctuation	Tinnitus	Vertigo	Goiter	Threshold (Rt) (dB)[a]	Threshold (Lt) (dB)[a]	Hearing level in the low frequencies[b]
4486	20	p. [1707 + 5G > A]; [1707 + 5G > A]	4	+	+	+	+	+	72.5	95	78.1
4490	25	p. [T410M];[T410M]	0	−	−	+	+	+	87.5	92.5	90.0
4508	29	p. [H723R];[H723R]	5	+	+	−	−	−	85	110	91.9
4518	26	p. [H723R];[919-2A > G]	0	+	+	+	+	−	105	97.5	98.1
4530	5	p. [H723R];[919-2A > G]	0	+	+	−	+	−	67.5	86.25	71.9
4545	12	p. [1707 + 5G > A];[H723R]	4	+	+	+	+	+	86.25	28.75	53.1
4549	13	p. [V659L];[1219delCT]	NA	+	+	+	+	−	38.75	50	38.1
4663	0	p. [1707 + 5G > A];[H723R]	0	−	+	NA	NA	−	68.75	68.75	99.2
4696	0	p. [V659L];[H723R]	0	+	−	NA	NA	−	NA	NA	97.5
4362	26	p. [H723R]; [=]	6	+	−	−	−	−	70	68.75	63.8
4513	34	p. [H723R]; [=]	NA	+	+	+	NA	−	71.25	53.75	61.3
4645	23	p. [919-2A > G]; [=]	14	+	−	+	−	−	96.25	105	93.8
723	NA	p. [H723R]; [=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
724	NA	p. [2111ins5bp]; [=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
742	NA	p. [H723R]; [=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
1975	3	p. [H723R];[H723R]	0	NA	NA	NA	NA	NA	80	70	62.5
2082	2	p. [H723R];[H723R]	0	−	−	−	−	−	NA	NA	NA
4735	9	p. [H723R];[919-2A > G]	0	+	+	+	+	−	107.5	110	103.8
195	20	p. [=];[=]	2	+	+	+	+	−	83.75	83.75	81.9
670	8	p. [=];[=]	3	+	−	+	−	−	26.25	107.5	62.5
1755	16	p. [=];[=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
2607	5	p. [=];[=]	0	−	+	−	−	−	97.5	105	98.8
3851	33	p. [=];[=]	0	+	+	+	−	+	103.75	103.75	100.6
4194	11	p. [=];[=]	NA	+	+	−	−	−	67.5	80	76.3
4215	5	p. [=];[=]	0	+	+	−	−	−	98.75	93.75	93.8
4216	55	p. [=];[=]	NA	+	+	+	+	NA	51.25	78.75	68.8
4258	30	p. [=];[=]	28	NA	−	+	−	−	17.5	7.5	13.8
4281	6	p. [=];[=]	2	−	−	−	−	−	57.5	61.25	63.1
4324	37	p. [=];[=]	6	−	−	−	−	−	10	27.5	22.5
4352	3	p. [=];[=]	0	+	+	−	−	−	86.25	88.75	88.1
4357	6	p. [=];[=]	4	+	+	+	−	−	71.25	72.5	67.5
4397	5	p. [=];[=]	0	−	−	−	−	−	102.5	105	100.6
4402	8	p. [=];[=]	0	+	−	−	−	−	100	90	88.8
4450	12	p. [=];[=]	NA	+	+	+	−	−	NA	NA	NA
4462	8	p. [=];[=]	7	+	−	+	−	−	63.75	20	41.3
4488	1	p. [=];[=]	0	−	−	NA	−	−	97.5	97.5	95.0
4671	2	p. [H723R];[600 + 1G > T]	0	+	−	−	+	−	NA	NA	NA
3253	NA	p. [I529S];[H723R]	NA	NA	NA	NA	NA	NA	NA	NA	NA
4949	0	p. [L727X];[H723R]	0	+	−	−	−	−	NA	NA	51.7
J27	NA	p. [H723R];[S448L]	NA	NA	NA	NA	NA	NA	NA	NA	90.6
3309	5	p. [919-2A > G];[P76S]	0	+	+	+	+	−	106.25	106.25	101.3
J15	0	p. [P123S];[H723R]	0	NA	NA	NA	NA	NA	NA	NA	NA
FUK2004	1	p. [H723R];[T94I]	0	NA	NA	N/A	NA	NA	NA	NA	85.0
1299	NA	p. [S610X];[S657N]	0	NA	NA	NA	NA	NA	NA	NA	NA
SNS5500	42	p. [919-2A > G];[919-2A > G]	4	+	+	+	+	+	70	81.3	64
SNS5503	37	p. [H723R];[1707 + 5G > A]	5	+	+	+	+	+	67.5	70	NA

Abbreviation: EVA, enlarged vestibular aqueduct; Lt, left; NA, not available; Rt, right.
[a]Average of 500, 1000, 2000 and 4000 Hz.
[b]Average of 125, 250 and 500 Hz.

retention of improperly folded Pendrin mutants in the endoplasmic reticulum has been suggested as the major pathological mechanism for Pendred syndrome.[19,20] In this study, we compared not only the difference between the T and NT mutations, but also compared the individual mutations and severity of hearing. However, there were no correlations (data not shown). Indeed, there was great variation regarding hearing loss severity even with the same mutations. For example, in the patients homozygous for the most prevalent mutation, p.H723R, hearing level at low frequency varied from 61 to 99 dB (Table 2). In addition, many reports have described intrafamilial

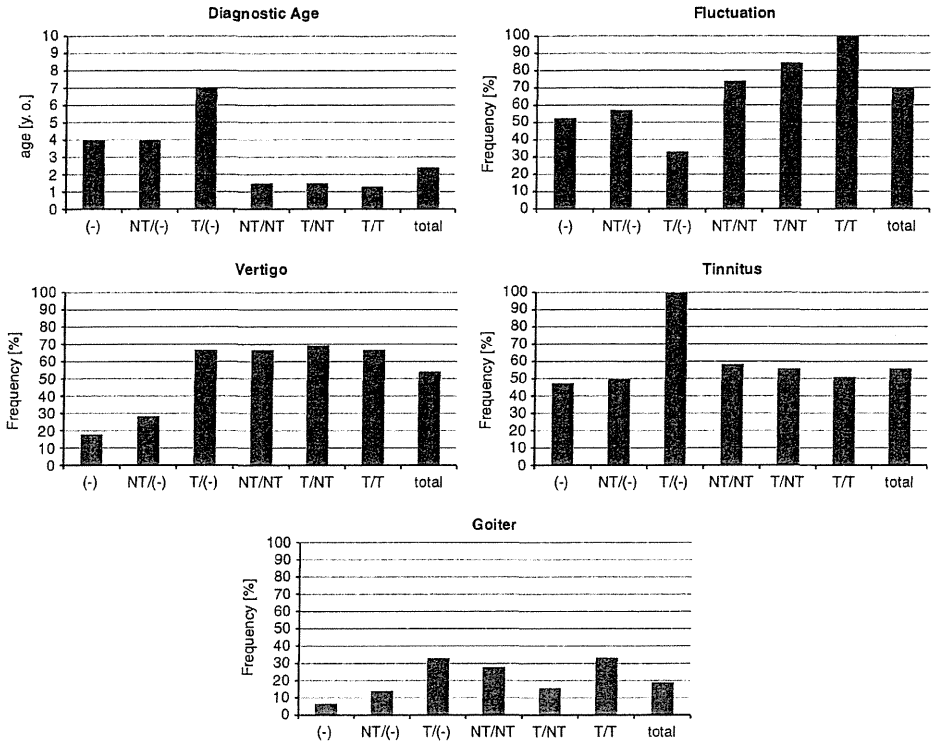

Figure 1 Genotypes and phenotypes (diagnostic age, fluctuation, vertigo, tinnitus and goiter) in the current study. NT/(−), heterozygote of nontruncating mutation; NT/NT, nontruncating/nontruncating; NT/T, nontruncating/truncating; T/(−), heterozygote of truncating mutation; T/T, truncating/truncating; (−), wild type.

