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ARTICLE

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

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Molecular diagnosis makes a substantial contribution to precise diagnosis, subclassification, prognosis, and selection of therapy. Mutations in the *PDS (SLC26A4)* gene are known to be responsible for both Pendred syndrome and nonsyndromic hearing loss associated with enlarged vestibular aqueduct, and the molecular confirmation of the *PDS* gene has become important in the diagnosis of these conditions. In the present study, *PDS* mutation analysis confirmed that *PDS* mutations were present and significantly responsible in 90% of Pendred families, and in 78.1% of families with nonsyndromic hearing loss associated with enlarged vestibular aqueduct. Furthermore, variable phenotypic expression by the same combination of mutations indicated that these two conditions are part of a continuous category of disease. Interestingly, the *PDS* mutation spectrum in Japanese, including the seven novel mutations revealed by this study, is very different from that found in Caucasians. Of the novel mutations detected, 53% were the H723R mutation, suggesting a possible founder effect. Ethnic background is therefore presumably important and should be noted when genetic testing is being performed. The *PDS* gene mutation spectrum in Japanese may be representative of those in Eastern Asian populations and its elucidation is expected to facilitate the molecular diagnosis of a variety of diseases.

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Introduction

The Pendred syndrome is defined as an autosomal recessive disorder characterized by congenital sensorineural hearing loss and goiter.[1] It is supposed that Pendred syndrome, the most common form of syndromic deafness, is as frequent as 7.5–10 per 100 000 persons and accounts for about 10% of hereditary hearing impairment.[2,3] In spite of such a high frequency, the precise clinical diagnosis has long been hampered by the variable phenotypes, the degree of involvement of goiter, appearance from congenital to complete absence, and the perchlorate discharge test

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which is nonspecific to Pendred syndrome.[3] However, the recent identification of the *PDS* gene[4] as being responsible for Pendred syndrome enables us to perform molecular evaluation of this syndrome. Simultaneously, the *PDS* gene has also been reported to cause 'nonsyndromic hearing loss with enlarged vestibular aqueduct (EVA)',[5] suggesting that the *PDS* gene may cover a wider range of diseases from typical Pendred syndrome to nonsyndromic hearing loss with EVA. Therefore, molecular genetic testing is becoming more important to diagnose this category of disease caused by the *PDS* gene.

The frequencies and nature of mutations are known to be influenced by ethnic differences and geographic isolation. Although much is known regarding the association of *PDS* mutations with deafness in populations of European descent, data about the ethnic groups that form the majority of the world's population is scarce. In the present study, screening of *PDS* mutations was carried out to confirm their contribution to the deafness in patients diagnosed with Pendred syndrome or nonsyndromic hearing loss with EVA, and to reveal the spectrum of the *PDS* gene in Japanese.

Materials and methods

A total of 10 Japanese families, in which some individuals had been diagnosed with Pendred syndrome, and 32 Japanese families, in which some individuals had bilateral sensorineural hearing loss associated with EVA but without goiter, participated in the current study. We defined Pendred syndrome patients as those having either a palpable goiter or abnormal perchlorate discharge. Computerized tomography scan was used to diagnose EVA patients (according to the criteria of EVA: a diameter greater than 1.5 mm at the midpoint between the common crus and the external aperture), and they were clinically well characterized by repeated auditory examinations and long follow-up periods. Briefly, the EVA patients had congenital, high frequency involved, fluctuating, sometimes progressive, sensorineural hearing loss.[6,7] There were no cases of Mondini malformation (cochlear hypoplasia) of the inner ear in the current subjects.

DNA samples from 96 unrelated Japanese, who had normal hearing, were used as controls. All participants gave informed consent for genetic analysis.

