CLINICAL STUDY

Clinical and molecular analysis of three Mexican families with Pendred's syndrome

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Abstract

Background: The autosomal recessive Pendred's syndrome is defined by congenital sensorineural deafness, goiter, and impaired iodide organification. It is caused by mutations in the Pendred's syndrome (*PDS*) gene that encodes pendrin, a chloride/iodide transporter expressed in the thyroid, the inner ear, and the kidney.

Objective: To perform a detailed clinical and molecular analysis of patients with Pendred's syndrome from four patients from three unrelated Mexican families.

Methods: Thyroid function tests, perchlorate test, thyroid scintigraphy, audiometry, computer tomography and magnetic resonance imaging were performed in all affected individuals. Haplotype analyses were performed using microsatellite markers flanking the *PDS* locus, and the *PDS* gene was submitted to direct sequence analysis.

Results: All patients presented with sensorineural deafness, Mondini malformations of the cochlea, an enlarged vestibular aqueduct, goiter, and a positive perchlorate test. Two patients were hypothyroid, two individuals were euthyroid. Sequence analysis revealed a complex homozygous deletion/insertion mutation at the end of exon 4 in the index patient of family 1 resulting in a premature stop codon at position 138. In family 2, the affected individuals were compound heterozygous for a splice acceptor mutation (IVS2 –1G > A) and a 1231G > C transversion substituting alanine 411 by proline (A411P). In family 3, the index patient was found to be homozygous for a transversion 412G > T in exon 4 replacing valine 138 by phenylalanine (V138F).

Conclusions: All patients included in this study presented with the classic Pendred syndrome triad and molecular analysis revealed pendrin mutations as the underlying cause. The identification of three novel mutations, one of them of complex structure, expands the spectrum of mutations in the *PDS* gene and emphasizes that they display marked allelic heterogeneity.

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Introduction

Pendred's syndrome, first recognized by Vaughan Pendred in two members of a large family in 1896 (1), is defined by the combination of sensorineural deafness, goiter and a positive perchlorate test (2–4). The most prominent and obligatory clinical sign in patients with Pendred's syndrome is profound sensorineural hearing impairment. In most individuals deafness is prelingual, but it may be progressive and become apparent only later in childhood (5, 6). The hearing loss appears to be particularly pronounced in the high tone range, and vestibular function may also be impaired (2). Patients with Pendred's syndrome

often have a malformation of the inner ear referred to as Mondini defect in which the cochlear turns are replaced by a rudimentary cochlea or a single cavity (7–9). The Mondini defect is, however, not specific for Pendred's syndrome and it is not present in all patients (5, 10). Malformations of the membranous labyrinth with a large vestibular aqueduct in association with an enlargement of the endolymphatic sac and duct may be a more specific diagnostic sign (10, 11).

The thyroid enlargement in Pendred's syndrome typically develops during childhood and is variable both between and within families (2, 3, 12, 13). Iodine deficiency has been recognized as a modifying factor (4), and it is possible that the prevalence of goiters may

be lower in patients with Pendred's syndrome living in iodine-replete regions (14).

The molecular basis of Pendred's syndrome has been unraveled through the cloning of the *PDS* (Pendred syndrome) gene located on chromosome 7q31 (15). The *PDS* gene encodes a highly hydrophobic membrane protein referred to as pendrin, which is predominantly expressed in the thyroid, the inner ear, and the kidney (15, 16). Functionally, pendrin is a chloride/iodide transporter (17). In thyroid follicular cells, pendrin is inserted into the apical membrane suggesting a possible role in iodide transport into the follicle (18), but its exact role in the physiology of the thyroid and the inner ear remains to be defined (19).

Pendred's syndrome may be one of the most common forms of syndromic deafness and it is thought to account for about 10% of all cases with hereditary deafness (4, 5, 13). Of note, *PDS* gene mutations have not only been identified in the classic Pendred's syndrome, but also in non-syndromic autosomal

recessive deafness DFNB4, and in non-syndromic familial enlarged vestibular aqueduct (20, 21). These non-syndromic forms of hearing impairment are thus allelic variants of the syndromic deafness characterizing Pendred's syndrome, and the incidence of sensorineural hearing impairment associated with alterations in the *PDS* gene may be even higher than previously thought (4).

In this study, we present clinical and molecular analyses of patients with classic Pendred's syndrome from three Mexican families.