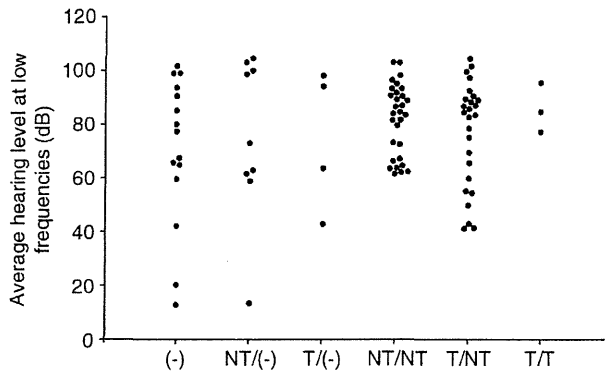

Figure 2 The relationship between hearing level at the lower frequencies and genotype. Hearing level was the average of 125, 250 and 500 Hz. NT/(−), heterozygote of nontruncating mutation; NT/NT, nontruncating/nontruncating; NT/T, nontruncating/truncating; T/(−), heterozygote of truncating mutation; T/T, truncating/truncating; (−), wild type.

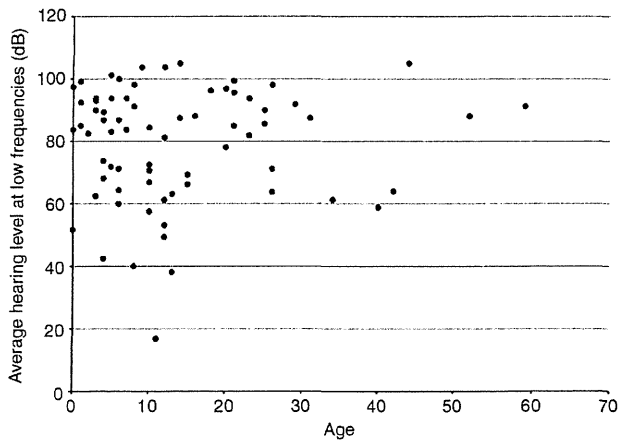

Figure 3 The relationship between hearing level and age in subjects with biallelic *SLC26A4* mutations. Hearing level was calculated as the average of 250, 500, 1000 and 2000 Hz in both sides.

phenotypic variation.[8–12] Therefore, phenotype may be determined not only by *SLC26A4* mutations but also other factors (genetic as well as environmental), contributing to such variability (Figure 2).

Unlike in the case of *GJB2*, phenotype cannot be predicted from the genotype;[6] however, the clarification of clinical features will enable more appropriate genetic counseling and proper medical management for these patients.

The present study confirmed clinical characteristics of 66 patients with EVA caused by biallelic *SLC26A4* mutations. These included congenital (5/63, 7.9%), fluctuated (42/52, 80.8%) and progressive (49/56, 87.5%) hearing loss usually associated with vertigo (35/52, 67.3%) and/or goiter (12/53, 22.6%) during long-term follow-up, in accordance with our previous study.[6] It is known that goiter sometimes becomes apparent between 10 and 20 years of age. The present cohort included young children, and therefore the frequency of goiter may be underestimated. As seen in Figure 3, in 66 patients with biallelic mutations for whom data were available, onset of hearing loss was likely to be early onset, and progressive with age.

CONCLUSIONS

Pendred syndrome and nonsyndromic hearing loss associated with EVA are a continuum of disease characterized as being associated with congenital, fluctuating and progressive hearing loss, and most patients have vertigo and/or goiter. However, in the present study, no genotype–phenotype correlation was found. The results obtained from the present study will facilitate accurate molecular diagnosis and better genetic counseling.

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1 Usami, S., Nishio, S., Nagano, M., Abe, S. & Yamaguchi, T. Deafness Gene Study Consortium. Simultaneous screening of multiple mutations by invader assay improves molecular diagnosis of hereditary hearing loss: a multicenter study. *PLoS One* **7**, e31276 (2012).
2 Tsukamoto, K., Suzuki, H., Harada, D., Namba, A., Abe, S. & Usami, S. Distribution and frequencies of PDS (*SLC26A4*) mutations in Pendred syndrome and nonsyndromic hearing loss associated with enlarged vestibular aqueduct: a unique spectrum of mutations in Japanese. *Eur. J. Hum. Genet.* **11**, 916–922 (2003).
3 Usami, S., Abe, S., Weston, M. D., Shinkawa, H., Van Camp, G. & Kimberling, W. J. Non-syndromic hearing loss associated with enlarged vestibular aqueduct is caused by PDS mutations. *Hum. Genet.* **104**, 188–192 (1999).
4 Namba, A., Abe, S., Shinkawa, H., Kimberling, W. J. & Usami, S. Genetic features of hearing loss associated with ear anomalies: PDS and *EYA1* mutation analysis. *J. Hum. Genet.* **46**, 518–521 (2001).
5 Iwasaki, S., Tsukamoto, K., Usami, S., Misawa, K., Mizuta, K. & Mineta, H. Association of *SLC26A4* mutations with clinical features and thyroid function in deaf infants with enlarged vestibular aqueduct. *J. Hum. Genet.* **51**, 805–810 (2006).
6 Suzuki, H., Oshima, A., Tsukamoto, K., Abe, S., Kumakawa, K., Nagai, K. *et al.* Clinical characteristics and genotype-phenotype correlation of hearing loss patients with *SLC26A4* mutations. *Acta Otolaryngol.* **127**, 1292–1297 (2007).
7 Tsukada, K., Nishio, S. & Usami, S. Deafness Gene Study Consortium. A large cohort study of *GJB2* mutations in Japanese hearing loss patients. *Clin. Genet.* **78**, 464–470 (2010).
8 Wang, Q. J., Zhao, Y. L., Rao, S. Q., Guo, Y. F., Yuan, H., Zong, L. *et al.* A distinct spectrum of *SLC26A4* mutations in patients with enlarged vestibular aqueduct in China. *Clin. Genet.* **72**, 245–254 (2007).
9 Park, H.-J., Lee, S.-J., Jin, H.-S., Lee, J. O., Go, S.-H., Jong, H. S. *et al.* Genetic basis of hearing loss associated with enlarged vestibular aqueducts in Koreans. *Clin. Genet.* **67**, 160–165 (2005).
10 Dai, P., Stewart, A. K., Chebib, F., Hsu, A., Rozenfeld, J., Huang, D. *et al.* Distinct and novel *SLC26A4*/Pendrin mutations in Chinese and U.S. patients with nonsyndromic hearing loss. *Physiol. Genomics* **38**, 281–290 (2009).
11 Albert, S., Blons, H., Jonard, L., Feldmann, D., Chauvin, P., Loundon, N. *et al.* *SLC26A4* gene is frequently involved in nonsyndromic hearing impairment with enlarged vestibular aqueduct in Caucasian populations. *Eur. J. Hum. Genet.* **14**, 773–779 (2006).
12 Pera, A., Villamar, M., Viñuela, A., Gandía, M., Medà, C., Moreno, F. *et al.* A mutational analysis of the *SLC26A4* gene in Spanish hearing-impaired families provides new insights into the genetic causes of Pendred syndrome and DFNB4 hearing loss. *Eur. J. Hum. Genet.* **16**, 888–896 (2008).
13 Yang, T., Vidarsson, H., Rodrigo-Blomqvist, S., Rosengren, S. S., Enerback, S., Smith, R. J. *et al.* Transcriptional control of *SLC26A4* is involved in Pendred syndrome and nonsyndromic enlargement of vestibular aqueduct (DFNB4). *Am. J. Hum. Genet.* **80**, 1055–1063 (2007).
14 Park, H. J., Shaukat, S., Liu, X. Z., Hahn, S. H., Naz, S., Ghosh, M. *et al.* Origins and frequencies of *SLC26A4* (PDS) mutations in east and south Asians: global implications for the epidemiology of deafness. *J. Med. Genet.* **40**, 242–248 (2003).
15 Pryor, S. P., Madeo, A. C., Reynolds, J. C., Sarlis, N. J., Arnos, K. S., Nance, W. E. *et al.* *SLC26A4*/PDS genotype-phenotype correlation in hearing loss with enlargement of the vestibular aqueduct (EVA): evidence that Pendred syndrome and non-syndromic EVA are distinct clinical and genetic entities. *J. Med. Genet.* **42**, 159–165 (2005).
16 Pera, A., Dossena, S., Rodighiero, S., Gandía, M., Bottà, G., Meyer, G. *et al.* Functional assessment of allelic variants in the SLC26A4 gene involved in Pendred syndrome and nonsyndromic EVA. *Proc. Natl Acad. Sci. USA* **105**, 18608–18613 (2008).
17 Yoon, J. S., Park, H. J., Yoo, S. Y., Namkung, W., Jo, M. J., Koo, S. K. *et al.* Heterogeneity in the processing defect of SLC26A4 mutants. *J. Med. Genet.* **45**, 411–419 (2008).
18 Dossena, S., Rodighiero, S., Vezzoli, V., Nofziger, C., Salvioni, E., Boccazzi, M. *et al.* Functional characterization of wild-type and mutated pendrin (SLC26A4), the anion transporter involved in Pendred syndrome. *J. Mol. Endocrinol.* **43**, 93–103 (2009).
19 Taylor, J. P., Metcalfe, R. A., Watson, P. F., Weetman, A. P. & Trembath, R. C. Mutations of the PDS gene, encoding pendrin, are associated with protein mislocalization and loss of iodide efflux: implications for thyroid dysfunction in Pendred syndrome. *J. Clin. Endocrinol. Metab.* **87**, 1778–1784 (2002).
20 Rotman-Pikielny, P., Hirschberg, K., Maruvada, P., Suzuki, K., Royaux, I. E., Green, E. D. *et al.* Retention of pendrin in the endoplasmic reticulum is a major mechanism for Pendred syndrome. *Hum. Mol. Genet.* **11**, 2625–2633 (2002).
21 Coucke, P. J., Van Hauwe, P., Everett, L. A., Demirhan, O., Kabakkaya, Y., Dietrich, N. L. *et al.* Identification of two different mutations in the PDS gene in an inbred family with Pendred syndrome. *J. Med. Genet.* **36**, 475–477 (1999).
22 Coyle, B., Reardon, W., Herbrick, J. A., Tsui, L. C., Gausden, E., Lee, J. *et al.* Molecular analysis of the PDS gene in Pendred syndrome. *Hum. Mol. Genet.* **7**, 1105–1112 (1998).