Mutation analysis

Intronic polymerase chain reaction (PCR) amplification primers flanking each exon[4] were used to detect mutations. Exons 1–21 of *PDS* were amplified from genomic DNA samples by PCR. A 5-min denaturation at 95°C was followed by 37 three-step cycles (95°C for 30 s, 55°C for 1 min, 72°C for 1 or 3 min), followed by 72°C for 10 min, and ending with a holding period at 4°C in a Perkin-Elmer thermal cycler. The PCR products were directly sequenced after removal of unincorporated dNTPs and primers by incubation at 37°C for 30 min with 50–100 ng PCR product with 0.1 μl exonuclease I (Amersham Life Science, Cleveland, USA) and 1 μl shrimp alkaline phosphatase (Amersham Life Science). The enzymes were heat-inactivated at 80°C for 15 min. An aliquot of 6 pmol of either the forward or the reverse primer was used in standard cycle sequencing reactions with ABI Big Dye terminators, and run on an ABI 377 sequencer.

Results

Table 1[5,8–23] summarizes all reported *PDS* mutations including the 18 mutations detected in the affected families in this study, which were 10 missense mutations, one stop mutation, four frameshift mutations, and three splice site mutations. The evolutionary conservation of the amino acids affected by the missense mutations, on the basis of the alignment of the closely related mouse pendrin protein and rat pendrin protein, are shown in Table 1 and Figure 1. All 10 missense mutations had occurred at the conservative residues among humans, mice, and rats (Figure 1). Mutations were identified in nine out of the 10 typical Pendred families (90%) and 25 out of the 32 families with sensorineural hearing loss associated with EVA (78.1%). Mutations were either homozygous, compound heterozygous, or heterozygous but with no other mutations detectable (Table 2).

Missense mutations

The 10 missense mutations detected were: P123S (367C>T in exon 4); M147V (439A>G in exon 5); K369E (1105A>G in exon 9); A372V (1115C>T in exon 9); N392Y (1174A>T in exon 10); C565Y (1694G>A in exon 15); S657N (1970G>A in exon 17); S666F (1997C>T in exon 17); T721M (2162C>T in exon 19), and H723R (2168A>G in exon 19).

Stop mutation

An 1829C>A substitution was found in exon 17, leading to S610X.

Frameshift mutations

Four frameshift mutations were detected: 322delC in exon 4 causing a frameshift at codon 108 and leading to a stop codon at position 139; 917delT in exon 7 causing a frameshift at codon 307 and leading to a stop codon at position 308; 1652insT in exon 15 causing a frameshift at codon 551 and leading to a stop codon at position 556; and a small 2111 insertion of GCTGC in exon 19 causing a frameshift leading to an amino-acid sequence change from codon 704, followed by a stop at codon 722.

Table 1 All reported PDS mutations (including the current ones)

