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Differential Diagnosis between Pendred and Pseudo-Pendred Syndromes: Clinical, Radiologic, and Molecular Studies

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ABSTRACT

The disease gene for Pendred syndrome has been recently characterized and named *PDS*. It codes for a transmembrane protein called pendrin, which is highly expressed at the apical surface of the thyroid cell and functions as a transporter of chloride and iodide. Pendrin is also expressed at the inner ear level, where it appears to be involved in the maintenance of the endolymph homeostasis in the membranous labyrinth, and in the kidney, where it mediates chloride-formate exchange and bicarbonate secretion. Mutations in the *PDS* gene and the consequent impaired function of pendrin leads to the classic phenotype of Pendred syndrome, *i.e.* dyshormonogenic goiter and congenital sensorineural hearing loss. In the present study, we performed a detailed clinical, radiologic, and molecular analysis of six families presenting with clinical diagnosis of Pendred syndrome. In two families a homozygous pattern for *PDS* mutations was found, whereas the affected members of the other four families were compound heterozygotes. One family did not harbor *PDS* mutations. Among the four novel mutations described, one is a transversion in exon 2 (84C>A), leading to the substitution S28R. Two other novel mutations lie in exon 4 (398T>A) and in exon 16 (1790T>C), leading to the substitutions S133T and L597S, respectively. The fourth novel mutation (1614+1G>A) is located in the first base pair of intron 14, probably affecting the splicing of the *PDS* gene. Clinically, all patients had goiter with positive perchlorate test, hypothyroidism, and severe or profound sensorineural hearing loss. In all the individuals harboring *PDS* mutations, but not in the family without *PDS* mutations, inner ear malformations, such as enlargement of the vestibular aqueduct and of the endolymphatic duct and sac, were documented. The pseudo-Pendred phenotype exhibited by the family without *PDS* mutations is likely caused by an autoimmune thyroid disease associated with a sensorineural hearing loss of different origin. **(*Pediatr Res* 51: 479–484, 2002)**

Abbreviations

PS, Pendred syndrome
ED, endolymphatic duct
ES, endolymphatic sac
SNHL, sensorineural hearing loss
CT, computed tomography
MRI, magnetic resonance imaging
VA, vestibular aqueduct
LVAS, large vestibular aqueduct syndrome
FSE MRI, fast spin-echo MRI

PS is an autosomal recessive disease first described in 1896 as the combination of congenital deafness and goiter (1). It is considered the most common cause of congenital deafness, but the actual incidence of the syndrome is difficult to establish because of the variable phenotype of affected subjects, even within the same family (2–4). In the last 3 y PS has been associated, with a homozygous or compound heterozygous pattern, to mutations in the *PDS* gene (5–14). *PDS* maps on chromosome band 7q22–31.1 and codes for pendrin (7), a transmembrane protein, which was first found to be expressed in the thyroid and in particular at the apical surface of the thyroid cell, where it likely functions as an iodide-chloride transporter (15–17). The effect of impaired pendrin function is thus a deficient transport of iodide into the colloid space and its accumulation into the cytoplasm. The consequent altered iodide gradient between thyroid and serum is revealed by the perchlorate test (18), which returns a positive result in the majority of affected patients. Indeed, the thyroid clearance of iodide, measured before and after the administration of per-

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chlorate, is notably higher in PS patients than in normal subjects (^{131}I discharge, 15–80% *versus* <10%, respectively). However, the defect is only partial, as in most patients the thyroid hormone status is normal or a subclinical hypothyroidism is shown (4). Furthermore, goiter is not a constant feature of the syndrome and can vary from a slight enlargement to a large multinodular goiter, with a time of onset ranging from the first months of life until puberty (3, 4, 19). Both endogenous and environmental factors could explain such variability in the expression of thyroid phenotype.

More recently, pendrin has been immunolocalized in the inner ear of the mouse and in particular in the ED and ES (20), both regulating endolymph secretion and resorption (21). Additional studies are needed to identify which anion(s) is transported by pendrin within the inner ear, although defects in chloride transport might produce both toxic and osmotic damages of the neuroepithelium (20), accounting for the SNHL of PS patients. In particular, in PS the deafness is generally profound (>60 dB) with a prelingual onset (19), and sometimes a fluctuating but worsening course (5, 22, 23), consistent with a progressive lesion of the sensory organ. Moreover, the increased osmotic pressure and volume of the endolymph leads to enlargement of the membranous labyrinth, *i.e.* the ED and ES, and of the surrounding bony structures such as the VA and the cochlea. Cochlear abnormalities (Mondini cochlea) are present in a minority of patients (24, 25), whereas the enlargement of the ED and ES and of the VA have been recently documented by high-resolution CT and MRI in 80–100% of patients affected with PS or LVAS, in which the SNHL is not associated with thyroid abnormalities (2, 6, 26–34).

