

Phenocopies for Deafness and Goiter Development in a Large Inbred Brazilian Kindred with Pendred's Syndrome Associated with a Novel Mutation in the *PDS* Gene*

P. KOPP, O. KARAMANOGLU ARSEVEN, L. SABACAN, T. KOTLAR, J. DUPUIS, H. CAVALIERE, C. L. S. SANTOS, J. L. JAMESON, AND G. MEDEIROS-NETO

Division of Endocrinology, Metabolism & Molecular Medicine (P.K., O.K.A. L.S., T.K., J.L.J.), Department of Preventive Medicine (J.D.), Northwestern University, Chicago, USA; Unidade de Tiroide, Hospital das Clinicas, University of Sao Paulo Medical School, Sao Paulo, Brazil (H.C., C.L.S.S., G.M.N.).

ABSTRACT

Pendred's syndrome is an autosomal recessive disease characterized by goiter, impaired iodide organification, and congenital sensorineural deafness. The gene mutated in Pendred's syndrome, *PDS* (Pendred's syndrome gene), was cloned very recently and encodes the putative sulfate transporter pendrin. Pendred's syndrome may account for up to 10% of the cases with hereditary hearing loss, and pendrin mutations have also been found in a kindred with non-syndromic deafness. In this study, 41 individuals from a large, highly inbred pedigree from Northeastern Brazil were examined for features of Pendred's syndrome. Linkage studies and sequence analysis of the coding region of the *PDS* gene were performed with DNA from 36 individuals. The index patient, with the classical triad of deafness, positive perchlorate test, and goiter, was found to be homozygous for a deletion of thymidine 279 in exon 3, resulting in a frameshift and a premature stop codon at amino acid 96. This alteration resulted in

truncation of the protein in the first transmembrane domain. Two other patients with deafness were found to be homozygous for this mutation; 19 were heterozygous and 14 were homozygous for the wild type allele. Surprisingly, 6 deaf individuals in this kindred were not homozygous for the *PDS* gene mutation; 3 were heterozygous and 3 were homozygous for the wild type allele, suggesting a probable distinct genetic cause for their deafness. All 3 homozygous individuals for the *PDS* mutation had goiters. However, goiters were also found in 10 heterozygous individuals and in 6 individuals without the *PDS* mutation and are most likely caused by iodine deficiency. In conclusion, we identified a novel mutation in the *PDS* gene causing Pendred's syndrome. The comparison of phenotype and genotype reveals, however, that phenocopies generated by distinct environmental and/or genetic causes are present in this kindred and that the diagnosis of Pendred's syndrome may be difficult without molecular analysis. (*J Clin Endocrinol Metab* 84: 336–341, 1999)

THE association of goiter and congenital deafness was described by Vaughan Pendred (1) in 1898, in a family in which two of five children were deaf mutes and had large goiters. Subsequent reports on families with Pendred's syndrome, many of them highly inbred, documented an autosomal recessive mode of inheritance (2, 3). As first demonstrated by Morgans and Trotter in 1958 (4), the administration of perchlorate in these patients results in a partial discharge of radiolabeled iodide from the thyroid, indicating an impaired organification of this trace element into thyroglobulin. Despite the presence of goiter and a mild organification defect, most Pendred patients are euthyroid. Therefore, it is unlikely that a lack of thyroid hor-

mones, which are essential for the development of the auditory system, are the cause of deafness in these individuals. The sensorineural deafness is typically, but not always, associated with a malformation of the inner ear, referred to as Mondini cochlea, in which the three-coiled structure of the cochlea is replaced by a single cavity in the apical region, and hair-cells and ganglion cells are largely absent. Deafness is most commonly present at birth, but may only become apparent during childhood. The incidence of Pendred's syndrome is thought to be as high as 7.5 to 10 in 100,000 individuals, and it has been estimated to account for about 10% of the cases with hereditary deafness (2, 5). If these estimates are correct, Pendred's syndrome may be the most common form of syndromic deafness.