Exon	*Nucleotide change*	*Amino-acid change*	*Japanese*	*Evolutionary conservation*	*Reference*
2	85G>C	E29Q		Yes	Campbell *et al*[8]
3	IVS2-2A>G	Splice acceptor		NA	Lopez-Bigas *et al*[9]
3	279delT	Stop at 96		NA	Kopp *et al*[10]
4	314A>G	Y105C		Yes	Campbell *et al*[8]
4	317C>A	A106D		Yes	Campbell *et al*[8]
4	322delC	Stop at 139	○	NA	This study
4	336insT	Stop at 180		NA	Coyle *et al*[11]
4	349C>T	L117F		Yes	Reardon *et al*[12]
4	367C>T	P123S	○	Yes	This study
4	395C>T	T132I		Yes	Lopez-Bigas *et al*[9]
4	406delTCTCA	Stop at 179		NA	Lopez-Bigas *et al*[9]
4	412G>T	*z*138z		Yes	Van Hauwe *et al*[13]
4	IVS4+7A>G	Splice donor		NA	Lopez-Bigas *et al*[9]
5	416G>C	G139A		Yes	Van Hauwe *et al*[13]
5	439A>G	M147V	○	Yes	This study
5	580C>T	T193I		Yes	Adato *et al*[15]
6	IVS5-1G>A	Splice acceptor	○	NA	This study
6	626G>T	G209V		Yes	Van Hauwe *et al*[13]
6	707T>C	L236P		Yes	Van Hauwe *et al*[13]
6	753delCTCT	Stop at 286		NA	Coyle *et al*[11]
6	754T>C	S252P		Yes	Park *et al*[16]
7	IVS6-2A>G	Splice acceptor		NA	Coucke *et al*[17]
7	783insT	Stop at 286		NA	Campbell *et al*[8]
7	811G>C	D271H		Yes	Van Hauwe *et al*[13]
7	917delT	Stop at 308	○	NA	Usami *et al*[5]
7	IVS7+1G>A	Splice donor		NA	Van Hauwe *et al*[13]
8	IVS7-2A>G	Splice acceptor	○	NA	Coucke *et al*[17]
8	IVS8+1G>A	Splice donor	○	NA	Coyle *et al*[11]
9	IVS8-2A>G	Splice acceptor		NA	Yong *et al*[18]
9	1003T>C	F335L		Yes	Campbell *et al*[8]
9	1105A>G	K369E	○	Yes	Usami *et al*[5]
9	1115C>T	A372V	○	Yes	Usami *et al*[5]
9	1146delC	Stop at 394		NA	Van Hauwe *et al*[13]
10	1151A>G	E384G		Yes	Coyle *et al*[11]
10	1174A>T	N392Y	○	Yes	Park *et al*[16]
10	1181delTCT	S394del		NA	Yong *et al*[18]
10	1197delT	Stop at 431		NA	Everett *et al*[4]
10	1226G>A	R409H		Yes	Van Hauwe *et al*[13]
10	1229C>T	T410M	○	Yes	Coyle *et al*[11]
10	1246A>C	T416P		Yes	Van Hauwe *et al*[13]
11	1284delTGC	A429del		NA	Coyle *et al*[11]
11	1334T>G	L445W		Yes	Van Hauwe *et al*[13]
11	1337A>G	Q446R		Yes	Reardon *et al*[12]
12	1334insAGTC	Stop at 467		NA	Coyle *et al*[11]
12	1341delG	Stop at 453		NA	Everett *et al*[4]
13	1439T>A	V480D		Yes	Scott *et al*[19]
13	1468A>C	I490L		Yes	Li *et al*[20]
13	1489G>A	G497S		Yes	Li *et al*[20]
13	1523C>A	T508N		Yes	Bogazzi *et al*[21]
13	1536delAG	Stop at 524		NA	Coyle *et al*[11]
13	IVS13+9C>G	Splice donor		NA	Yong *et al*[18]
14	1584insC	Stop at 526		NA	Park *et al*[16]
14	1588T>C	Y530H		Yes	Coyle *et al*[11]
15	IVS14-7A>G	Splice acceptor		NA	Park *et al*[16]
15	1652insT	Stop at 556	○	NA	Namba *et al*[22]
15	1666T>C	Y556H		Yes	Lopez-Bigas *et al*[9]
15	1667A>G	Y556C		Yes	Coyle *et al*[11]
15	1694G>A	C565Y	○	Yes	Van Hauwe *et al*[13]
16	1790T>C	L597S		Yes	Campbell *et al*[8]
17	1829C>A	S610X	○	NA	This study
17	1898delA	Stop at 634		NA	Van Hauwe *et al*[13]
17	1958T>C	V653A		Yes	Scott *et al*[19]
17	1970G>A	S657N	○	Yes	This study
17	1997C>T	S666F	○	Yes	This study
17	2000T>G	F667C		Yes	Everett *et al*[4]
17	2015G>A	G672E		Yes	Coyle *et al*[11]

Table 1 Continued

Exon	*Nucleotide change*	*Amino-acid change*	*Japanese*	*Evolutionary conservation*	*Reference*
17	2027T>A	L676Q		Yes	Park *et al*[16]
17	2111insGCTGG	Stop at 722	○	NA	Usami *et al*[5]
19	2127delT	Stop at 719		NA	Coyle *et al*[11]
19	2162C>T	T721M	○	Yes	Usami *et al*[5]
19	2168A>G	H723R	○	Yes	Van Hauwe *et al*[13]
19	2182insG	Y728X		NA	Fugazzola *et al*[23]
21	2343A>G	X781W		NA	Lopez-Bigas *et al*[9]

○: mutations found in Japanese.