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RESEARCH ARTICLE Open Access

Pathogenic substitution of IVS15 + 5G > A in *SLC26A4* in patients of Okinawa Islands with enlarged vestibular aqueduct syndrome or Pendred syndrome

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Abstract

Background: Pendred syndrome (PS) and nonsyndromic hearing loss associated with enlarged vestibular aqueduct (EVA) are caused by *SLC26A4* mutations. The Okinawa Islands are the southwestern-most islands of the Japanese archipelago. And ancestral differences have been reported between people from Okinawa Island and those from the main islands of Japan. To confirm the ethnic variation of the spectrum of *SLC26A4* mutations, we investigated the frequencies of *SLC26A4* mutations and clinical manifestations of patients with EVA or PS living in the Okinawa Islands.

Methods: We examined 22 patients with EVA or PS from 21 unrelated families in Okinawa Islands. The patient's clinical history, findings of physical and otoscopic examinations, hearing test, and computed tomography (CT) scan of the temporal bones were recorded. To detect mutations, all 21 exons and the exon–intron junctions of *SLC26A4* were sequenced for all subjects. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for *SLC26A4* and calculations using the comparative CT ($2^{-\Delta\Delta CT}$) method were used to determine the pathogenicity associated with gene substitutions.

Results: *SLC26A4* mutations were identified in 21 of the 22 patients. We found a compound heterozygous mutation for IVS15 + 5G > A/H723R in nine patients (41%), a homozygous substitution of IVS15 + 5G > A in six patients (27%), and homozygous mutation for H723R in five patients (23%). The most prevalent types of *SLC26A4* alleles were IVS15 + 5G > A and H723R, which both accounted for 15/22 (68%) of the patients. There were no significant correlations between the types of *SLC26A4* mutation and clinical manifestations. Based on qRT-PCR results, expression of *SLC26A4* was not identified in patients with the homozygous substitution of IVS15 + 5G > A.

Conclusions: The substitution of IVS15 + 5G > A in *SLC26A4* was the most common mutation in uniquely found in patients with PS and EVA in Okinawa Islands. This suggested that the spectrum of *SLC26A4* mutation differed from main islands of Japan and other East Asian countries. The substitution of IVS15 + 5G > A leads to a loss of *SLC26A* expression and results in a phenotype of PS and EVA.

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Background

Profound hearing loss affects about 1 in 300 to 1 in 1000 newborns [1-4], and about one-half of these cases can be attributed to genetic factors [5]. About 51% of these cases are due to single nucleotide polymorphisms [5]. As to inheritance pattern among monogenic probands, about 1% is X-linked, 22% is autosomal dominant, and 77% is autosomal recessive [5]. Pendred syndrome (PS) is an autosomal recessive disorder characterized by congenital sensorineural hearing loss and goiter [6]. The causative gene for PS and EVA was identified to be *SLC26A4* [7,8]. Enlarged vestibular aqueduct (EVA) is a common inner ear malformation that can be diagnosed radiographically in patients with impaired hearing (Figure 1). EVA is frequently associated with PS [9-11]. In addition to PS, *SLC26A4* mutations also cause nonsyndromic hearing loss with EVA in the absence of a thyroid phenotype [12,13].

Previous studies revealed that the spectrum of *SLC26A4* mutations varied on the basis of ethnic background [14,15]. Tsukamoto et al. [15] demonstrated that *SLC26A4* mutations occurred in 90% of families with a history of PS and in 78% of families with a history of EVA in Japan. Among these *SLC26A4* mutations, H723R was suggested to have a founder effect in the Japanese population.

The Okinawa Islands are the southwestern-most islands of the Japanese archipelago (Figure 2). Previous studies suggested that there were substantial ancestral differences between Okinawa Islands the main islands of Japan [16]. In this study, we examined patients with EVA or PS from the Okinawa Islands to determine the frequencies and the genotypes of *SLC26A4* mutations and their clinical manifestations.

Methods

Subjects

From May 2008 to July 2012, 22 patients (8 males, 14 females; age range: 0–33 years; mean age: 5.8 years; median age: 8.5 years; Table 1) were diagnosed with PS or EVA in the Department of Otorhinolaryngology, Head and Neck Surgery of the University of the Ryukyus, Japan.