Finally, pendrin has been recently immunolocalized in the proximal tubule of the kidney, where it seems to function as a chloride-formate exchanger (35), and in the intercalated cells of cortical collecting ducts, where it has an essential role in bicarbonate secretion (36). Probably because of the redundancy of compensatory mechanisms all along the nephron, no phenotypic alterations have been reported at the renal level in patients with *PDS* mutations.

In the present study, we report the clinical and radiologic investigations and the genetic analyses in six Italian families referred to our institution with a clinical diagnosis of PS.

METHODS

Patients. We studied nine patients from six unrelated Italian families and from nonconsanguineous parents referred to us with a clinical diagnosis of PS (Table 1). The affected members showed childhood deafness related to a sensorineural defect and goiter, with positive perchlorate discharge test. Three of the affected patients are males (family 1 and 5). The age range was 14–41 y (mean, 26.7 y) at the time of the present study. The two sisters of family 6 are monozygotic twins. Thyroid function analyses showed, in patients from families 1–5, a subclinical hypothyroidism with normal values of free thyroid hormones and TSH concentrations ranging between 5 and 6.5 mU/L (normal values, 0.25–4.5 mU/L). The two sisters of family 6 were subclinical hypothyroid at the diagnosis (9 y of age) with TSH levels of 5.3 and 6 mU/L, although a worsening of hypothyroid state was seen during levothyroxine withdrawal at 16 y of age (TSH, 40 and 36 mU/L, respectively). Antithy-

Table 1. *Clinical and genetic characteristics of the six families studied**

No.	Age	Sex	Goiter (mL)	Thyroid hormone status	Degree of SNHL	Enlarged VA	Enlarged ED/ES	*PDS* mutations	Other
1	14	M	30	Subclinical hypothyroidism	Severe	Yes	Yes	S28R S28R	Language disturbances
2a	39	F	35	Subclinical hypothyroidism	Severe/ profound	Yes	Yes	1001 + 1G > A L597S	Language disturbances
2b	41	F	55	Subclinical hypothyroidism	Severe/ profound	Yes	Yes	1001 + 1G > A L597S	Language disturbances
3	19	F	60	Subclinical hypothyroidism	Severe	Yes	Yes	FS400 1614 + 1G > A	Language disturbances
4	29	F	50	Subclinical hypothyroidism	Severe/ profound	Yes	Yes	R409H R409H	Vestibular syndrome
5a	24	M	28	Subclinical hypothyroidism	Severe	Yes	Yes	T410M S133T	Language disturbances
5b	27	M	30	Subclinical hypothyroidism	Severe	Yes	Yes	T410M S133T	Language disturbances
6a	24	F	22	Hypothyroidism	Severe	No	No	No	Severe language disturbances Positive TPO and Tg-Ab Hypoechoic thyroid pattern Low IQ
6b	24	F	18	Hypothyroidism	Severe	No	No	No	Severe language disturbances Positive TPO and Tg-Ab Hypoechoic thyroid pattern Low IQ

* All patients had a positive perchlorate test.

Abbreviations used: TPO, thyroid peroxidase; Tg-Ab, thyroglobulin antibody.

roglobulin and antithyroid peroxidase antibodies were positive (350 IU/mL and 649 IU/mL, respectively; normal values, <100 IU/mL) only in family 6. In all patients a multinodular goiter of different sizes was documented at ultrasound examination (from 18 to 60 mL). A hypoechoic pattern was shown in patients from family 6. It is worth noting that all patients developed goiter after the first months or years of life. The perchlorate test, performed by measuring radiolabeled iodide thyroid uptake before and after the oral administration of 1 g of $KClO_4$, was positive in all patients with discharge rates of 40–60% of basal radioiodine uptake.