Initial reports on linkage of Pendred's syndrome to chromosome 7q22–31.1 were confirmed in subsequent studies and demonstrated genetic homogeneity (6–9). These studies also excluded the genes encoding thyroglobulin (chromosomal location 8q24), thyroperoxidase (2p25), or the thyroid hormone receptor α (17q11.2) and β (3p24.3) in the pathogenesis of this syndrome. By studying additional consanguineous kindreds, Everett *et al.* (10) further refined the critical region from about 1.7 centiMorgans (cM) to a genetic distance of 1.1 cM and ultimately identified the gene found to be defective in patients affected with Pendred's syndrome.

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Address correspondence and requests for reprints to: Peter Kopp, M.D., Division of Endocrinology, Metabolism and Molecular Medicine, Northwestern University, Tarry 15, 303 Chicago Avenue, Chicago, Illinois 60611. E-mail: p-kopp@nwu.edu.

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† Current address: Josée Dupuis, Genome Therapeutics Corporation, 100 Beaver Street, Waltham, Massachusetts 02154.

The Pendred syndrome gene (*PDS*) contains an open reading frame of 2343 bp and encompasses 21 exons. The predicted gene product, pendrin, is a highly hydrophobic 780 amino-acid protein with 11 putative transmembrane domains. Based on its high homology to several sulfate transporters found in yeast, plants, and animals, pendrin is thought to exert a similar functional role (10, 11). Remarkably, mutations have not only been found in patients with Pendred's syndrome, but also in a family with non-syndromic deafness (12).

The present study on a large and highly inbred kindred with numerous individuals with deafness and goiter revealed that only a minority of them have true Pendred's syndrome, and that other pathogenic mechanisms generated an identical clinical phenotype in several family members. Because of the possibility of phenocopies, the diagnosis of Pendred's syndrome may therefore be difficult if it is established solely on clinical findings.

Material and Methods

Clinical studies

Some of the clinical features of this pedigree have been presented previously (3, 13). This complex and highly inbred kindred lives in the remote and poorly developed Serra Talhada region of Northeastern Brazil (Fig. 1). Of note, further information led to some revisions of the family trees. The index patient (VI-22), a single female of 37 yr, had congenital deafness, but a normal somatic development and no signs of hypothyroidism. Her parents are first cousins (Fig. 1). Clinically she was found to have a goiter, and on ultrasonographic examination, several small cysts and microcalcifications were found in the enlarged thyroid gland with an estimated weight of 27 g. Computer tomography of the inner ear revealed the presence of a Mondini cochlea. The perchlorate test was positive with a discharge of 26% of the incorporated iodide 2 h after administration of 1 gram KClO₄. Her thyroid function tests confirmed a euthyroid metabolic state: T4 9 µg/dL (4.5–10.5), free T4 1.3 ng/dL (0.95–2.93), T3 120 ng/dL (80–200), TSH 2 mU/L (0.5–4). Her serum thyroglobulin was 23.5 ng/mL, and both anti-thyroglobulin and anti-thyroperoxidase antibodies were normal. A TRH stimulation test (200 µg) showed a relatively pronounced TSH-response: basal TSH 2 mU/L, 30 min after TRH 36.4 mU/L.

After obtaining informed consent, blood was collected from 41 members of the pedigree. Thyroid function was determined with the following assays: serum TSH (GammaCoat IRMA Kit, INCSTAR Corp.,

Stillwater, MN; norm 0.5–4 mU/L); total T4 and T3 (GammaCoat RIA Kit, INCSTAR Corp.; norm 4.5–10.5 µg/dL and 80–200 ng/dL); free T4 (GammaCoat RIA Kit, INCSTAR Corp.; norm 0.95–2.93 ng/dL); thyroglobulin (RIA, Diagnostic Products, Los Angeles, CA; norm 5–25 ng/mL); anti-thyroglobulin antibodies (Diagnostic Products, Los Angeles, CA; norm: negative); and anti-thyroperoxidase antibodies (Dynotest anti-TPO, Brahms Diagnostica, Berlin, Germany; norm < 60 U/mL).