Prior to enrollment, all subjects provided a written informed consent. Our research protocol was approved by the Ethical Review Board of the University of the Ryukyus.

Clinical manifestations of PS and EVA

Clinical history of 22 patients with neuro-otologic symptoms was recorded. A physical examination, including otoscopy, hearing level test, computed tomography (CT) scan of the temporal bones, and examination for thyroid goiter was conducted.

Depending on a subject's ability, hearing level was determined using auditory brainstem response, conditioned orientated response, or pure tone audiogram. Hearing level was defined as the average of the hearing threshold at 0.5, 1.0, 2.0, and 4.0 kHz. Hearing was described as: normal, < 20 dB; mild impairment, 21–40 dB; moderate impairment, 41–70 dB; severe impairment, 71–90 dB; and profound impairment, >91 dB.

Neck palpation or echography of the neck was performed in all patients, to determine thyroid goiter. In addition, their serum levels of thyroid-stimulating hormone (TSH) and free thyroxine (FT4) were measured to evaluate thyroid function (normal values: 0.9–1.6 ng/dl and 0.5–5.0 mU/l, respectively). A perchlorate test was not performed.

High-resolution temporal bone CT was performed in all patients to determine if there were any other inner ear malformations in addition to EVA. EVA was defined as a vestibular aqueduct with a diameter of >1.5 mm at the midpoint between the common crus of the semicircular canal and the external aperture of the vestibular aqueduct on CT [17].

Mondini dysplasia was defined when the cochlea consisted of 1.5 turns in which the middle and apical turns had coalesced to form a cystic apex due to the absence of the interscalar septum [18,19].

Vestibular enlargement was defined when the ratio of the membranous vestibule diameter to the inner ear diameter of the lateral semicircular canal was >1.2 [20].

Vertigo was investigated based on spontaneous nystagmus, caloric vestibular test or patients' self-reporting of past episode. The spontaneous nystagmus was evaluated

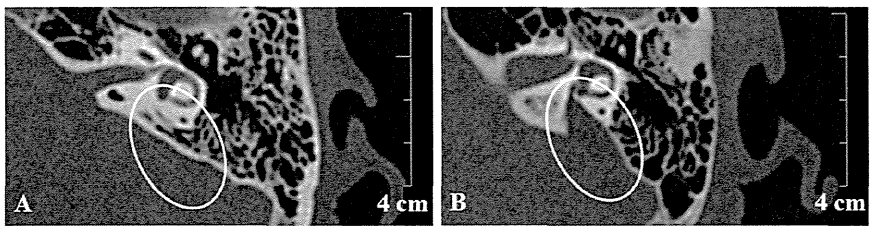

Figure 1 Computed tomography of the temporal bone showing an enlarged vestibular aqueduct. Circles show the vestibular aqueduct. The vestibular aqueduct is not identified in control subject (**A**). The enlarged vestibular aqueduct is identified in a patient with EVA (**B**).

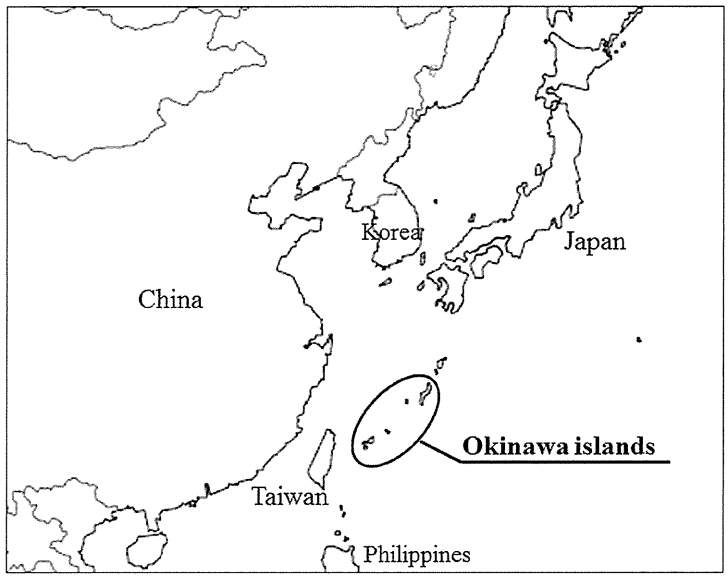

Figure 2 Location of the Okinawa islands in relation to East Asia. The Okinawa islands are located between Taiwan and the Japanese island of Kyushu. The Japanese archipelago comprises Hokkaido, Honshu, Kyusyu, and the Okinawa islands, as well as some smaller islands.

using Frenzel's glass or infrared CCD camera (IRN-1, Morita, Kyoto, Japan).

SLC26A4 genotyping

Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). To detect mutations, all 21 exons and the exon–intron junctions of *SLC26A4* were sequenced for all subjects. A 35 step cycle of Polymerase chain reactions (PCR) was performed as follows: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 40 s, 60°C for 40 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. PCR reactions were run using a programmable thermal cycler (Verti™ 96-Well Thermal Cycler, Applied Biosystems, CA, USA).

PCR products were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, WI, USA) and directly sequenced using an ABI PRISM 3130 × l Genetic Analyzer (Applied Biosystems). The sequences obtained were aligned and compared using the BLAST program with known human genome sequences available in the GenBank database.

We surveyed the substitution IVS15 + 5G > A in 100 healthy objects as control.

The genotype of the IVS15 + 5G > A was detected by digestion of the PCR product with the restriction enzyme SspI (New England Biolabs, Ipswich, MA, U.S.A).

Total RNA isolation and reverse-transcription

Total RNA was isolated from leukocytes using a QIAamp RNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. Before cDNA synthesis, residual DNA was removed by incubation with RNase-free DNase I (Ambion Inc., City, TX, USA). Then, total RNA was reverse transcribed using a TaKaRa Prime Script High Fidelity RT® Kit (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. Possible contaminating genomic DNA in RNA samples was determined by electrophoresis.

Quantitative nested real-time PCR

Nested real-time quantitative (q) PCR was performed to investigate the level of *SLC26A4* expression in the blood.

First-step PCR (conventional PCR)

A conventional PCR assay was performed in a 10 μl reaction mixture that included 2 μl of cDNA, 0.5 units of DNA Taq polymerase (TaKaRa), 2.5 mM deoxynucleotide triphosphates (dNTPs), 1 μM forward and reverse primers for first-step PCR (Table 2), 10 × buffer, and 1.875 mM $MgCl_2$, with distilled water (H_2O) for the final reaction volume of 10 μl. A 33 step cycle of PCR were performed as follows: 94°C for 5 min, 33 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 40 s, and a final extension at 72°C for 5 min.

