CLINICAL STUDY

Phenotypes associated with replacement of His⁷²³ by Arg in the Pendred syndrome gene

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Abstract

Background: Pendred syndrome is often associated with inner ear malformations, especially enlarged vestibular aqueduct (EVA). Recently, mutations in the Pendred syndrome gene (PDS) have been reported in patients with EVA, in addition to those with classical Pendred syndrome.

Objective: The aim of this study was to investigate the genotype–phenotype correlations of PDS.

Methods: Each of the 21 exons and flanking splice regions of PDS was analysed by direct DNA sequencing in nine patients with EVA; allele-specific amplification was performed to confirm the mutation. Genetic analyses were compared with thyroid function tests, perchlorate discharge tests, thyroid volume and pure-tone audiogram. Magnetic resonance imaging was used to determine the volume of the endolymphatic duct and sac of each patient.

Results: A missense mutation, H723R, was identified in the homozygous state in three patients and in the heterozygous state in another three. Although none of the patients had goitre, increased serum thyroglobulin and an abnormal degree of iodide release were correlated with the number of mutant alleles identified. However, there was no relationship between the degree of hearing loss and the number of mutant alleles.

Conclusion: The present study reveals that the number of mutant alleles correlates with the degree of subclinical thyroid abnormality, but not with the degree of hearing loss in Japanese patients with the PDS missense mutation H723R.

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Introduction

Pendred syndrome is characterized by sensorineural hearing loss, goitre, and an iodine organification defect (1, 2). This syndrome is one of the most common forms of syndromic deafness, and may account for up to 10% of congenital deafness. Hearing loss is prelingual in the majority of the cases (3); only a subset of patients have a progressive hearing loss later in life. Radiologically detectable inner ear malformations, such as an enlarged vestibular aqueduct (EVA) and Mondini dysplasia, are recognized associated features. EVA has been reported to be present in all affected patients (3, 4), but Mondini dysplasia is not always associated with Pendred syndrome (4). In Pendred syndrome, goitre develops around puberty, and its severity is variable, from a slight enlargement to a large multinodular form, depending on the degree of the dietary iodide intake (5, 6). The most common thyroid disorder in Pendred syndrome is characterized by an abnormal release of iodide from the thyroid after perchlorate challenge, which is due to an iodine organification defect (7).

Pendred syndrome is caused by mutations in the Pendred syndrome gene (*PDS*) (8). Mutations in *PDS* cause not only classic Pendred syndrome, but also non-syndromic deafness with EVA (9–12). This gene has been localized to chromosomal region 7q31. It encodes a transmembrane glycoprotein, pendrin, which contains 11 or 12 transmembrane domains (13) and acts as an iodide and chloride transporter. Functional analysis in *Xenopus* oocytes revealed that the mutant alleles found in individuals with ‘classical’ Pendred syndrome cause a complete loss of pendrin-induced iodide and chloride transport. In contrast, the mutant alleles found in individuals with only EVA cause an incomplete loss of iodide and chloride transport (14). However, there are few reports of phenotypic variability arising from the same mutation.

Recently, *PDS*-knockout mice were generated by Everett *et al.* (15). *PDS*^{−/−} mice are profoundly deaf, with vestibular dysfunction. The inner ears of these mice develop normally until embryonic day 15, after which endolymphatic dilatation occurs. Ultrastructural studies have revealed severe degeneration of the sensory

hair cells in the cochlea and the maculae in the utricle and saccule. Data on a perchlorate discharge test and the amount of iodide intake were not available in these mice; however, there is no histological or biochemical evidence of thyroid abnormality. These findings (the onset of hearing loss, the presence of vestibular dysfunction, and the absence of goitre) are not relevant to all human patients with *PDS* mutations (3, 16); the knockout destroys both *PDS* alleles whereas, in contrast, some human patients may have partially functioning alleles and consequently a less severe phenotype. Homozygosity for some *PDS* mutations may result in a severe phenotype.