Subjects and methods

Clinical studies

Family 1 The male patient (II-1) had a small goiter at birth, and he has prelingual deafness (Fig. 1, Table 1). His somatic development was otherwise normal. At the age of 14, he was evaluated at the Thyroid Clinic,

Figure 1 (A) Pedigree and haplotype of family 1. Individual II-1 presented with sensorineural deafness with a Mondini defect, goiter, a positive perchlorate test, and hypothyroidism. He was homozygous for polymorphic markers flanking the *PDS* locus suggesting that his parents have a common ancestor. (B) Mutation in the *PDS* gene in family 1. Direct sequence analysis of the *PDS* gene revealed a complex mutation at the boundary of exon 4 and intron 4 consisting of a deletion of 25 basepairs that is combined with an insertion of 5 basepairs (wild type (wt) sequence in upper chromatogram, homozygous mutation (mut) in lower chromatogram). This mutation results in a premature stop codon at position 138 (V138X) and is thought to truncate pendrin in the third transmembrane domain.

Table 1 Synopsis of clinical, biochemical and molecular findings in the index patients of three Mexican families with Pendred's syndrome.

	Family 1	Family 2		Family 3
	Individual II-1	Individual II-4	Individual II-5	Individual II-4
TSH (0.3–3.5 mU/l)	19	–	–	2.6
T ₄ (77–154 nmol/l)	35	–	–	94
TG (<60 ng/l)	170	–	–	100
Protein bound iodine (4–8 µg/dl)	–	6	2.4	–
Perchlorate test	>50%	30%	90%	20%
Thyroid enlargement	Small goiter at birth. Diffuse goiter on scintigraphy at age 14	Large goiter at age 20	Goiter first noted at age 9	Goiter first noted at age 18. Multinodular goiter 60 g at age 33
Deafness	Prelingual	Prelingual	Prelingual	Prelingual
Audiometry	Sensorineural	Sensorineural	Sensorineural	Sensorineural
CT/MRI	Mondini cochlea	Mondini cochlea	Mondini cochlea	Mondini cochlea
	Enlarged vestibular aqueduct	Enlarged vestibular aqueduct	Enlarged vestibular aqueduct	Enlarged vestibular aqueduct
(Normal axial width of vestibular aqueduct ≤1.5 mm (10))	Axial width: right 3.5, left 4 mm	Axial width: right 4, left 4 mm	Axial width: right 4, left 4 mm	Axial width: right 4, left 3.5 mm
	Enlarged endolymphatic sac	Enlarged endolymphatic sac	Enlarged endolymphatic sac	Enlarged endolymphatic sac
Comments	Thyroid antibodies negative	Clinically euthyroid Thyroidectomy at age 24	Clinically hypothyroid with mental retardation, stunted growth and retarded bone age	
Mutations	del/ins exon 4/intron 4: V138X	Allele 1: IVS2 –1G > A (intron 2) Allele 2: 1231 G > C (exon 10): A411P		412G > T (exon 4): V138F

del, deletion; ins, insertion.

Figure 2 Computer tomography with Mondini cochlea. The coronal section of the inner ear of individual II-1 from family 1 reveals that the normal cochlear turns are replaced by a cavity consisting of one and a half rudimentary turns.

Instituto Nacional Nutrición S. Zubiran, Mexico City. His serum thyrotropin (TSH) was elevated at 19 mU/l (norm: 0.3–3.5), his total thyroxine (T_4) was 35 nmol/l (norm: 77–154), his tri-iodothyronine (T_3) was 3.31 nmol/l (norm: 1.16–3.85), and his thyroglobulin (TG) was 170 ng/ml (norm: <60). Anti-thyroperoxidase and anti-thyroglobulin antibodies were negative. A thyroid scan showed a diffuse goiter. After administration of 1 g perchlorate, he had a discharge of radioiodine of > 50% during the first three hours. The volume of his goiter decreased under treatment with 150 µg levothyroxine per day, and follow-up evaluations documented a euthyroid metabolic state.

Audiometric studies confirmed profound sensorineural deafness with a hearing loss of ≥ 100 dB. Computer tomography (CT) revealed a typical Mondini cochlea (Fig. 2), an enlarged vestibular aqueduct, and magnetic resonance imaging (MRI) showed an enlarged endolymphatic sac. His sister (II-2) has a small goiter and mild hearing impairment.

Family 2 In this family, two individuals presented with Pendred's syndrome (Fig. 3, Table 1). Deafness was diagnosed at the age of 6 months in the affected girl (II-4). At the age of twenty, she had a large goiter of PAHO class III (22), and the perchlorate test was positive with 30% discharge. Clinically she was euthyroid and her protein-bound iodine was in the normal range. Audiological tests confirmed sensorineural deafness. Radiological evaluations documented a Mondini malformation, an enlarged vestibular aqueduct, and an enlarged endolymphatic sac (Table 1). Because of a cold nodule in her right thyroid lobe, she underwent total thyroidectomy at the age of 24 years. The histology of her non-nodular tissue showed heterogeneous microfollicular and macrofollicular alterations with dense colloid (Fig. 4). The cold nodule had the characteristic features of a follicular adenoma and there were no signs of capsular or vascular infiltration.