In all patients a bilateral SNHL was diagnosed during childhood. The degree of deafness, assessed by pure-tone audiometry in each ear, was bilaterally severe (60–80 dB) in the affected members of families 1, 3, 5, and 6 and severe or profound (>80 dB) in families 2 and 4. In the patient of family 4, the SNHL showed a characteristic fluctuating and progressive pattern. In addition, she suffered from a vestibular syndrome. Mild language disturbances, related to the SNHL, were observed in families 1, 2, 3, and 5. In the twins of family 6 a diagnosis of SNHL with severe language disturbances and mental retardation was reached at the age of 5.5 y. Subclinical hypothyroidism with goiter was incidentally discovered at 9 y of age, therefore leading to the diagnosis of PS. In monitoring control subjects (age, 15.5 y), neurologic examination revealed a global cognitive retardation (IQ, 68 on WISC-R test) and mental age of 10.5 y in both twins. It is likely that such mental retardation derives from a not readily cured deafness, consistent with the poor level of instruction and social background of the family. Nevertheless, clinical history revealed that their father was deaf-mute but without evidence of thyroid disease, suggesting a possible genetic origin of the hearing defect.

The six families come from different Italian regions with moderate iodine deficiency.

Radiologic studies. In all patients, the presence of alterations of the cochlea and the VA was tested by high-resolution CT of temporal bones in coronal and axial planes (1-mm contiguous sections). The VA was considered enlarged when its diameter at the midpoint between the common crus and the external aperture was ≥1.5 mm or more on thin CT sections (32).

High-resolution FSE T_2-weighted MRI was performed (in axial and coronal planes) to study the membranous labyrinth and in particular the ED and ES. The size of the normal ED at its midpoint ranges from not visible to 1.4 mm (29). ES is rarely seen in normal subjects, despite high-quality FSE MR images, and it is considered enlarged when >2.5 mm (29, 31). All the images were photographed individually and as a three-dimensional composite.

Molecular studies. DNA was extracted by standard methods from whole blood of all patients and their available parents. The experimental studies were approved by the ethics committees of each institution. An informed consent was obtained for all screened subjects.

In each affected member all 20 coding *PDS* exons (2–21) were amplified using primers flanking each exon (7). Samples were subjected to 5 min of denaturation at 98°C, followed by 35 three-step cycles (55°C for 1 min, 72°C for 2 min, 94°C for 1 min), and 72°C for 10 min in a TouchDown Thermal Cycler (Hybaid, Middlesex, U.K.). PCR products were directly sequenced after removal of unincorporated deoxynucleotides and primers by GFX PCR DNA purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden). An aliquot of 3–10 ng/100 bp of purified DNA and 3.2 pmol of either the forward or reverse primer were used in standard cycle-sequencing reactions with ABI PRISM Big Dye terminators and run on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The cycle-sequencing conditions consisted of 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. One sequence read from each direction across the entire coding region and including intron-exon boundaries was obtained for each patient.

RESULTS

Radiologic examination. In all patients from families 1–5, malformations of the inner ear were found. At CT scan, the VA was enlarged bilaterally, with a diameter ranging from 2 to 4 mm. No cochlear alterations were found. The ED and ES were enlarged in all patients at MRI examination, with an ES diameter ranging from 4 to 11 mm (Table 1). The higher dimensions belong to the patient from family 4, who exhibited in the left ear a huge ES (11 mm), leading to the complete disruption of the corresponding VA, in which the endolymphatic system is contained (Fig. 1). In the two sisters of family 6, no abnormalities of the inner ear structures were detected by either CT or FSE MRI.

Mutation analysis. *PDS* sequence analyses of the entire 2–20 exons and of the coding portion of exon 21 showed gene mutations in all patients but those of family 6. All mutations have been classified according to the nomenclature recommendations, with the A of the ATG of the initiator Met codon denoted as +1 (37). In the proband of family 1, a homozygous missense mutation in exon 2 (84C>A) was found (Fig. 2). This is a novel mutation leading to the substitution of a serine for an arginine at codon 28 (S28R). No mutations were found in all the other *PDS* coding exons. The parents of the proband showed the same mutation in heterozygosity. In the two sisters of family 2, a compound heterozygous pattern was found: a known mutation in intron 8 (1001+1G>A) and a novel missense mutation in exon 16 (1790T>C; Fig. 2). The first mutation affects a splicing site and the second leads to the substitution of a leucine for a serine at codon 597 (L597S). The proband of family 3 was compound heterozygote for a known single base pair deletion in exon 10 (1197delT) and for a novel intronic mutation in intron 14 (1614+1G>A) (Fig. 2). In particular the exon 10 deletion leads to a frameshift at codon 400 (FS400) with the creation of a stop codon at 431, whereas the intronic mutation affects a splicing site.