Thyroid weight was estimated by inspection and palpation using the criteria established by the Pan American Health Organization for public health studies conducted in the field (14). In six patients of this kindred it was also possible to examine the thyroid by ultrasonography using a portable ALOKA SSD500 instrument with a 7.5 MHz probe (Aloka Instruments, Tokyo, Japan), confirming the presence of an enlarged thyroid gland in four of these six patients.

The perchlorate test could only be performed in a subset of the patients (VI-4, VI-9, VI-17, VI-20, VI-22, VI-24, VII-15). Two hours after administration of ¹³¹I-iodine (50 µCi), 1 g KClO₄ was administered, and the discharge was determined after 1 and 2 h.

Linkage studies

DNA was extracted from peripheral leukocytes by standard techniques. PCR was performed with fluorescently labeled primers flanking polymorphic loci (PE Applied Biosystems, Foster City, CA; Research Genetics, Inc., Huntsville, AL) in 15 µL reactions containing 50 ng of 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, PE Applied Biosystems) and analyzed using the Genescan 672 Software (PE Applied Biosystems, Foster City, CA).

Statistical analysis

Linkage analysis was performed using the software LINKAGE 5.1, with the subroutines I LINK and M LINK from the FASTLINK 3.0 package (15–17). Two-point logarithm of the odds (LOD) scores were computed separately for the left and right side of the pedigree because of the highly inbred structure. The analysis was performed assuming a fully penetrant autosomal recessive gene with an allele frequency of 0.01. Allelic frequencies of the markers were set to 1/n, where n is the number of distinct alleles present in the pedigree. The analysis was also performed independently for various definitions of the affection status.

DNA sequencing

Exons 2 to 21 of the *PDS* gene were amplified with primers and conditions reported by Everett *et al.* (10). The PCR products were purified with Centricon 100 columns (Amicon, Beverly, MA), and both strands were sequenced directly using FS AmpliTaq DNA polymerase