In the present study, we examined the clinical features of nine independent Japanese patients with EVA and undertook corresponding molecular analyses of their *PDS* genes, to investigate the genotype–phenotype correlations of *PDS*.

Materials and methods

Patients

Nine Japanese patients with EVA (two males and seven females; age range 7–36 years), from independent families, were included in this study. Eight patients had bilateral EVA and one patient had unilateral EVA. A diagnosis of EVA was established when the diameter of the endolymphatic duct and sac was 1.5 mm or more at its mid-portion, using magnetic resonance imaging (MRI). No palpable goitre was present in any individual. This study was approved by the institutional research board, and all the patients gave informed consent before participating in the study.

Audiological findings and endolymphatic duct and sac volumes

All the patients were evaluated for onset of hearing loss (or the age at which hearing loss was first noted), average hearing level, and the presence of vertigo. The same audiometer (Rion, Model AA-61BN, Tokyo, Japan) was used in the same sound-insulated chamber for all pure-tone audiometry in all patients. Average hearing level is expressed as the average at three frequencies (500 Hz, 1 kHz and 2 kHz).

Heavily T2-weighted three-dimensional fast spin-echo MRI (Visart; Toshiba, Tokyo, Japan) was used to determine the volume of the endolymphatic duct and sac. Figure 1 shows transverse images of the right petrous temporal bone in a normal individual and in a patient with EVA. The vestibular aqueduct is not visualized by MRI, but its contents, the endolymphatic duct and sac, are clearly visible when it is enlarged. The sum of the area of the endolymphatic duct and sac was multiplied by the section thickness, 0.8 mm. The normal volume of endolymph is about 34.0 mm³ (17). This method has previously been described in detail (18).

Thyroid evaluation

Thyroid function was evaluated by free tri-iodothyronine (FT₃) and free thyroxine (FT₄) (Amarex-MAB Kit, Ortho Clinical Diagnostics, Tokyo, Japan), thyroid stimulating hormone (TSH), serum thyroglobulin (TFB, Tokyo, Japan), and thyroglobulin antibody (anti-Tg Ab) (RSR Limited, Cardiff, UK). All blood samples were obtained in the morning after overnight fasting.

Figure 1 Heavily T2-weighted transverse magnetic resonance image of the right petrous temporal bones in a normal individual (A) and in patient No. 3 (B). Endolymphatic duct and sac cannot be seen in the normal individual, but endolymphatic duct and sac are enlarged, with a diameter of more than 1.5 mm (arrow), in patient No. 3.

Thyroglobulin concentrations of patients Nos 3, 4 and 6 were measured in duplicate and the mean values were calculated. Normal ranges are: FT₃ 2.47–4.34 pg/ml; FT₄ 0.97–1.79 ng/ml; TSH 0.50–6.0 µU/ml; thyroglobulin 0–35 ng/ml; anti-Tg Ab undetectable. Thyroid ultrasonography was performed with LOGIQ 500MD version 4.1 and a 50 mm 9 MHz linear transducer, effective length 40 mm (GE Medical System). The volume of each lobe was calculated using the formula for an ovoid ($\text{width} \times \text{depth} \times \text{length} \times \pi/6$). The isthmus was not included in the calculated volume (19). The potassium perchlorate discharge test was performed in six patients, after their informed consent had been obtained. Two hours after the oral administration of iodine-123 (1.85 GBq), 1 g potassium perchlorate was administered orally, and the discharge was determined after 2 h. If the discharge exceeded more than 10%, the perchlorate test was defined as positive.

DNA sequencing and allele-specific amplification analysis

DNA was extracted from whole blood using DNA Quick II (Dainippon, Osaka, Japan). Each of the 21 exons and flanking splice sites of *PDS* was amplified by PCR using primers described previously (8, 20). The cycling conditions consisted of 95 °C for 5 min followed by 37 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 50 s or 2 min 40 s; then 72 °C for 7 min (TP2000, Takara Biomedicals, Tokyo, Japan). PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Both strands were directly sequenced using ABI Prism Big Dye Terminators on an ABI Prism 310 Genetic Analyzer according to the manufacturer's procedure (Perkin–Elmer Applied Biosystems, CA, USA).