Second-step PCR (quantitative nested PCR)

Following the first PCR, a second PCR was performed using a set of internal primers (Table 2). The reaction mixture contained 1 μl of the first PCR product (diluted 10-fold), 10 μl of SYBR Premix Ex Taq, and 0.2 μM of the internal forward and reverse primers; the final

Table 1 Summary of clinical features of 22 patients

Age (years)			CT			PTA		Vertigo	Thyroid	
			EVA	MD	VE	HL (dB)	Conductive hearing loss		Goiter	Thyroid function
1	3	R	+	+	+	SO	unknown	-	-	normal
		L	+	+	+	SO	unknown			
2	14	R	+	+	-	105	+	-	+	normal
		L	+	+	-	96	+			
3	21	R	+	+	+	73	+	+	+	normal
		L	+	+	+	91	+			
4	21	R	+	-	-	81	+	+	+	normal
		L	+	-	-	85	+			
5	28	R	+	+	+	96	+	+	+	normal
		L	+	+	+	SO	+			
6	33	R	+	+	-	101	+	+	+	normal
		L	+	+	+	106	+			
7	1	R	+	+	-	SO	unknown	-	-	normal
		L	+	+	+	SO	unknown			
8	1	R	+	-	-	SO	unknown	-	-	normal
		L	+	-	-	103	unknown			
9	2	R	+	+	-	101	unknown	-	-	normal
		L	+	+	-	100	unknown			
10	12	R	+	-	-	95	+	-	+	normal
		L	+	-	-	100	+			
11	29	R	+	+	+	85	+	-	-	
		L	+	+	+	110	+			
12	0	R	+	-	-	55	unknown	+	-	normal
		L	+	-	-	73	unknown			
13	3	R	+	-	+	85	unknown	+	-	normal
		L	+	+	+	58	+			
14	5	R	+	+	+	95	+	+	-	normal
		L	+	+	+	93	+			
15	5	R	+	+	+	103	+	-	-	normal
		L	+	+	+	100	unknown			
16	6	R	+	-	-	81	+	+	-	normal
		L	+	-	-	91	+			
17	7	R	+	-	-	83	+	-	-	normal
		L	+	-	+	81	+			
18	14	R	+	+	+	96	+	-	+	normal
		L	+	+	+	91	+			
19	16	R	+	-	+	91	+	-	+	normal
		L	-	-	+	21	-			
20	26	R	+	-	-	98	+	+	-	normal
		L	+	-	+	103	+			
21	5	R	+	+	+	85	+	-	-	normal
		L	+	+	-	97	+			
22	10	R	+	-	-	53	+	-	-	normal
		L	-	-	-	15	-			

EVA enlarged vestibular aqueduct, *MD* Mondini malformation, *VE* vestibular enlargement, *PTA* pure tone audiogram, *HL* hearing level, *SO* scale out, *NA* no available data.

Table 2 Primer sequences used for nested real-time PCR

Nested PCR assay			Sequence	PCR product size (bp)
First-step PCR (external primer)	Exon 14	forward	TCTTGGAATGGCCTTGGAAGC	282
	Exon 17	reverse	TGAAACAGCATCACTTATGATGC	
Second-step PCR (internal primer)	Exon 15	forward	TGAAGAACCTCAAGGAGTGAAG	154
	Exon 16	reverse	TTTCTGTATTTTCCTCAGCGCT	

reaction volume was adjusted to 20 μl with distilled H_2O. A Light Cycler real-time quantitative PCR system (Roche, Basel Switzerland) was used for amplification and detection of the PCR products. A 40 step cycle of thermal cycler program was performed as follows: denaturation at 95°C for 5 min; 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 40 s; followed by recording the fluorescence values after each elongation step and melting curve analysis with denaturation at 95°C for 5 s, annealing at 65°C for 1 min, and redenaturation by increasing the temperature to 95°C. The second-step PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination. For this analysis, we used three control subjects with no mutations (wild type), three patients compound heterozygous for IVS15 + 5G > A/H723R, and three patients homozygous for IVS15 + 5G > A.

Validation of comparative CT ($2^{-\Delta\Delta CT}$) method and calculations for quantifying *SLC26A4* mRNA

We used the CT ($2^{-\Delta\Delta CT}$) method by assuming approximately equal amplification efficiencies for both target and reference genes. This prerequisite was verified by performing a validation experiment using both *SLC26A4* and a housekeeping gene. Calculations were made using the comparative CT ($2^{-\Delta\Delta CT}$) method. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), *PGK-1* (phosphoglycerate kinase 1), and *ACTB* (actin beta) were used as internal reference genes for PCR normalization with regard to the amount of RNA added to the reverse transcription reactions. Normalized results were expressed as the mean ratio of *SLC26A4* mRNA to *GAPDH* mRNA, *PGK-1* mRNA, and *ACTB* mRNA. To evaluate relative transcript levels, the threshold cycle value (Ct) of each sample was used to calculate and compare the ΔCt of each sample to that of the control subject and patients with a compound heterozygous for IVS15 + 5G > A/H723R, and a homozygous for IVS15 + 5G > A. ΔΔCT was also calculated to compare the transcript levels in the control subject, and patients with a compound heterozygous for IVS15 + 5G > A/H723R, and a homozygous for IVS15 + 5G > A. The transcript levels were calculated in each genotype with three subjects and each subject was calculated in triplicate.

Results

Mutation analysis for *SLC26A4*

By direct DNA sequence analysis, *SLC26A4* mutations were observed in 21 of 22 patients. Among the 21 patients with mutations, a compound heterozygous mutation for IVS15 + 5G > A/H723R was identified in nine patients (Figure 3C, D), a homozygous mutation for H723R was identified in five patients (Figure 3E), and a homozygous substitution of IVS15 + 5G > A was identified in six patients (Figure 3F). A compound heterozygous substitutions for IVS15 + 5G > A/T527P was identified in one subject. We could not identify any *SLC26A4* mutations in one subject (Table 3). We could not find the substitution IVS15 + 5G > A in 100 control objects.

Clinical characteristics

Table 1 summarizes the clinical characteristics of all 22 subjects. High-resolution temporal bone CT scans revealed that bilateral EVA was present in 20 patients and unilateral EVA was present in other two. Mondini dysplasia and vestibular enlargement was observed in 17 ears (17/44; 39%) and 22 ears (22/44; 50%), respectively.

Hearing loss grades in the affected ears ranged from moderate to profound in the patients with EVA (Table 1). The hearing levels of the two unaffected ears were normal and mild hearing loss, respectively. Table 4 shows the hearing level distributions based on genotypes. No significant differences were expected in the distributions for hearing level among the five genotype groups due to the small sample of only 22 patients.

Neck examinations revealed thyroid goiters in 8 of 22 patients. Overall, 0% (0/11) and 73% (8/11) of the patients younger and older than 10 years of age, respectively, had a thyroid goiter. Their serum FT4 and TSH levels were within the normal ranges. There is no relation between occurrence of goiter and mutation genotypes.

SLC26A4 expression in patients with IVS15 + 5G > A

Electrophoretic separation of the real-time PCR products did not exhibit any bands in patients with the homozygous substitution for IVS15 + 5G > A (Figure 4C).

Because the *SLC26A4* expression levels were not high in blood samples, we investigated its expression using nested real-time qPCR for three control subjects, three

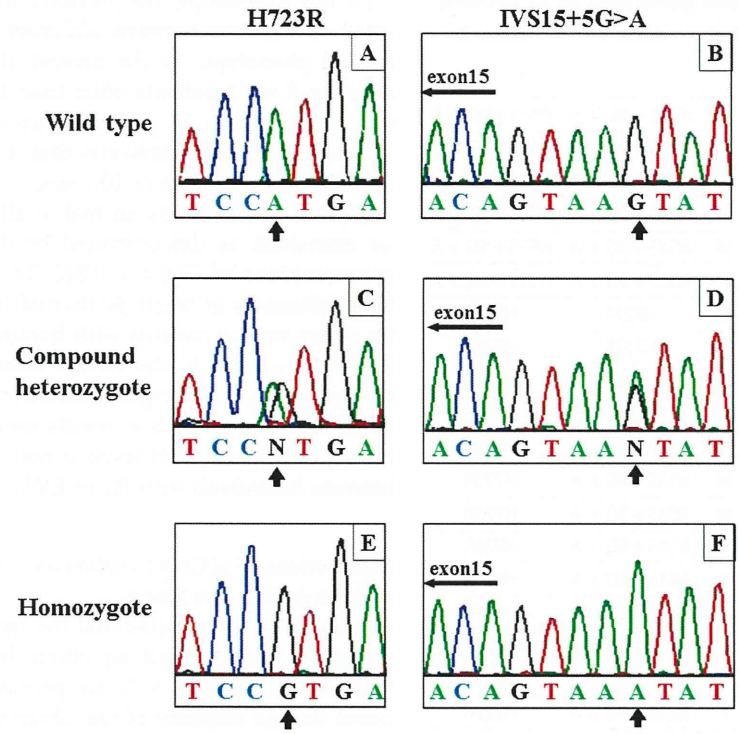

Figure 3 Examples of direct sequence analysis of the *SLC26A4* gene. Representative results of H723R and the IVS15 + 5G > A mutation analysis are shown. Genomic sequences of the *SLC26A4* gene in normal individuals (**A**), (**B**). A compound heterozygous mutation for IVS15 + 5G > A/H723R (**C**), (**D**). A homozygous mutation for H723R (**E**). A homozygous substitution of IVS15 + 5G > A (**F**). The arrows indicate the variant nucleotide.