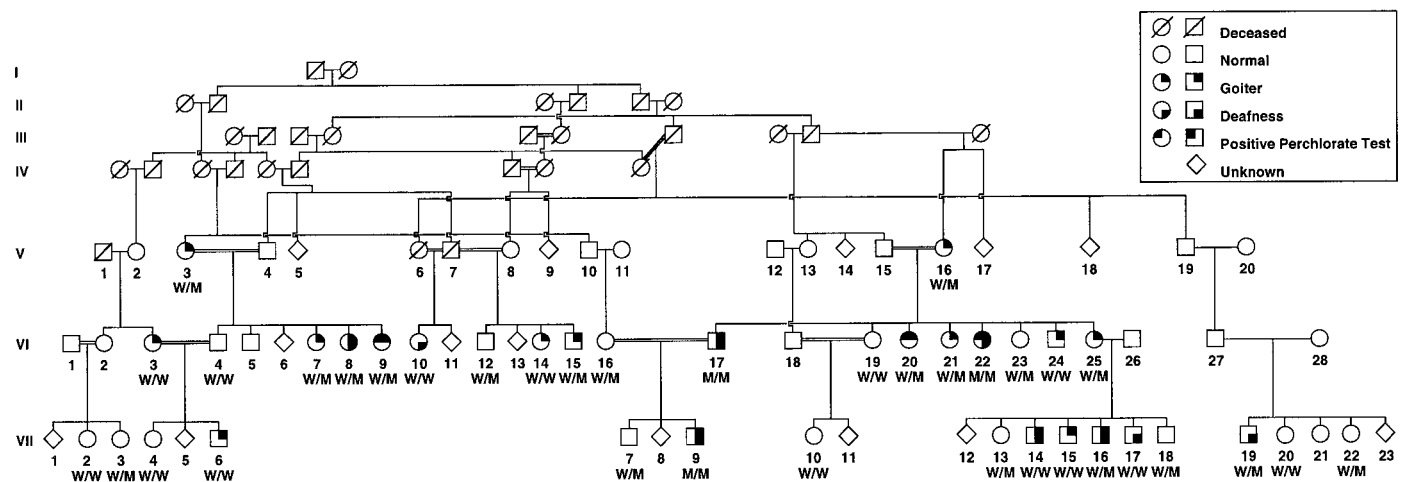


FIG. 1. Pedigree. Phenotypes and *PDS* gene genotypes of a highly inbred kindred from Northeastern Brazil with multiple individuals with prelingual deafness and goiter. The pedigree differs slightly from previous versions (3, 13). Symbols indicating goiter, deafness, and positive perchlorate test are shown in the insert. W = wild type allele for the *PDS* gene; M = mutated allele for the *PDS* gene.

with an ABI prism rhodamine dye primer cycle sequencing kit, following the protocol of the supplier. Sequencing products were analyzed on a 373A Sequencer (PE Applied Biosystems).

ScaI restriction analysis

The detected mutation (see *Results*) eliminates a *ScaI* restriction site at 279 bp of the coding sequence. The presence of the mutation was therefore independently confirmed by restriction analysis with *ScaI* (Promega Corp., Madison, WI). A 411 bp fragment encompassing exon 3 was generated by PCR. Gel-purified PCR product (1 μ g) was digested with 20 U *ScaI* overnight at 37 C. After digestion, the DNA fragments were separated on a 1% agarose gel and visualized with ethidium bromide. Digestion of the wild type allele results in two fragments of 288 and 123 bp.

Results

Clinical studies, correlation of genotype and phenotype

The phenotypes and genotypes of the individuals of this highly inbred pedigree are summarized in Fig. 1. Deafness was found in nine individuals and was of prelingual onset in all of them. Six deaf individuals of this kindred were not homozygous for the *PDS* gene mutation; three were heterozygous, and three were homozygous for the wild type allele.

Results of the thyroid function tests are shown in Fig. 2. All individuals had normal peripheral hormone levels. TSH was minimally elevated with 4.2 mU/L in individual VII-10, a female homozygous for the *PDS* wild type allele, and slightly below the normal range (0.3 mU/L) in the unaffected female VII-21, whose genotype is not known. In addition, serum thyroglobulin levels, which may be elevated in patients with Pendred's syndrome (3), were within normal limits in almost all patients and minimally elevated in three individuals (Fig. 2). The thyroperoxidase antibodies were elevated in a patient homozygous for the wild type allele and normal thyroid function tests (VII-4), but they were normal in all other individuals (data not shown).

Goiters (PAHO class Ib and II) were found in all three individuals homozygous for the mutation, in ten individuals heterozygous for the mutation, and in six individuals homozygous for the wild type allele (Fig. 1). The perchlorate test was performed in seven patients. Using a perchlorate

discharge of over 15% as cut-off, the test was positive (26%) in the index patient (VI-22), but not in patient VI-17, who was also found to be homozygous for the *PDS* mutation (discharge 7%). The low specificity of this test is illustrated by the fact that the heterozygous individuals VI-9, VI-20, and VII-15 had positive perchlorate tests of 22%, 23% and 15%. In two individuals homozygous for the wild type allele (VI-4 and VI-24), the perchlorate tests were normal (0 and 11%).

DNA sequencing and ScaI restriction analysis

Sequence analysis of the *PDS* gene revealed that the index patient (VI-22) with the classical triad deafness, positive perchlorate test, and goiter is homozygous for a deletion of thymidine 279 in exon 3, resulting in a frameshift and a premature stop codon at amino acid 96 (Fig. 3). This premature stop results in truncation of the protein in the first transmembrane domain (Fig. 4). Two other patients with deafness (VI-17, VII-9) were also found to be homozygous for this mutation (Fig. 1). The mutation eliminates a restriction site for *ScaI* at 279 bp of the complementary DNA. This allowed independent confirmation of the mutation by restriction analysis (Fig. 3). In all 36 patients analyzed at the molecular level, the presence or absence of the mutation was determined both by sequence and restriction analysis in 2 separate batches of DNA.