The proband of family 4 resulted homozygous for an already described missense mutation in exon 10 (1226G>A). This mutation leads to the substitution of an arginine for a histidine at codon 409 (R409H) and was found in heterozygosity in the mother of the proband, although the father was not available for investigation.

In the two brothers of family 5 a compound heterozygous pattern has been found: a known missense mutation in exon 10

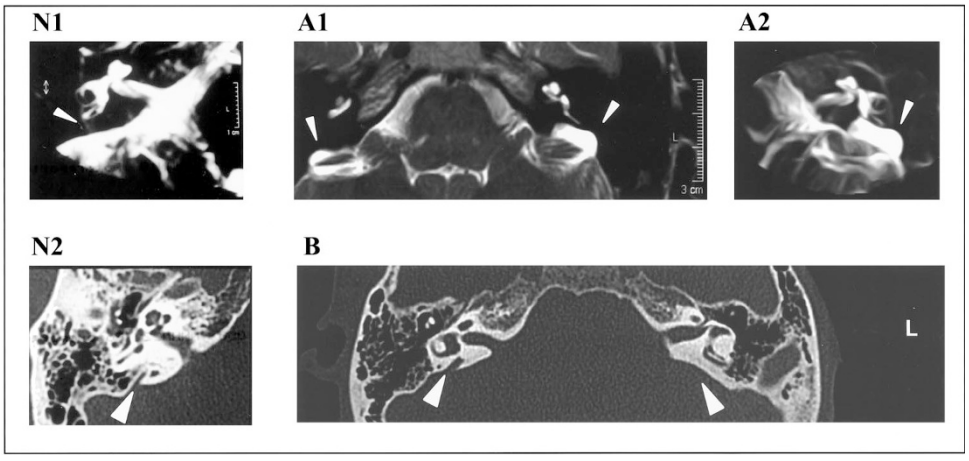

Figure 1. Radiologic studies of the inner ear in a normal subject and in a patient of family 4. *N1*, high-resolution FSSE T_2-weighted MRI scan of the petrous temporal bones. In the normal subject the endolymphatic sac cannot be identified. *A1*, in the proband the ES is enlarged in both ears, with a diameter of 5 mm in the right and 11 mm in the left ear (*arrows*). *A2*, a particular of the left ES in which axial, contiguous, 1-mm images have been summated into a single composite three-dimensional image. *N2*, high-resolution CT scan of the petrous temporal bones (*axial view*). Normal VA. *B*, in the proband the VA of the right ear (*arrow*) is enlarged (2.4 mm). The left VA is disrupted by the very enlarged endolymphatic system (*arrow*).

(1229C>T), leading to the substitution of a threonine for a methionine at codon 410 (T410M), and a novel mutation in exon 4 (398T>A), leading to the substitution of a serine for a threonine at codon 133 (S133T; Fig. 2). The parents showed a heterozygous pattern, T410M/wild-type for the mother, and S133T/wild-type for the father.

Besides the amino terminal mutation (S28R) and the splice site substitutions, the other mutations are located in the II transmembrane domain (S133T), in the IX transmembrane domain (FS400), in the V extracellular domain (R409H and T410M), and in the C- terminus (L597S), according to the model proposed by Everett and collaborators (7).