Allele-specific amplification was performed with either a wild-type allele-specific primer (sense, 5'-ACAT-TCTTTTGGACGGTCCA-3') or a mutant allele-specific primer (sense, 5'-ACATTCCTTTTGGACGGTCCG-3') in combination with the exon 19 antisense primer (5'-CTGATGAAAAAAGTGAAGTC-3'). Each PCR reaction mixture contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.25 mM each dNTP, 0.25 µM primers, and 0.25 units TaKaRa *Taq* DNA polymerase in a total volume of 10 µl. Amplification was performed on a GeneAmp PCR System 9700 (Perkin–Elmer/Applied Biosystems) programmed for an initial 10 min denaturation at 95 °C followed by 25 cycles of 20 s at 95 °C, 20 s either at 58 °C (wild-type allele) or at 60 °C (mutant allele), and 20 seconds at 72 °C; with a final 5 min at 72 °C. The PCR producted a fragment of 228 bp.

Results

Mutation analysis of *PDS* revealed one missense mutation in exon 19 in six patients. This mutation

Figure 2 Allele-specific amplification analysis of exon 19 of *PDS*. Results for patients Nos 1, 4 and 7 are shown. The PCR fragment is 228 bp. In patient 1, only the mutant fragment (M) was amplified. However, both the wild-type fragment (W) and mutant fragment were amplified in patient 4. MWM, molecular weight markers.

was an A→G transition at nucleotide 2168, resulting in a predicted His→Arg substitution at residue 723 (H723R). This mutation is believed to be located in the intracellular carboxy terminus. Three patients were homozygous for this mutation, and another three were heterozygotes. No other mutation in the studied region of *PDS* was found in these individuals, although a secondary mutation might exist in regulatory or intronic sequences. There were no sequence abnormalities in the studied region of *PDS* in the remaining three patients.

Allele-specific amplification was used to confirm the H723R mutation in *PDS*. Figure 2 demonstrates a typical assay illustrating homozygous H723R, heterozygous H723R, and wild-type genotypes.

The clinical and genetic features of the patients are listed in Table 1. The patients were classified according to each *PDS* genotype: homozygous H723R and heterozygous H723R. Because three individuals who had no mutation in the studied region of *PDS* had a normal perchlorate discharge test, their EVA may have another pathogenesis. They were excluded from statistical analysis. The age of onset of hearing loss was in early childhood (<5 years old) in four patients. However, in two homozygous individuals, hearing loss onset occurred after 10 years of age. The average hearing levels for the homozygous and heterozygous groups were 91.2 ± 16.5 dB (mean $\pm$ standard deviation) and 91.2 ± 18.9 dB respectively, which do not differ significantly ($P = 0.631$, Mann–Whitney *U* test). The mean endolymphatic duct and sac volumes were 429 ± 234 mm³ and 384 ± 144 mm³ for homozygous and heterozygous patients, respectively. The difference in volumes of the endolymphatic duct and sac was not significant ($P = 0.631$, Mann–Whitney *U* test). Four patients suffered vertigo (33%), but the incidence of vertigo did not correlate with homozygous mutant *PDS*.

Serum FT₃, FT₄ and TSH concentrations were within the normal range in all the patients. Figure 3 shows the serum thyroglobulin concentrations, perchlorate discharge test results, and thyroid volumes in each group. The average serum thyroglobulin was 70.4 ± 24.0 ng/ml and 16.8 ± 14.3 ng/ml for the homozygous

Table 1 Clinical findings in the patients with EVA.