Linkage studies

In the initial phase of the project, linkage studies were performed with polymorphic markers located within or in proximity to the loci for thyroid hormone receptor β , thyroid hormone receptor α , thyroglobulin, and thyroperoxidase. After publication of reports on linkage of Pendred's syndrome to chromosome 7q22-31.1 (6, 7), this chromosomal region was analyzed as well. However, these studies showed no statistically significant linkage to this chromosomal region (data not shown).

After detection of the mutation in the *PDS* gene in the index patient and mutational analysis of the entire kindred, it became apparent that numerous individuals homozygous for the wild type allele, or only heterozygous for the muta-

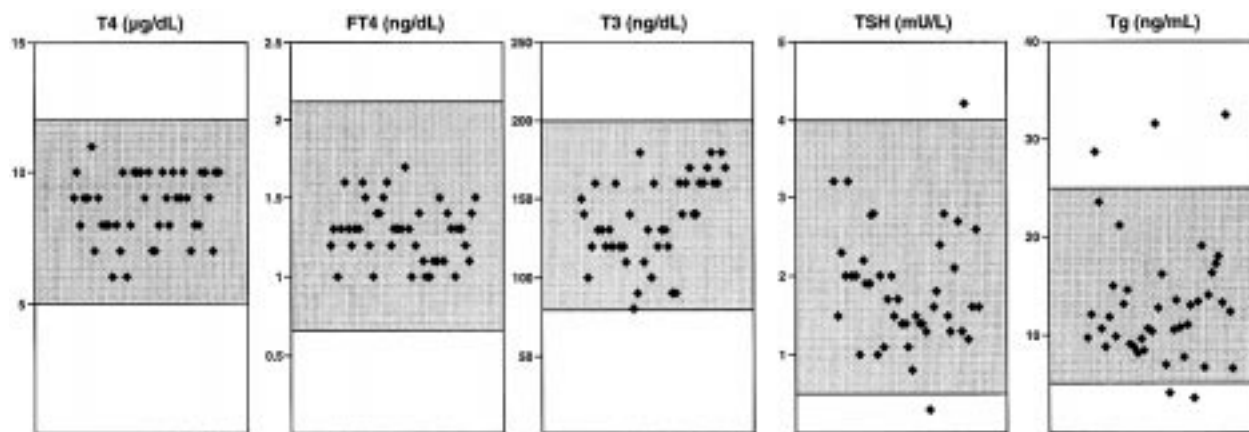


FIG. 2. Thyroid function tests. Serum hormone levels were determined as indicated in *Material and Methods*. All individuals had normal peripheral hormone levels. TSH was minimally elevated, with 4.2 mU/L in individual VII-10, a female homozygous for the *PDS* wild type allele, and slightly below the normal range (0.3 mU/L) in the unaffected female VII-21, whose genotype is not known. Serum thyroglobulin was minimally elevated in three individuals.