Her younger brother, II-5, has congenital deafness and is mute. He was only able to stand at the age of 3, and to walk at the age of 5 years. A large goiter was first noted at the age of 9 years and he was allegedly treated with iodine solution. At age 14, he presented with overt clinical hypothyroidism with mental retardation. His height was 132 cm (<3%) and his bone age was retarded by two years. Consistent with these findings, his protein-bound iodine was 2.4 µg/dl (norm: 4–8). His perchlorate test was positive with 90% discharge. Therapy with levothyroxine resulted in reduction of goiter size, normal pubertal development, and acceleration of growth with normalization of his bone age; his adult height is 160 cm (<3%). A Mondini cochlea and an enlarged vestibular aqueduct were found on CT scan, and MRI documented enlargement of the endolymphatic sac, confirming the clinical diagnosis of Pendred's syndrome.

Figure 3 (A) Pedigree and haplotype of family 2. One girl (II-4) and one boy (II-5) were affected and presented the classic Pendred syndrome triad. The boy (II-5) is mentally retarded and he had stunted growth and hypothyroidism. The two affected individuals are compound heterozygous for mutations in the *PDS* gene, the other four siblings are heterozygous for one of the two mutations. wt, wild type. (B) Mutation in the *PDS* gene in family 2. One allele carried a transition of G to A in the splice acceptor site of intron 2 (upper panel). The second allele harbors a transversion of 1231G > C in exon 10 resulting in the substitution of alanine 411 by proline (A411P) (lower panel).

Family 3 The female patient II-4 suffers from congenital deafness (Fig. 5, Table 1). A goiter was first documented at the age of about 18 years and treated with unknown medications. Later, she was treated with levothyroxine or levothyroxine/liothyronine in variable doses. At the age of 31, she treated herself with iodine solutions, but stopped this therapy because of a pruritic skin rash. At the age of 33 years, she was referred to the Thyroid Clinic, Instituto Nacional Nutrición S. Zubiran, Mexico City. She was found to have a multinodular goiter of about 60 g. Her thyroid function tests were in the euthyroid range with a TSH of 2.6 mU/l (0.3–3.5) and a total T_4 of 94 nmol/l (77–154). Her serum thyroglobulin was elevated at 100 ng/l (<60). Thyroid scintigraphy showed the

Figure 4 Thyroid histology of individual II-4 from family 2. Section of non-nodular thyroid parenchyma with multiple follicles of varying sizes that contain dense colloid. Other regions contained tall follicular epithelium with papillary budding (hematoxylin-eosin stain; magnification $\times 100$).

typical findings of a multinodular goiter, the 24-h uptake was normal, and the perchlorate test was positive with a discharge of 20%. Audiometry documented bilateral sensorineural deafness. CT of the inner ear revealed a Mondini cochlea and an enlarged vestibular aqueduct, and MRI documented enlargement of the endolymphatic sac.

Haplotyping

After obtaining informed consent, blood was collected and DNA was extracted from peripheral leukocytes using standard techniques. PCR was performed with fluorescently labeled primers flanking the *PDS* locus (Applied Biosystems, Foster City, CA, USA; Research Genetics, Huntsville, AL, USA) in 15 μ l reactions containing 50 ng genomic DNA. The samples were electrophoresed on 6% denaturing gels (0.4 mm) at 800 V/40 mA/28 W on a DNA sequencer (ABI 373A, Applied Biosystems) and analyzed using the Genescan 672 Software (Applied Biosystems).

DNA sequencing and restriction analysis Exons 2 to 21 of the *PDS* gene were amplified with primers reported by Everett *et al.* (15). The PCR products were purified with Centricon 100 columns (Amicon, Beverly, MA, USA) and both strands were sequenced directly using FS AmpliTaq DNA polymerase with an ABI prism rhodamine dye primer cycle sequencing kit

following the protocol of the supplier (Applied Biosystems). Sequencing products were analyzed on a 373A Sequencer.