Patient No.	Sex	Age (years)	Onset age of hearing loss (years)	Ear	Hearing level (dB) [<20]	Endolymphatic duct and sac volume (mm ³) [neg]	Vertigo	FT 3 (pg/dl) [2.47–4.34]	FT 4 (pg/dl) [0.97–1.79]	T S H (μ U/ml) [0.5–6.0]	T G (ng/ml) [0–35]	Anti-Tg (U/ml) [neg]	Perchlorate discharge test (%) [<10]	Thyroid volume (ml) [5–20]	Mutation
1	F	36	17	Right Left	100 83	501 362	–	2.95	1.33	0.74	90	neg	12.6	13	H723R homozygous
2	F	7	3	Right Left	67 115	709 359	–	4.21	1.38	2.91	77.54	neg	ND	5	H723R homozygous
3	M	30	11	Right Left	85 97	600 40	–	3.74	1.25	0.94	43.59	neg	18	14	H723R homozygous
4	F	24	3	Right Left	107 88	143 303	+	4.29	1.17	1.9	10.4	neg	12.7	15	H723R heterozygous
5	F	16	2	Right Left	57 105	554 478	–	3.11	1.35	1.04	33.14	neg	ND	14	H723R heterozygous
6	F	12	2	Right Left	103 87	419 406	+	3.71	1.23	2.72	6.79	neg	8	7	H723R heterozygous
7	M	22	3	Right Left	70 97	207 413	+	3.14	1.24	1.57	22.92	neg	ND	16	–
8	F	32	20	Right Left	8 65	58 56	+	3.71	1.18	1.23	16.97	neg	4.3	14	–
9	F	23	4	Right Left	100 115	102 neg	–	3.72	1.23	1.88	32.64	0.57	5	8	–

Normal values are given in square brackets. neg, undetected; –, absent; +, present. ND, not done.

and heterozygous groups respectively. Serum thyroglobulin was significantly increased in patients homozygous for the mutation ($P = 0.0495$, Mann–Whitney U test). Average perchlorate discharges were $15.3 \pm 3.1\%$ and $10.4 \pm 2.7\%$ for the homozygous and heterozygous groups respectively. Perchlorate discharge also tended to be greater in patients homozygous for the mutation, and mildly increased in heterozygous individuals. Thyroid volumes ranged from 5 ml to 15 ml (mean $\pm$ standard deviation, 11.3 ± 4.2 ml), remaining within the normal range.

Discussion

Mutations in *PDS* have been identified in several families with classical Pendred syndrome, and in families with non-syndromic deafness. We studied the sequences of *PDS* in nine independent Japanese individuals affected with EVA. The missense mutation, H723R, was identified in six of them. According to previous reports, this mutation accounts for up to 75% of *PDS* mutations in Japanese families with EVA (10, 12), making it the most frequent mutation in these families. Although we did not perform precise microsatellite analysis, the frequency of this mutation may be attributable to an early founder effect.

In thyrocytes, pendrin is located in the apical membrane (21). The thyroidal iodide trapping system relies on active transport via the sodium–iodide symporter, which is functional in patients with Pendred syndrome. Pendrin functions as an iodide–chloride transporter

(22), and is believed to transport iodide within the thyrocyte to the colloid space, where it is rapidly bound to thyroglobulin in a reaction catalysed by thyroid peroxidase (TPO); impaired iodide transport caused by mutation of *PDS* may result in decreased iodide flux into the colloid space. *PDS* mutations may not cause the iodide organification defect directly, but rather by delaying the reaction catalysed by TPO, because iodide passage via the mutated transporter is abnormal. The *PDS* mutations associated with Pendred syndrome differ from those associated with EVA in the degree to which ion transport is impaired by the mutated pendrin (14). The secondary structure caused by some mutations may result in intracellular trafficking defects of different degrees. Other mutant *PDS* proteins may, however, be inserted normally into the membrane, but with altered anion transport.

Increased serum thyroglobulin has been reported in Pendred syndrome (5, 23). This might mirror the activity of the thyroid gland in terms of hormone production. These factors are responsible for the stimulation of TSH and hypoiodination. However, all our patients had normal serum TSH concentrations and no goitre. The mechanism by which serum thyroglobulin is increased, therefore, remains unresolved by the available data.