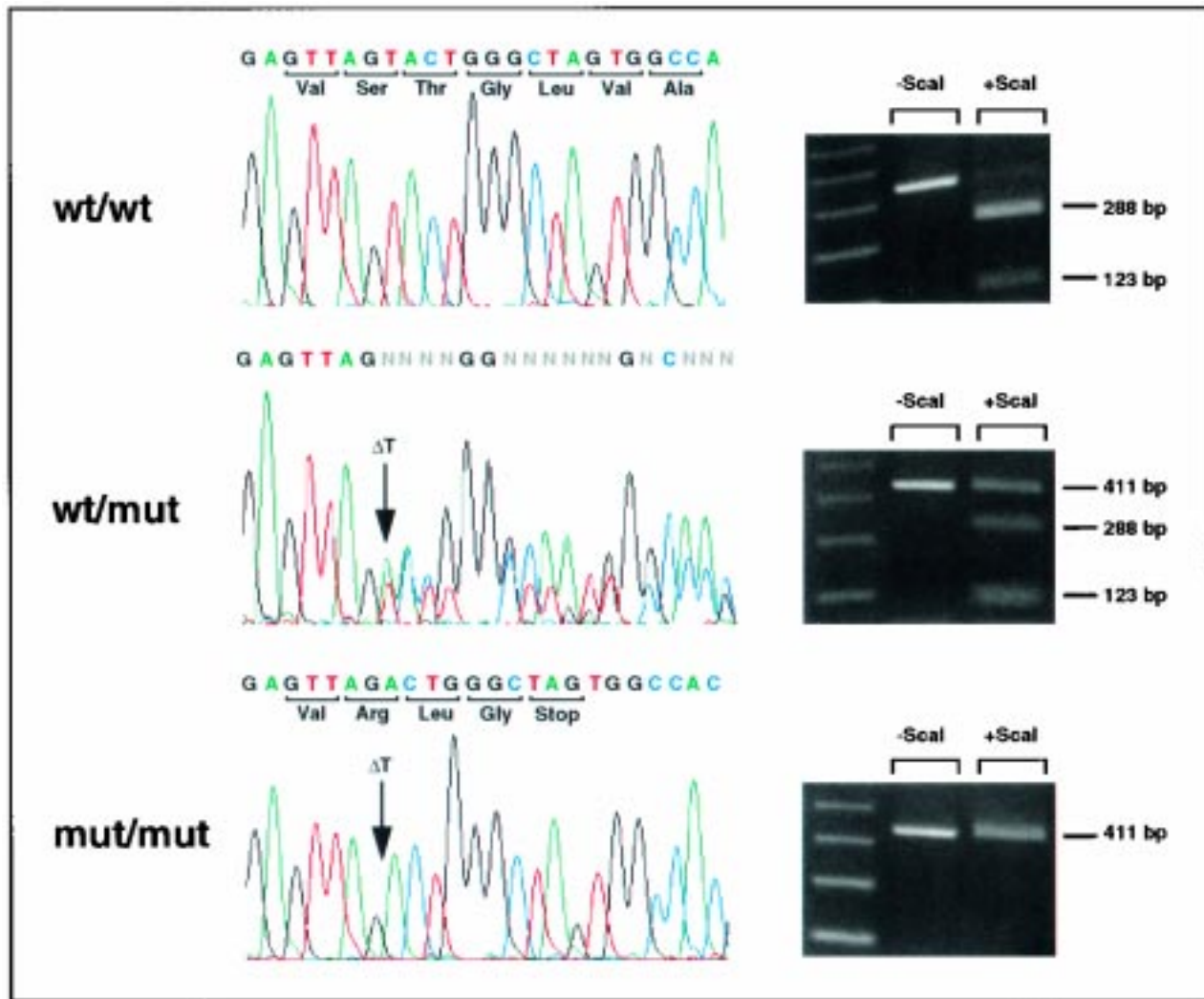


FIG. 3. Sequence and restriction analysis of exon 3 of the *PDS* gene. *Upper panel*: individual homozygous for the wild type allele (VI-19). *Middle panel*: individual heterozygous for the mutated allele (VI-16). *Lower panel*: The index patient (VI-22) with the classical triad deafness, positive perchlorate test, and goiter is homozygous for a deletion of thymidine 279 in exon 3, resulting in a frameshift and a premature stop codon at amino acid 96. The detected mutation eliminates a *Scal* restriction site at 279 bp of the coding sequence. Digestion of the wild type allele results in two fragments of 288 and 123 bp.

tion, also presented with prelingual deafness and goiter (Fig. 1). Haplotype analysis indicated that the three subjects homozygous for the mutation (VI-17, VI-22, VII-9) were homozygous for all determined alleles between D7S501 and D7S523 (Fig. 5). The haplotype of the mother (V-16) of the index patient (VI-22) was homozygous for these same alleles between D7S501 and GATA21H01, but differed between D7S525 and D7S486. Based on this haplotype constellation, it is therefore likely that the heterozygous individuals VI-19, VI-21, VI-23, and VI-25 inherited the affected allele from the mother, whereas VI-20 inherited the mutated allele from the father. Important is the observation that the four brothers VII-14, VII-15, VII-16, and VII-17 with prelingual deafness, have a clearly distinct haplotype (Fig. 5). The fact that three of the seven siblings of this nuclear family are deaf suggests that a distinct genetic defect may be causing the hearing loss in these patients.