patients with the compound heterozygous mutation for IVS15 + 5G > A/H723R, and three patients with the homozygous substitution for IVS15 + 5G > A. The control subjects had normal hearing without any malformations of the inner or middle ear and no family history of hearing loss. After obtaining a written informed consent, blood samples were collected from each subject and were subjected to Real-time PCR with SYBR Green and the expression level was evaluated using the comparative CT ($2^{-\Delta\Delta CT}$) method. The relative *SLC26A4* expression levels in the control no.1, control no.2 and control no.3 with no *SLC26A4* mutations were 9089 ± 441.5 (standard deviation), 2417 ± 189.5, and 4956 ± 260.4 respectively. In patient no.12, patient no.14 and patient no.16 with a compound heterozygous mutation for IVS15 + 5G > A/H723R were 979.5 ± 79.12, 2846 ± 206.5 and 1183 ± 33.93 respectively. In patient no.1, patient no.2 and patient no.4 with a homozygous substitution for IVS15 + 5G > A were $1.96 \times 10^{-4} \pm 7.66 \times 10^{-5}$, $5.76 \times 10^{-5} \pm 3.37 \times 10^{-6}$ and $4.35 \times 10^{-5} \pm 8.09 \times 10^{-6}$ respectively (Figure 5).

Based on the results of both electrophoresis and RT-nested qPCR, no *SLC26A4* expression was observed in patients with homozygous substitution of IVS15 + 5G > A.

Discussion

Correlations between *SLC26A4* genotypes and hearing phenotypes

Hearing loss in patients with EVA and PS is usually apparent at the pre- or perilingual stage [6,21]. Hearing loss in EVA and PS is sensorineural with some mixed hearing loss in the low-frequency range [22-27]. The hearing level sometimes deteriorates suddenly and may be followed by a partial recovery, such as with fluctuating hearing loss [28,29]. In our study, hearing loss was detected at the pre- or perilingual stage in all cases except for two cases of unilateral EVA. However, in all cases, hearing levels eventually deteriorated to severe or profound loss (Table 1) and were permanent with or without hearing fluctuation or stepwise hearing deterioration. No significant differences were observed in the hearing levels among the five genotypes (Table 4).

Correlations between *SLC26A4* genotypes and thyroid phenotype

SLC26A4 encodes for the 86 kDa transmembrane protein pendrin [7,30]. In the thyroid, this protein acts as co-transporter of chloride and iodine in the thyroid

Table 3 Distribution of *SLC26A4* genotypes of 22 patients

	Age at onset of hearing loss (years)	Age at genetic test (years)	Sex	Allele 1	Allele 2
1	0	3	M	IVS15 + 5G > A	IVS15 + 5G > A
2	2	14	F	IVS15 + 5G > A	IVS15 + 5G > A
3	3	21	F	IVS15 + 5G > A	IVS15 + 5G > A
4	2	22	F	IVS15 + 5G > A	IVS15 + 5G > A
5	0	23	M	IVS15 + 5G > A	IVS15 + 5G > A
6	0	29	F	IVS15 + 5G > A	IVS15 + 5G > A
7	0	1	F	H723R	H723R
8	1	1	F	H723R	H723R
9	4	2	M	H723R	H723R
10	0	12	F	H723R	H723R
11	5	29	M	H723R	H723R
12	0	0	M	IVS15 + 5G > A	H723R
13	2	3	M	IVS15 + 5G > A	H723R
14	0	5	F	IVS15 + 5G > A	H723R
15	1	5	F	IVS15 + 5G > A	H723R
16	0	6	F	IVS15 + 5G > A	H723R
17	2	7	F	IVS15 + 5G > A	H723R
18	2	14	F	IVS15 + 5G > A	H723R
19	7	16	F	IVS15 + 5G > A	H723R
20	5	26	M	IVS15 + 5G > A	H723R
21	1	5	M	H723R	T527P
22	7	10	F	ND	ND

ND not determined.

[31,32]. In PS patients, a mutation in *SLC26A4* results in reduced pendrin-induced chloride and iodide transport and, ultimately, goiter [33].

Goiter usually develops around the end of the first decade of life or during young adulthood, although the time of onset and severity vary considerably among patients [12,34], and even within families [35]. Despite an impaired incorporation of iodide, most patients with PS are clinically and biochemically euthyroid [21,34,36].

To our knowledge, no previous studies have investigated correlations between *SLC26A4* genotypes and the thyroid phenotype. In the present study, PS was diagnosed in 8 of 11 patients older than 10 years of age, but not in any of the 11 patients who were younger than 10 years of age. This indicates that it is difficult to diagnose PS before the age of 10 years.

Thyroid function was normal in all of the 21 patients we examined, as demonstrated by their normal serum concentrations of FT4 and TSH. There were no significant differences in serologic thyroid test results and goiter status among patients with homozygous substitution for IVS15 + 5G > A, the H723R homozygous mutation, or compound heterozygous mutation for IVS15 + 5G > A/H723R. Therefore, our results indicate that serologic testing of FT4 and TSH levels is not useful to distinguish between individuals with PS or EVA.

Distributions of *SLC26A4* mutations in EVA and PS patients in Okinawa Islands

It was previously reported that the spectrum of *SLC26A4* mutations varied based on ethnic background [35,36]. H723R and IVS7-2A > G are prevalent alleles that account for the majority of the observed *SLC26A4* mutations in East Asian populations [35]. In the Japanese population, H723R was the most common mutation [15,36,37]. In Chinese and Taiwanese populations, IVS7-2A > G was the most common mutation [38-40], whereas in the Korean population, H723R and IVS7-2A > G were the most frequent and accounted for 60.2% (47/78) and 30.7% (24/78) of the mutated alleles, respectively [41].

Ancestral differences have been reported between people from Okinawa Islands and those from the main islands of Japan based on single-nucleotide polymorphism genotypes [16]. We analyzed *SLC26A4* mutations among 22 patients with EVA or PS from 21 unrelated families. H723R have been reported as the most common mutation found in the main islands of Japan. As with H723R mutation, IVS15 + 5G > A substitution was

Table 4 Clinical features in different genotype groups

Genotype	Hearing level					CT		Vertigo
	Normal	Mild	Moderate	Severe	Profound	MD	VE	
IVS15 + 5 G > A homozygous (n = 6)	0	0	0	3	9	6/12	6/12	4/6
H723R homozygous (n = 5)	0	0	0	1	9	4/10	3/10	0/5
IVS15 + 5 G > A/H723R (n = 9)	0	1	2	4	11	5/18	11/18	4/9
IVS15 + 5G > /T527P (n = 1)	0	0	0	1	1	2/2	1/2	0/1
No mutation (n = 1)	1	0	1	0	0	0/2	0/2	0/1
Subtotal	1	1	3	9	30	17/44	21/44	8/22
Total	44							

Normal: ≤20 dB; Mild: 21–40 dB; Moderate: 41–70 dB; Severe: 71–90 dB; Profound: >91 dB.
MD Mondini malformation, *VE* Vestibular enlargement, *CT* computed tomography.