One of the detected mutations in family 2 (1231G > C) eliminates an HaeIII restriction site (GGCC > GCCC) and the mutation in family 3 (412G > T) eliminates a DdeI restriction site (CTCAG > CTCAT). The presence of these mutations was therefore independently confirmed by restriction analysis with HaeIII or DdeI (Promega, Madison, WI, USA). One microgram gel-purified PCR product was digested with 20 U of the respective enzyme overnight at 37 °C. The DNA fragments were then resolved on a 1% agarose gel and visualized with ethidium bromide.

Results

Clinical and radiological findings

The pedigrees are shown in Figs 1, 3 and 5 and the main clinical and biochemical findings of the index patients are summarized in Table 1. The affected patients of family 1 and patient II-5 of family 2 were hypothyroid. Given the absence of antibodies against thyroperoxidase and thyroglobulin, it seems unlikely that the patient of family 1 had concomitant autoimmune thyroid disease, a combination that has been reported in other patients with Pendred's syndrome (23, 24). Autoantibodies could not be measured in the hypothyroid patient of family 2, and therefore it is

Figure 5 (A) Pedigree and haplotype of family 3. Haplotype analysis revealed that the index patient II-4 is homozygous for microsatellite markers flanking the *PDS* locus. The genotype of the father is deduced from the findings in the offspring and is therefore shown in italics. wt, wild type. (B) Mutation (mut) in the *PDS* gene in family 3. The index patient harbored a homozygous transversion 412G > T in exon 4 leading to the substitution of valine 138 by phenylalanine (V138F), the mother and the sister were heterozygous for this alteration. This mutation has previously been reported in patients with Pendred's syndrome with compound heterozygosity (26, 27). (C) Restriction analysis with DdeI. Exon 4 of the *PDS* gene was amplified with intronic primers resulting in a product of 272 base pairs. Digestion of the wild type allele results in two fragments of 177 and 95 base pairs. The mutation abolishes the DdeI site.

unknown whether the hypothyroid metabolic state is due to concomitant autoimmune thyroiditis, or the organification defect itself. Patient II-4 from family 2, and patient II-4 from family 3 were euthyroid. The perchlorate test was positive in all individuals with deafness and goiter, and radiographic examinations of the inner ear with CT and MRI demonstrated classic Mondini malformations, an enlarged vestibular aqueduct, and an enlarged endolymphatic sac (Table 1).

Genetic and mutational analysis

Family 1 Direct sequence analysis of the *PDS* gene revealed that the index patient was homozygous for a complex mutation at the boundary of exon 4 and intron 4 (Fig. 1). It consists of a deletion of 25 basepairs, that is combined with an insertion of 5 basepairs, and results in a premature stop codon at position 138 of pendrin, V138X (nomenclature of mutations according to (25)). This residue is thought to be located in the third transmembrane domain according to the model proposed by Everett *et al.* (15), a model that is currently under revision. Pendrin was initially thought to have eleven transmembrane domains, but more recent studies suggest that the carboxy terminus, initially thought to be extracellular, is located within the cell and that the number of transmembrane domains may thus differ from the initial prediction (18; J Zheng & P Kopp, unpublished results).

The parents and the sister were heterozygous for the mutation. Although the parents were not known to be related, the complex nature of this deletion/insertion suggested a common ancestor, a notion that is supported by homozygosity by descent for microsatellite markers flanking the *PDS* locus (Fig. 1). The sister of the patient, who has a small goiter and mild hearing impairment (<30 dB between 125 and 1000 Hz), was heterozygous for the mutation. We cannot formally exclude the possibility that she carries a second mutation in regulatory or intronic sequences. However, the absence of overt sensorineural deafness suggests that her hearing impairment is not caused by true Pendred's syndrome although proper radiological testing was not possible.

Family 2 The two affected members of this family were found to be compound heterozygous for two novel *PDS* mutations. One allele harbored a transition of G to A in the splice acceptor site of intron 2 (IVS2 -1G > A), and the second allele harbored a transversion 1231G > C in exon 10 resulting in substitution of alanine 411 by proline (A411P), an amino acid thought to be located in the fifth extracellular loop based on the model proposed by Everett *et al.* (Fig. 3) (15). The four unaffected brothers were all heterozygous for the splice acceptor mutation (II-2, II-3) or the A411P substitution (II-1, II-6). The presence of the

1231G > C mutation was independently confirmed by restriction analysis with HaeIII (data not shown).

Family 3 Only the mother, the index patient and her sister were available for genetic studies. Haplotype analysis revealed that the index patient II-4 is homozygous for polymorphic markers flanking the *PDS* locus (Fig. 5A). Sequence analysis of the *PDS* gene demonstrated a homozygous transversion 412G > T in exon 4 leading to the substitution of valine 138 by phenylalanine (V138F) (Fig. 5B). The mother and the unaffected sister were both found to be heterozygous for this mutation. The presence of the mutation was independently confirmed by restriction analysis with DdeI (Fig. 5C).