Discussion

After the recent cloning of the *PDS* gene, mutations have been described in families with classical Pendred's syndrome (10) and in a kindred with non-syndromic deafness, but without goiters or positive perchlorate tests (12). In the present kindred with a high prevalence of goiters and deafness, sequence analysis led to the identification of a novel mutation in the *PDS* gene causing Pendred's syndrome. The comparison of phenotype and genotype revealed, however, that several individuals who were homozygous for the wild type alleles or heterozygous for the mutation, also have congenital deafness and/or goiters. The presence of these phenocopies explains retrospectively why convincing logarithm of the odds (LOD) scores were not obtained with markers flanking the *PDS* locus on chromosome 7q. Distinct genetic and/or environmental causes resulting in deafness and goi-

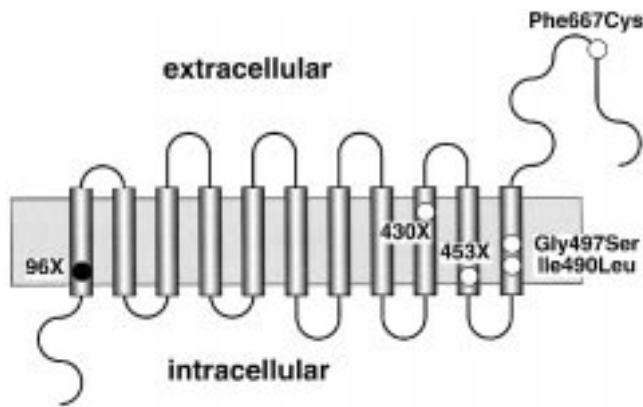


FIG. 4. Location of the mutations identified in syndromic and non-syndromic deafness in the pendrin molecule. The mutation found in this kindred truncates pendrin in the first transmembrane domain (96X; ●). Previously reported mutations (○) in the *PDS* gene of patients with Pendred's syndrome resulted in premature truncations of the protein in transmembrane domain 9 or 10 (453X; 430X), or disruption of a highly conserved region (Phe667Cys) in the extracellular carboxyterminus (10). In the family with non-syndromic deafness associated with a pendrin mutation, a point mutation Gly497Ser in transmembrane domain 11 is thought to have resulted in functional alteration of the protein (12). The mutated allele harbored an additional amino acid change, Ile490Leu.

ter thus occur simultaneously with Pendred's syndrome in this kindred.

Goiter is very frequent in this region of Northeastern Brazil, and the major etiologic factor is iodine deficiency (18). Iodine deficiency has been appreciated as a modifying factor of Pendred's syndrome in several clinical studies (18–20). The high degree of consanguinity in this kindred, together with the pronounced genetic heterogeneity for deafness, make it likely that the prelingual hearing defect is also due to a hereditary cause in the individuals without *PDS* mutations. This is supported by the fact that at least 50% of the patients with prelingual deafness are thought to have a genetic etiology (21), and about 70% are caused by single gene mutations (22).

The mutation found in this kindred truncates pendrin in the first transmembrane domain (Fig. 3). The previously reported mutations in the *PDS* gene of patients with Pendred's syndrome resulted in premature truncations of the protein in transmembrane domain 9 or 10 (frameshift at amino acid 446 with premature stop at 453; frameshift at amino acid 400 with premature stop at 430) or disruption of a highly conserved region (Phe667Cys) in the extracellular carboxyterminus (Fig. 4). In the family with non-syndromic deafness associated with a pendrin mutation, a point mutation Gly497Ser in transmembrane domain 11 is likely to