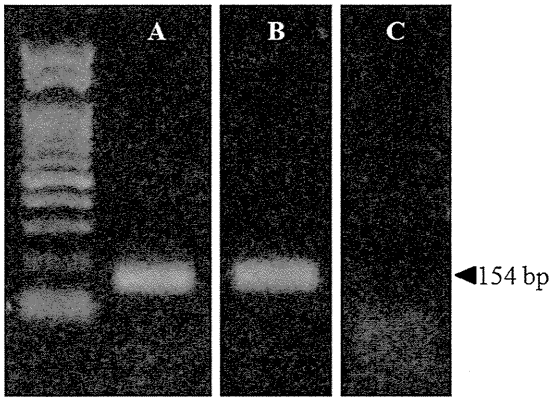

Figure 4 Expression of the *SLC26A4* gene in patients with PS or EVA. The expected RT-nested PCR amplification product of *SLC26A4* was 154 base pairs (bp) in length. Agarose gel electrophoresis shows the 154 bp band for the control subject (**A**) and the patient with IVS15 + 5G > A/H723R compound heterozygous mutation (**B**); however, there was no band for the patient with IVS15 + 5G > A homozygous substitution (**C**).

also identified most frequently in 15 of 22 of our Okinawa patients. The substitution of IVS15 + 5G > A in one allele have been reported only 10 cases in Asian populations [36,42-45]. Thus, IVS15 + 5G > A was the characteristic *SLC26A4* gene mutation among patients in Okinawa Islands, indicating a difference in the spectrum of *SLC26A4* mutations among patients in Okinawa Islands compared with patients in other

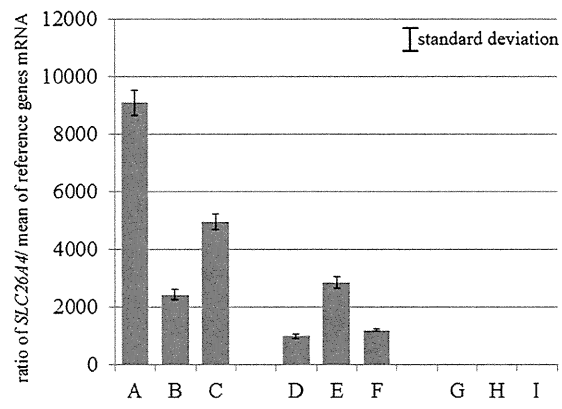

Figure 5 Relative expression of the *SLC26A4* gene in control subjects and in patients with a homozygous mutation of IVS15 + 5G > A or compound heterozygous mutation of IVS15 + 5G > A/H723R. The ratio of SLC26A4 mRNA to GAPDH mRNA is shown in three control subjects (**A**, **B**, **C**), three patients with compound heterozygous mutation of IVS15 + 5G > A/H723R (**D**, **E**, **F**), and three patients with IVS15 + 5G > A homozygous substitution (**G**, **H**, **I**). No expression of *SLC26A4* was observed in the three patients with the IVS15 + 5G > A homozygous substitution (**G**, **H**, **I**). All experiments were done in tripricate.

populations. These results suggest that this *SLC26A4* mutation may have originated from a common ancestor.

Pathogenic effect of IVS15 + 5G > A substitution

The heterozygous substitution of IVS15 + 5G > A has been assumed to cause aberrant splicing [36,42-45]. However, Yang et al. [42] could not find any abnormal RT-PCR products related to the size for SLC26A4 sequence analysis in patients with splice mutation. Because its pathogenicity was only implicated on the basis of uncommon polymorphisms, the pathogenic potential of IVS15 + 5G > A still remains unknown.

Substitutions near the canonical splice sites are difficult to classify as pathogenic or non-disease causing. Because such substitutions affect proper RNA splicing but some substitutions do not cause any effect [46-48]. Thus, it is important to determine the pathogenic effect of a particular substitution near the donor site by mRNA analysis [48]. We investigated *SLC26A4* expression in patients with compound heterozygous mutation for IVS15 + 5G > A/H723R and homozygous substitution for IVS15 + 5G > A by RT-PCR and RT-real time PCR by targeting genes around these mutations. No aberrant PCR products were detected in the patient with heterozygous substitution of IVS15 + 5G > A (Figure 4B), which suggests that IVS15 + 5G > A does not cause aberrant splicing, as also argued by Yang et al. However, in patients with the homozygous substitution of IVS15 + 5G > A, *SLC26A4* was not expressed, as shown in Figure 4. In addition, for patients with the heterozygous substitution, *SLC26A4* expression was reduced from the normal control level. These findings suggest that IVS15 + 5G > A disrupts pre-mRNA splicing and causes the loss of *SLC26A4* expression. The patients in Yang et al. [42] were heterozyote so that Yang et al. [42] most likely amplified the non-mutated allele. Taken together, our results indicate that the substitution of IVS15 + 5G > A is a loss-of-function mutation caused by a loss of *SLC26A4* expression.

Conclusions

We found no correlations between the type of *SLC26A4* mutation and hearing levels or the thyroid phenotype. Moreover, thyroid testing using serum FT4 and TSH levels was not useful for distinguishing between individuals with PS and EVA.

The substitution of IVS15 + 5G > A in the *SLC26A4* was unique and the most common in PS and EVA patients from Okinawa Islands. This supports that the spectrum of *SLC26A4* mutations differs by geographic area in East Asia. Our qPCR results for *SLC26A4* indicate that the substitution of IVS15 + 5G > A should be a pathogenic mutation that leads to a loss of *SLC26A4* expression and results in a phenotype of PS and EVA.

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

Authors' contributions
AG diagnosed the patients, collected clinical data, performed the experiments, and wrote the manuscript. TK, KY, and SU carried out data analysis. KN, TT, and MS edited the manuscript and supervised the project. All authors read and approved the final manuscript.