Discussion

After the cloning of the *PDS* gene in late 1997, mutational analysis of patients with Pendred's syndrome revealed marked intragenic heterogeneity (11, 15, 26–31). Moreover, mutations in the *PDS* gene were also found in families classified as having non-syndromic autosomal recessive deafness DFNB4, or hearing loss associated with non-syndromic familial enlarged vestibular aqueduct (20, 21). The majority of the reported *PDS* mutations are simple missense mutations, a smaller subset consists of nonsense mutations with or without preceding frameshift, and there are several mutations affecting splice donor or acceptor sites (11, 32). Recent functional studies suggest that mutations associated with the classic Pendred syndrome may affect the pendrin-induced chloride and iodide transport more severely than mutations found in patients with non-syndromic deafness (33).

In family 1, the unusually complex nature of the mutation, together with the fact that the patient is homozygous for microsatellite markers flanking the *PDS* locus, strongly suggest that the parents have a common ancestor (Fig. 1). The affected patients in family 2 were compound heterozygous for a splice acceptor mutation and a substitution of A411P. The consequences of the splice site mutation are unclear and could not be further addressed in the absence of RNA from thyroid tissue. The substitution of alanine 411 by proline probably disrupts the secondary structure and may thus lead to altered insertion into the plasma membrane, and/or biochemical inactivation of pendrin. The mutation found in family 3, V138F, has previously been reported in patients with Pendred's syndrome (26, 27). In both instances, the V138F substitution was only found in one allele and the patients were compound heterozygous for missense mutations. The detection of this mutation in both alleles in the affected individual further supports the view that this alteration is causally involved in the inactivation of pendrin.

Clinically, all patients of the three families analyzed in this study presented with the triad consisting of congenital sensorineural deafness, goiter, and a positive perchlorate test. On radiological examination, they had Mondini malformations, enlarged vestibular aqueducts and enlarged vestibular sacs. Although the exact function of pendrin in the inner ear is currently unclear, mRNA expression of the mouse ortholog *Pds* was recently documented in the developing vestibular system (16). It was found throughout the endolymphatic duct and sac, in areas of the utricle and saccule, and in the external cochlear sulcus region. This expression pattern involves several regions thought to be important for endolymphatic fluid resorption suggesting that the enlargement of the endolymphatic system is caused by a defect in anion and fluid transport (16). Thyroid enlargement was significant in the four patients included in this study. In one patient (II-1 in family 1), a small goiter was already noted at birth, and it had been documented in all individuals before the age of 20. Whether scarce iodine supplementation, a dietary factor that may have an impact on the degree of thyroid enlargement in patients with Pendred's syndrome, was present in these patients is unknown. Of note, two patients (II-1 in family 1; II-5 in family 2) had overt hypothyroidism. The absence of thyroid antibodies in individual II-1 of family 1 suggests that the hypothyroidism was caused by the organification defect itself, and not by concomitant autoimmune thyroid disease, a recognized confounding entity in patients with Pendred's syndrome (23, 24). Autoimmune thyroid disease could not be excluded in individual II-5 of family 2.

In the affected individuals of the families presented here, the clinical and biochemical findings were consistent with classic Pendred's syndrome, and this diagnosis could be confirmed by malformations of the inner ear and the presence of *PDS* mutations. However, it is important to remember that the diagnosis of Pendred's syndrome is not reliable if it is based solely on clinical findings because of intrafamilial variability in the phenotype and non-syndromic allelic variants (5, 11, 20, 21, 26, 28, 29). In some families, Pendred's syndrome may concur with autoimmune thyroid disease and simple goiter (24), and deafness and goiter caused by distinct genetic and environmental causes have been documented in relatives of patients with Pendred's syndrome (28). Therefore, radiological studies of the inner ear and/or genetic analysis are needed to definitely establish the diagnosis of Pendred's syndrome and its allelic variants.

In conclusion, the detection of three novel mutations in the *PDS* gene in this study of patients with Pendred's syndrome from three Mexican families further emphasizes the large allelic heterogeneity of mutations in this gene. The fact that *PDS* mutations are very diverse, together with the recognition that they are not associated solely with the classic Pendred's triad,

suggests that mutations in this gene may account for an even larger proportion of congenital deafness than anticipated. Molecular analysis can be essential in confirming the diagnosis of Pendred's syndrome or its allelic variants, and will contribute to determine the true prevalence of these disorders.

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