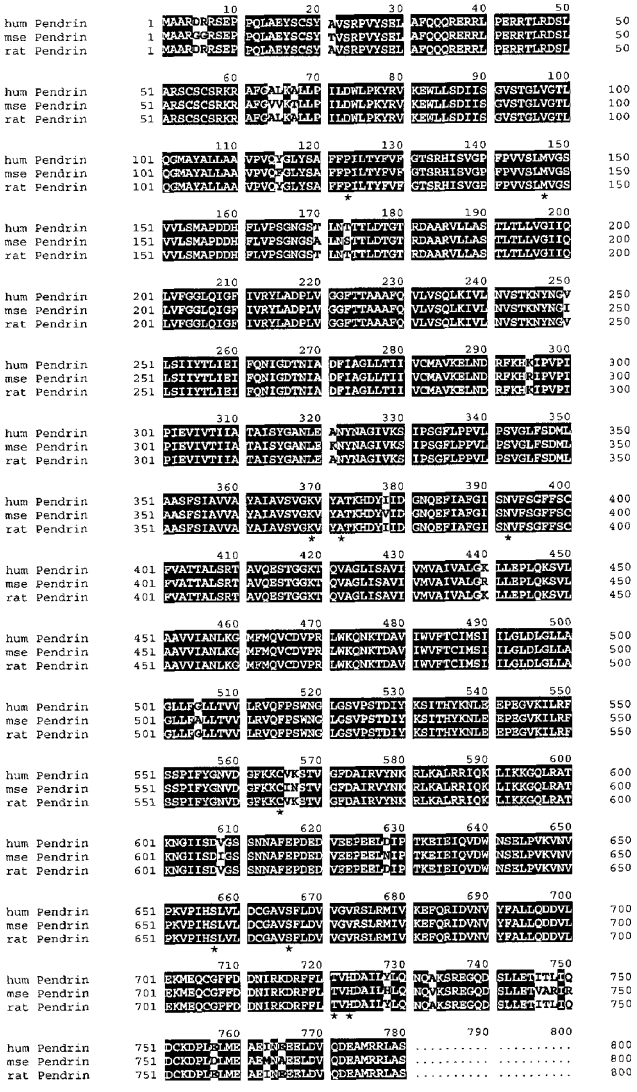

Figure 1 Multiple-sequence alignment of selected proteins with significant sequence homology to human pendrin. The amino-acid sequence of human pendrin (hum-pendrin) is aligned relative to the sequences of the mouse pendrin (mse-pendrin) and rat pendrin (rat-pendrin). Positions exhibiting absolute identity among the three proteins are shown with a black background. The black background regions lead evolutionary conservation. The regions with asterisks (*) denote the missense mutations detected in our study.

Splice site mutations

The three splice site mutations found were: IVS5-1G>A (exon 6 acceptor splice site); IVS7-2A>G (exon 8 acceptor splice site); and IVS8+1G>A (exon 8 donor splice site).

Mutations found in unaffected controls

Of the 18 mutations identified in the Pendred syndrome and EVA families, only the H723R mutation was found in a single control subject (one out of 192 alleles).

Differences between Japanese and those with Caucasoid ancestry

Figure 2 shows a schematic representation of the *PDS* gene and the approximate positions of mutations found in Japanese, and those found in families with European ancestry.