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References

1. Downs MP: **Universal newborn hearing screening–the Colorado story.** *Int J Pediatr Otorhinolaryngol* 1995, **32**:257–259.
2. Mehl AL, Thomson V: **Newborn hearing screening: the great omission.** *Pediatrics* 1998, **101**:E4.
3. Mehl AL, Thomson V: **The Colorado newborn hearing screening project, 1992–1999: on the threshold of effective population-based universal newborn hearing screening.** *Pediatrics* 2002, **109**:E7.
4. Bitner-Glindzicz M: **Hereditary deafness and phenotyping in humans.** *Br Med Bull* 2002, **63**:73–94.
5. Morton NE: **Genetic epidemiogy of hearing impairment.** *Ann NY Acad Sci* 1991, **630**:16–31.
6. Pendred V: **Deaf-mutation and goiter.** *Lancet* 1896, **2**:532.
7. Everett L, Glaser B, Beck J, Idol J, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Baxevanis A, Sheffield V, Green E: **Pendred syndrome is caused by mutations in a putative sulphate transporter gene (*PDS*).** *Nat Genet* 1997, **17**:411–422.
8. Li XC, Everett LA, Lalwani AK, Desmukh D, Friedman TB, Green ED, Wilcox ER: **A mutation in PDS causes non-syndromic recessive deafness.** *Nat Genet* 1998, **18**:215–217.
9. Johnsen T, Jørgensen MB, Johnsen S: **Mondini cochlea in Pendred's syndrome.** *Acta Otolaryngol* 1986, **102**:239–247.
10. Nakagawa O, Ito S, Hanyu O, Yamazaki M, Urushiyama M: **Female siblings with Pendred's syndrome.** *Int Med* 1994, **33**:369–372.
11. Reardon W, Trembath RC: **Pendred syndrome - 100 years of underascertainment?** *Q J Med* 1997, **90**:443–447.
12. Campbell C, Cucci RA, Prasad S, Green GE, Edeal JB, Galer CE, Karniski LP, Sheffield VC, Smith RJ: **Pendred syndrome, DFNB4, and PDS/SLC26A4 identification of eight novel mutation and possible genotype–phenotype correlations.** *Hum Mutat* 2001, **17**:404–411.
13. Usami S, Abe S, Weston MD, Shinkawa H, Camp GV, Kimberling WJ: **Non-syndromic hearing loss associated with enlarged vestibular aqueduct is caused by PDS mutations.** *Hum Genet* 1999, **104**:188–192.
14. Park HJ, Shaukat S, Liu XZ, Hahn SH, Naz S, Ghosh M, Kim HN, Moon SK, Abe S, Tsukamoto K, Riazuddin S, Kabra M, Erdenetungalag R, Radnaabazar J, Khan S, Pandya A, Usami S-I, Nance WE, Wilcox ER, Riazuddin S, Griffith AJ: **Origins and frequencies of SLC26A4 (PDS) mutations in east and south Asians: Global implications for the epidemiology of deafness.** *J Med Genet* 2003, **40**:242–248.
15. Tsukamoto K, Suzuki H, Harada D, Namba A, Abe S, Usami S: **Distribution and frequencies of PDS (SLC26A4) mutations in Pendred syndrome and nonsyndromic hearing loss associated with enlarged vestibular aqueduct: a unique spectrum of mutations in Japanese.** *Eur J Hum Genet* 2003, **11**:916–922.
16. Japanese Archipelago Human Population Genetics Consortium, Jinam T, Nishida N, Hirai M, Kawamura S, Oota H, Umetsu K, Kimura R, Ohashi J, Tajima A, Yamamoto T, Tanabe H, Mano S, Suto Y, Kaname T, Naritomi K, Yanagi K, Niikawa N, Omoto K, Tokunaga K, Saitou N: **The history of human populations in the Japanese Archipelago inferred from genome-wide SNP data with a special reference to the Ainu and the Ryukyuan populations.** *J Hum Genet* 2012, **57**:787–795.
17. Valvassori GE, Clemis JD: **The large vestibular aqueduct syndrome.** *Laryngoscope* 1978, **88**:723–728.
18. Phelps PD: **The basal turn of the cochlea.** *Br J Radiol* 1992, **65**:370–374.
19. Goldfeld M, Glaser B, Nassir E, Gomori JM, Hazani E, Bishara N: **CT of the ear in pendred syndrome.** *Radiology* 2005, **235**:537–540.
20. Davidson HC, Harnsberger HR, Lemmerling MM, Mancuso AA, White DK, Tong KA, Dahlen RT, Shelton C: **MR evaluation of vestibulocochlear anomalies associated with large endolymphatic duct and sac.** *AJNR Am J Neuroradiol* 1999, **20**:1435–1441.
21. Fraser GR: **Association of congenital deafness with goiter (Pendred's syndrome): a study of 207 families.** *Ann Hum Genet* 1965, **28**:201–249.
22. Valvassori GE: **The large vestibular aqueduct and associated anomalies of the inner ear.** *Otolaryngol Clin North Am* 1983, **16**:95–101.
23. Jackler RK, de la Cruz A: **The large vestibular aqueduct syndrome.** *Laryngoscope* 1989, **99**:1238–1243.
24. Levenson MJ, Parisier SC, Jacobs M, Edelstein DR: **The large vestibular aqueduct syndrome in children.** *Arch Otolaryngol Head Neck Surg* 1989, **115**:54–58.
25. Arcand P, Desrosiers M, Dube J, Abela A: **The large vestibular aqueduct syndrome and seisorineural hearing loss in the pediatric population.** *J Otolaryngol* 1991, **20**:247–250.
26. Belenky WM, Madgy DN, Leider JS, Becker C, Hotaling AJ: **The enlarged vestivular aqueduct syndrome (EVA syndrome).** *ENT J* 1993, **72**:746–751.
27. Okumura T, Takahashi H, Honjo I, Takagi A, Mitamura K: **Sensorineural hearing loss in patients with large vestibular aqueduct.** *Laryngoscope* 1995, **105**:289–294.
28. Stinckens C, Huygen PL, Joosten FB, Van Camp G, Otten B, Cremers CW: **Fluctuant, progressive hearing loss associated with Meniere like vertigo in three patients with the Pendred syndrome.** *Int J Pediatr Otorhinolaryngol* 2001, **61**:207–215.
29. Cremers CW, Admiraal RJ, Huygen PL, Bolder C, Everett LA, Joosten FB, Green ED, van Camp G, Otten BJ: **Progressive hearing loss, hypoplasia of the cochlea and widened vestibular aqueducts are very common features in Pendred's syndrome.** *Int J Pediatr Otorhinolaryngol* 1998, **45**:113–123.
30. Dossena S, Rodighiero S, Vezzoli V, Nofziger C, Salvioni E, Boccazzi M, Grabmayer E, Botta G, Meyer G, Fugazzola L, Beck-Peccoz P, Paulmichl M: **Functional characterization of wildtype and mutated pendrin (SLC26A4), the anion transporter involved in Pendred syndrome.** *J Mol Endocrinol* 2009, **43**:93–103.
31. Fugazzola L, Cerutti N, Mannavola D, Vannucchi G, Beck-Peccoz P: **The role of pendrin in iodide regulation.** *Exp Clin Endocr Diab* 2001, **109**:18–22.
32. Scott D, Wang R, Kreman T, Sheffield V, Karniski L: **The Pendred syndrome gene encodes a chloride-iodide transport protein.** *Nat Genet* 1999, **21**:440–443.
33. Scott DA, Wang R, Kreman TM, Andrews M, McDonald JM, Bishop JR, Smith RJH, Karnishki LP, Sheffield VC: **Functional differences of the PDS gene product are associated with phenotypic variation in patients with Pendred syndrome and non-syndromic hearing loss (DFNB4).** *Hum Mol Genet* 2000, **9**:1709–1715.
34. Reardon W, Coffey R, Chowdhury T, Grossman A, Jan H, Britton K, Kendall-Taylor P, Trembath R: **Prevalence, age of onset, and natural history of thyroid disease in Pendred syndrome.** *J Med Genet* 1999, **36**:595–598.
35. Napiontek U, Borck G, Muller-Forell W, Pfarr N, Bohnert A, Keilmann A, Pohlenz J: **Intrafamilial variability of the deafness and goiter phenotype in Pendred syndrome caused by a T416P mutation in the SLC26A4 gene.** *J Clin Endocrinol Metab* 2004, **89**:5347–5351.
36. Iwasaki S, Tsukamoto K, Usami S, Misawa K, Mizuta K, Mineta H: **Association of SLC26A4 mutation with clinical features and thyroid function in deaf infants with enlarged vestibular aqueduct.** *J Hum Genet* 2006, **51**:805–810.
37. Kitamura K, Takahashi K, Noguchi Y, Kuroishikawa Y, Tamagawa Y, Ishikawa K, Ichimura K, Hagiwara H: **Mutations of the Pendred syndrome gene (PDS) in patients with large vestibular aqueduct.** *Acta Otolaryngol* 2000, **120**:137–141.