The present study investigated, for the first time, the genotype–phenotype correlation between homozygous and heterozygous H723R mutations. Although the number of patients in this study was small, the impairment in iodine organification tended to be greater in those homozygous for H723R, and mildly increased in

Figure 3 Serum thyroglobulin concentrations, perchlorate discharge tests, and thyroid volumes in the patients homozygous for H723R (M/M), those heterozygous for H723R (M/W), and the wild-type individuals (W/W). Serum thyroglobulin concentrations in the patients homozygous for the mutant allele were significantly increased. The perchlorate discharge test was positive in all patients homozygous for H723R and in one heterozygous individual. Thyroid volumes were within the normal range in all patients. The dotted lines represent the upper limit of normal values.

heterozygous individuals. Serum thyroglobulin concentrations were also increased in individuals homozygous for H723R. Given that there is no evidence for haplo-insufficiency or a dominant negative mechanism for any of the pendrin mutations, it is likely that individuals heterozygous for H723R have a second mutation in a regulatory or intronic region. However, the thyroid data for patients homozygous for H723R typify the classical Pendred syndrome more closely than the data for those heterozygous for H723R. Because no patient in this study with iodide organification defect had goitre, impaired iodide transport may not be the direct cause of goitre. Furthermore, an identical missense mutation has been reported in a Dutch family with Pendred syndrome (24). This can be explained by differences in iodine intake between the Japanese and Dutch. Even where an impaired iodide transporter exists, high iodine intake may prevent the development of goitre.

RNA *in situ* hybridization on the inner ears of mice, from 8 days post-coitum to postnatal day 5, revealed that *PDS* expression is detectable in the endolymphatic duct and sac (25). To investigate the mechanism underlying the inner-ear defect associated with mutations of *PDS* in more detail, Everett *et al.* (25) generated a *PDS*-knockout mouse. The inner ears of *PDS*^{-/-} mice develop normally until embryonic day 15, after which severe endolymphatic dilatation occurs. This dilatation may not arise from simple developmental arrest. The endolymphatic duct and sac are involved in the resorption of endolymph, and the *PDS*-expressing region in the endolymphatic duct and sac may also maintain inner-ear fluid homeostasis. A *PDS* mutation may result in an abnormality in endolymph resorption during embryonic development. Impaired anion transport may change the volume of the endolymph, and this may cause enlargement of the endolymphatic duct and sac.

Furthermore, in the second postnatal week, severe degeneration of the sensory cells occurs. Lack of Preyer's reflex and the auditory-evoked brainstem response revealed that *PDS*^{-/-} mice have profound hearing loss. However, EVA-associated hearing loss is not congenital in humans, even in those individuals with the *PDS* mutation. A hypothesis explaining the mechanism of hearing loss is that hyperosmolar protein-rich fluids in the endolymphatic sac reflux to the cochlear duct through the enlarged vestibular aqueduct (26–28). Biochemical abnormalities of the endolymph may trigger the degeneration of normally developed sensory hair cells of the inner ear (15). There was no correlation between hearing loss and the size of the endolymphatic sac ($r = 0.157$) in the present study. *PDS*^{-/-} mice also display signs of vestibular dysfunction, which may be caused by malformation of the otoconia. In the present study, vertigo was apparent in only two of six subjects with the *PDS* mutation. Reflux of hyperosmolar fluid from the endolymphatic sac to the vestibule has also been considered to cause vertigo. In humans, however,

vertigo does not always occur when the hearing level decreases.

Finally, we have demonstrated phenotypic variability associated with the H723R mutation. Increased serum thyroglobulin was observed in the patients homozygous for H723R. Abnormal perchlorate discharge tended to be greater in those homozygous for H723R than in heterozygous patients. Our findings suggest that the number of the mutant alleles may correlate with thyroglobulin values and with the degree of the iodide organification defect, but not with the degree of hearing loss.

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