Genotype–phenotype correlation

Table 2 summarizes phenotype–genotype correlation. One mutation, IVS5-1G>A, was found in the Pendred syndrome family, whereas 11 mutations, P123S, M147V, K369E, C565Y, S657N, S666F, S610X, 322delC, 917delT, IVS7-2A>G, and IVS8+1 G>A were detected in families with nonsyndromic hearing loss associated with EVA. The other six mutations, A372V, N392Y, T721M, H723R, 1652insT, and 2111ins5bp, were found in both EVA and Pendred syndrome families.

Discussion

Recent advances in molecular genetics as well as radiology have provided reliable diagnostic criteria for Pendred syndrome. After identification of the gene responsible for Pendred syndrome, *PDS*,[4] it has become possible to diagnose this syndrome from the molecular genetic viewpoint. This is noteworthy because of the possible molecular diagnostic applications for affected individuals. Furthermore, mutations in *PDS* were found in nonsyndromic hearing loss associated with EVA, suggesting that this gene may give rise to different pathologic phenotypes.[5]

The appearance of EVA by CT/MRI is demonstrated to be a reliable radiological marker, and has become one of the reliable diagnostic criteria for Pendred syndrome.[24] According to Phelps *et al*,[24] EVA was found in a majority of Pendred syndrome patients, but Mondini deformity, which

Table 2 Genotype–phenotype correlations in the current study

Family-patient #	*Phenotype*	*Allele 1*	*Allele 2*
#1	Pendred	–	
#2	NSEVA	917delT	
#3	NSEVA	–	
#4-P	NSEVA	T721M	H723R
#4-B	NSEVA	T721M	H723R
#4-F	Normal	T721M	
#4-M	Normal	H723R	
#5-P	Pendred	A372V	H723R
#5-B	Pendred	A372V	H723R
#5-M	Normal	H723R	
#6-P	NSEVA	A372V	2111ins5bp
#6-F	Normal	A372V	
#6-M	Normal	2111ins5bp	
#7	NSEVA	H723R	
#8	NSEVA	H723R	
#9	NSEVA	–	
#10-P	NSEVA	K369E	H723R
#10-F	NA	K369E	
#10-M	NA	H723R	
#11	Pendred	A372V	1652insT
#12	NSEVA	H723R	
#13	NSEVA	2111ins5bp	
#14	NSEVA	H723R	
#15	NSEVA	N392Y	H723R
#16	Pendred	H723R (homo)	
#17-P	NSEVA	S610X	S657N
#17-M	NA	S610X	
#17-A	NSEVA	S610X (homo)	
#18	NSEVA	–	
#19	NSEVA	–	
#20	NSEVA	H723R	
#21	NSEVA	–	
#22-P	NSEVA	IVS7-2A>G	H723R
#22-S	NSEVA	IVS7-2A>G	H723R
#22-F	Normal	H723R	
#22-M	Normal	IVS7-2A>G	
#23	NSEVA	T721M	
#24-P	NSEVA	IVS8+1G>A	H723R
#24-F	Normal	H723R	
#24-M	Normal	IVS8+1G>A	
#25	NSEVA	P123S (hetero)	
#26	NSEVA	IVS7-2A>G	H723R
#27	NSEVA	–	
#28	NSEVA	H723R (homo)	
#29-P	NSEVA	S666F	H723R
#29-M	NSEVA	S666F (homo)	
#30	NSEVA	C565Y	H723R
#31-P	NSEVA	H723R (homo)	
#31-S	NSEVA	H723R (homo)	
#31-F	Normal	H723R	
#31-M	Normal	H723R	
#32-P	Pendred	IVS5-1G>A	H723R
#32-F	Normal	H723R	
#32-M	Normal	IVS5-1G>A	
#33-P	NSEVA	H723R (homo)	
#33-B	NSEVA	H723R (homo)	
#33-M	NA	H723R	
#34	Pendred	H723R (homo)	
#35	Pendred	H723R (homo)	
#36	Pendred	T721M	H723R
#37	Pendred	A372V	H723R
#38	Pendred	H723R (homo)	
#39	NSEVA	—	
#40	NSEVA	322delC	
#41	NSEVA	M147V	H723R
#42-P	NSEVA	H723R (homo)	
#42-F	Normal	H723R	
#42-M	Normal	H723R	