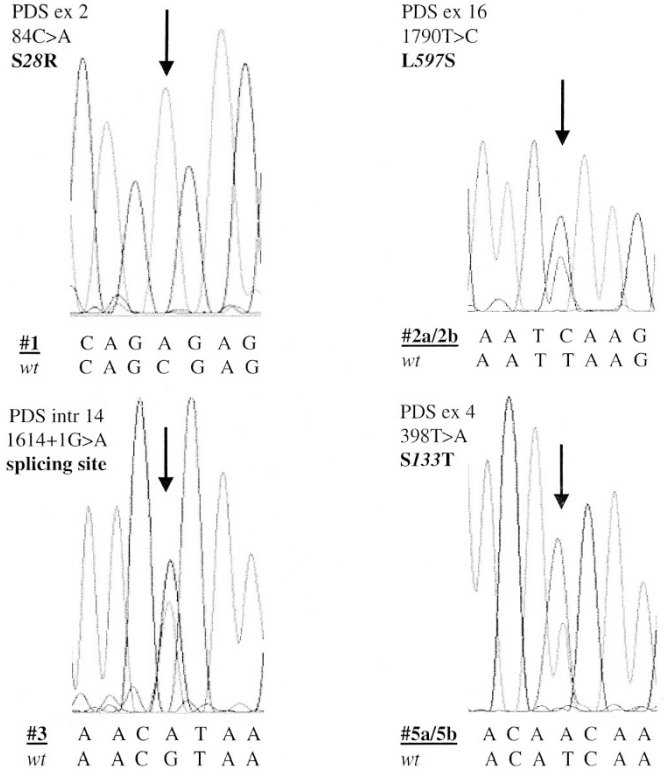

Figure 2. Sequence analysis of the four novel mutations described in patients of familes 1, 2 (2a, 2b), 3, and 5 (5a, 5b). The homozygous 84C>A mutation and the other three heterozygous mutations are indicated by *arrows*.

DISCUSSION

After the characterization of the PDS gene in 1997, more than 40 different mutations have been described scattered all along the 20 coding exons, in families with PS or LVAS (5–13, 34, 38, 39). The majority of the mutations (approximately 60%) are missense substitutions and are localized in the second half of the protein. However, neither hot-spot regions nor phenotype-genotype correlation have been clearly identified. The present study, reporting the characterization of four novel *PDS* mutations and the precise description of the associated clinical picture, aims to give more insights on mutations clustering and genotype-phenotype relationship.

The three novel substitutions described in the present work have not been observed in the normal control population and affect conserved amino acids, as results from the alignments of the *PDS* sequence with two highly homologous human genes, such as *DRA* (down-regulated in adenoma) and *DTD* (diastrophic dysplasia) (7). Thus, it can be expected that these mutations result in impairment of pendrin function. In particular, S133T is located in the II transmembrane domain and likely impairs the stability of the protein at the cell membrane level, as previously reported for mutations affecting other transmembrane domains (8). The L597S lies in the C-terminus, where the majority of *PDS* mutations have been localized (6), therefore suggesting an important role of this domain in pendrin function. Furthermore, after submission of this paper, Campbell *et al.* (39) reported the same mutation in three families with enlarged VA. The substitution leading to the amino-terminal mutation (S28R) was heterozygous in the unaffected parents, and the proband inherited both mutated alleles. The change of an uncharged amino acid (serine) with a bulkier, highly basic, and positively charged amino acid (arginine) is predicted to affect the protein function. Moreover, an amino

acid change in the adjacent residue (E29Q) has been very recently reported in a patient affected with enlarged VA (39). The fourth novel mutation (1614+1G>A, family 3) is located in the first base pair of intron 14, and most probably affects splicing of the *PDS* gene. As no tissues expressing the *PDS* gene were available from this patient, we were unable to analyze the effect of the mutation at the mRNA or protein level. However, there are several examples of mutations occurring at AG/GT invariant intronic sequences of splice sites in other genes, which have been found to impair protein expression or function and therefore cause genetic diseases [reviewed in Krawczak *et al.* (40)]. Intronic mutations affecting splice sites seem to be frequent also for *PDS* gene, and involve intron 4 (12), intron 7 (10), intron 8 [(8, 13), present study], and intron 14 (present study). Moreover, the splice site mutation in intron 8 (1001+1G>A) belongs to the group of the most common mutations reported to date. Indeed, the revision of the literature shows that seven *PDS* mutations are recurrent: L236P [$n = 21$ families; (8, 9, 38, 39)], T416P [$n = 18$; (8, 9, 38, 39)], 1001+1G>A [$n = 18$; (8, 13, 39)], E384G [$n = 7$; (5, 8)], L445W [$n = 6$; (5, 9, 10, 14, 38)], FS400 [$n = 5$; (6, 7, 9)], and T410M [$n = 4$; (8, 38)]. Consistent with these findings, our families harbored, besides the novel, also the mutations 1001+1G>A, FS400, and T410M. Only one mutation (T410M) is a transition in CpG dinucleotides that are frequent sites of point mutation in several other genes (41), and no mutations in G-C–rich regions (defined as four or more consecutive Gs or Cs) have been recorded. Although a cluster of mutations appears to localize in exon 10 (Fig. 3), a founder effect for some of these mutations cannot be ruled out, as most of the families studied originate from Northern Europe.