Pendred, Pendred syndrome; NSEVA, nonsyndromic hearing loss with EVA; NA, patient less than 1 year of age and cannot be accurately diagnosed; P, proband; B, brother; S, sister; F, father; M, mother; A, aunt.

had been thought to be a characteristic radiological feature of Pendred syndrome, was not found. Based on molecular genetics and radiological evaluation, Pendred syndrome and nonsyndromic hearing loss associated with EVA are currently thought to be a continuum of disease caused by *PDS* mutations; that is, hearing loss associated with EVA with/without goiter.

In the present study, causative mutations have been identified in 90% of typical Pendred families and 78.1% of those with sensorineural hearing loss associated with EVA. The results confirmed a new category of disease caused by mutations in *PDS*, encompassing a range from 'classic' Pendred syndrome to nonsyndromic hearing loss associated with EVA.

To date, 66 mutations causing Pendred syndrome and nonsyndromic hearing loss with EVA have been reported in the *PDS* gene (Table 1). We have previously reported seven mutations, K369E, A372V, T721M, H723R, 917delT, 1652insT, and 2111ins5bp, in Japanese families with nonsyndromic hearing loss with EVA or Pendred syndrome.[5,22] Kitamura *et al*[25] reported the T410M mutation in a family with nonsyndromic hearing loss with EVA. The present study added 12 mutations including seven novel *PDS* mutations: 322delC, P123S, M147V, IVS5-1G>A, S610X, S657N, and S666F. It is likely that these are pathologic mutations rather than rare or functionally neutral polymorphic changes because: (1) none of the novel mutations represented here were found in any of the controls, (2) these mutations were highly associated with affected subjects in which mutations were found to be homozygous or compound heterozygous, also indicating that they may be disease-causing mutations, and (3) all of the missense mutations found in our study had occurred at amino-acid residues that were conserved in the rat and the mouse (Figure 1). The present study revealed a unique spectrum of *PDS* mutations quite different from that found in the populations with European ancestry. Of the 19 mutations found in Japanese, only five mutations were also found in European populations (Figure 2), further suggesting a founder effect of these mutations as demonstrated in frequent mutations in *GJB2*.[26,27] In the Caucasoid popula-