No phenotypic differences have been found among individuals with different mutations, even considering the patient showing a very premature truncated protein (family 1) and those harboring mutations in the C terminus (family 2). However, although all the patients reported in the present study showed a congenital, severe or profound hearing loss, only one patient (family 4) shared a fluctuating and progressive course associated with a vestibular syndrome. Moreover, in this case the enlargement of the ED and ES of the left ear was so huge to determine the complete disruption of the VA. Further studies are needed to establish whether the amino acid substitution found in this patient (R409H) is associated with an extremely severe ear phenotype accompanied by vestibular symptoms.

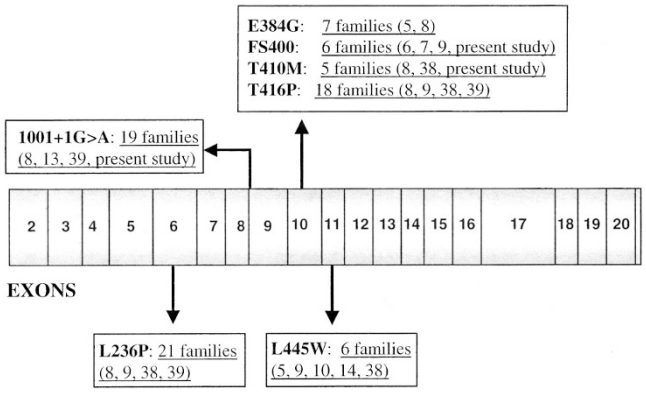

Figure 3. Schematic representation of the *PDS* gene (all but exon 1 and 21 are drawn to scale). The seven more common mutations reported in the literature are shown in *bold* characters, and their distribution on the *PDS* gene is indicated by *arrows*. The number of families harboring each mutation is also reported (references in parentheses). The mutation located in intron 8 (1001+1G>A) is predicted to lead to an altered mRNA splicing. All the other mutations are within the coding sequence and are located in exon 6 (L236P), in exon 10 (E384G, FS400, T410M, and T416P), and in exon 11 (L445W). All mutations are uniformly classified according to the recent nomenclature recommendations (37).

As far as the two sisters without *PDS* mutations (family 6) is concerned, the radiologic examinations and the genetic analysis allowed us to exclude the initial clinical diagnosis of PS. They are likely to be affected with Hashimoto's thyroiditis, as suggested by the presence of antithyroid antibodies and a typical pattern at the ultrasound. We have recently reported a similar case (6) in which, despite a clinical diagnosis of PS, no *PDS* mutations were found, although biochemical and ultrasonography signs of thyroid autoimmunity were present. In both cases an SNHL was shown without inner ear malformations detectable at CT and MRI. Thus, among a total of nine families presenting with a clinical diagnosis of PS [(6) and present study], two exhibit a pseudo-Pendred condition, in which the association of thyroid and hearing impairment is not caused by *PDS* mutations. Because congenital deafness is extremely common, affecting 1 of 2000 newborns (4), and >50 different genes have been found to be involved in it (42), the origin of hearing loss in these cases may be owing to mutations in other deafness genes. However, considering the high frequency of pseudo-Pendred in our series, the hypothesis of a correlation between the thyroid and the ear disorders should be considered. Indeed, it has been postulated that the hearing loss is associated with autoimmunity (43). Positive anti-Tg antibodies and thyroid-stimulating antibodies were found in PS patients by Friis *et al.* (44), and Mayot *et al.* (45) reported anti-thyroid as well as anti-cochlear antibodies in a group of 40 patients with progressive SNHL, suggesting a link between autoimmune inner ear disease and autoimmune thyroid disease. More studies are needed to establish the importance of these observations, but we postulate that in the two families presenting with pseudo-Pendred, the hearing loss may be linked to the thyroid disorder on an autoimmune basis. Clinically, inasmuch as the perchlorate test is not necessarily conclusive of PS as positive tests (20–80%) are found in Hashimoto's thyroiditis (46), the differential diagnosis between PS and pseudo-PS should be based on the study of inner ear malformations that are extremely specific signs and can be considered the only clinical feature always corresponding to the presence of *PDS* mutations.

In conclusion, our data confirm that molecular genetic analysis of the *PDS* gene is an essential requisite to the identification of PS, mostly because of the extreme high variability of the phenotype making the clinical diagnosis difficult to secure. However, as confirmed by our data, *PDS* mutations are widely distributed along the gene, highlighting how time- and money-consuming genetic analysis can be. Hence, efforts should be made to recognize the full range of presentation of the syndrome and to make a correct diagnosis before performing the

molecular studies, taking into account the more specific clinical criteria.

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