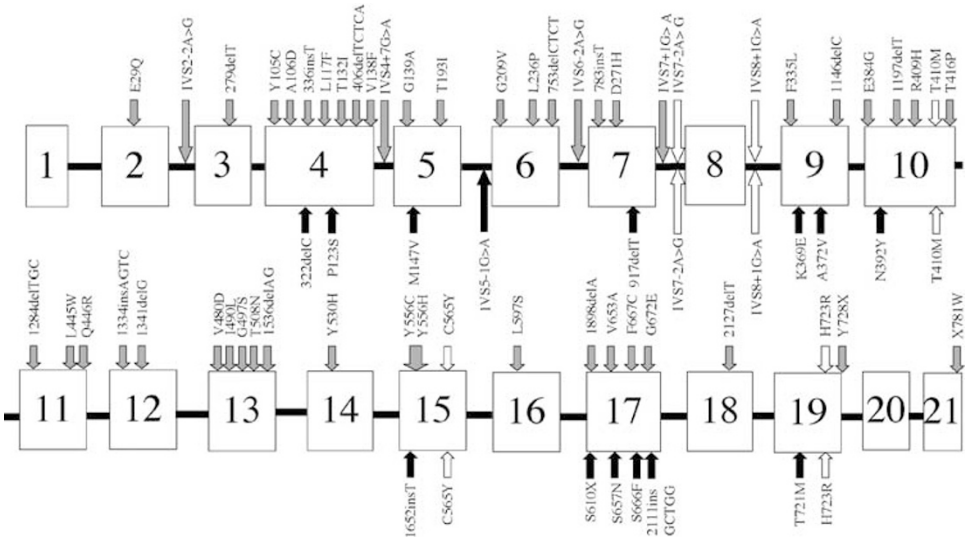

Figure 2 Differences in *PDS* mutation spectrums between Japanese and the populations with European ancestry. Shaded arrows indicate approximate localization of *PDS* mutations found in the Caucasoid populations, filled arrows are localization of mutations found in Japanese and white arrows are the mutations that have been reported in both Caucasoid and Japanese families.

tions, three frequent mutations, L236P (16%), T416P (15%), and IVS8 + 1G > A (14%), account for nearly half of all *PDS* mutant alleles.[8] In contrast, these mutations are rare in Japanese (L236P, 0%; T416P, 0%; and IVS8 + 1G > A, 2%), whereas H723R accounted for 53% of *PDS* mutations. A recent report suggested that the cause of the frequent mutation (H723R) was the existence of a common ancestor rather than a mutational hot spot.[16] The spectrum of *PDS* mutations revealed by the present study will facilitate the effective molecular diagnosis of a category of disease caused by the *PDS* gene.

Pendrin, the transmembrane protein encoded by the *PDS* gene, is expressed in the inner ear, thyroid gland, and kidney.[4,19,28] In Xenopus oocytes and Sf9 cells, it has been demonstrated that pendrin transports chloride and iodine, and mediates the exchange of chloride and formate.[19,29] The mutations associated with Pendred syndrome showed complete loss of pendrin-induced chloride and iodide transport, whereas alleles associated with nonsyndromic sensorineural hearing loss with EVA showed partial transport function, which may be sufficient to maintain thyroid function and eliminate goiter.[19] With regard to genotype–phenotype correlation, four mutations, P123S, S610X, S666F, and H723R, were found to be homozygous (Table 2). In these cases, the phenotype would reflect the function of the respective mutations. The former three mutations caused nonsyndromic sensorineural hearing loss with EVA, and therefore may have residual transport function. However, the homozygous H723R mutation caused either Pendred syndrome or nonsyndromic sensorineural hearing loss with EVA, making the story more complex. Actually, patient #38 showed a huge goiter and tracheostomy was necessary because of dyspnea,[30] whereas three out of eight patients did not show any goiter. Phenotypic variability has also been reported in two families carrying the same *PDS* missense mutation (L445W).[31] A modifier gene or environmental factor (iodine uptake, nutrition, etc.) may contribute to such variability.

In the present study, one out of 10 families with Pendred syndrome and seven out of 32 families with nonsyndromic hearing loss associated with EVA did not show any mutations. This suggests that mutations possibly exist in the activate cryptic splice sites in the introns or that they occur in the promoter region, which was not analyzed in this study. An alternative explanation may be that other genes were responsible for those families without *PDS* mutations. According to Hulander *et al*,[32] Foxi1 is an upstream regulator of pendrin, and defective pendrin-mediated chloride ion resorption in the endolymphatic duct/sac epithelium accounts for the phenotype seen in Foxi1-null mice. Therefore, it is possible that mutations in *FOXI1* cause Pendred syndrome in humans.

In conclusion, the present study confirmed that a range of disease from 'classical' Pendred syndrome to nonsyndromic hearing loss associated with EVA is dependent on the *PDS* mutations. Our findings also represented a unique spectrum of the *PDS* gene in Japanese, which is distinct from that found in the populations with European ancestry. The present study clearly indicates that the establishment of databases for different populations will facilitate the molecular diagnosis of a clinically complex disease.

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