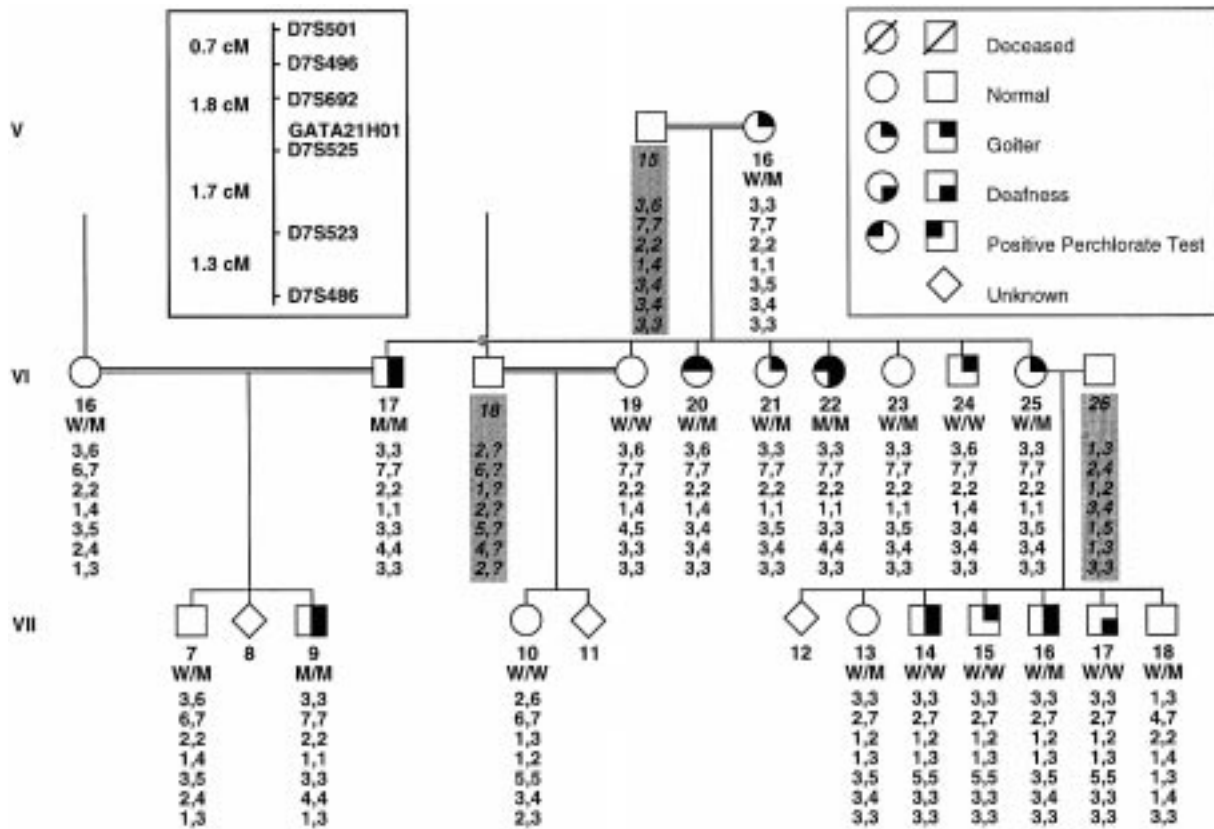


FIG. 5. Haplotype analysis. Haplotype analysis of a part of the kindred with prelingual deafness and goiters. The three subjects homozygous for the mutation (VI-17, VI-22, VII-9) were homozygous for all determined alleles between D7S501 and D7S523. The haplotype of the mother (V-16) of the index patient (VI-22) was homozygous for these alleles between D7S501 and GATA21H01, but differed between D7S525 and D7S486. The heterozygous individuals VI-19, VI-21, VI-23, and VI-25 are thought to have inherited the affected allele from the mother, whereas VI-20 inherited the mutated allele from the father. The three brothers VII-14, VII-16, and VII-17 with prelingual deafness have a distinct haplotype constellation. Haplotypes that have been deduced based on the alleles in the offspring are *in italics* on grey background.

result in a functional alteration of the protein (12) (Fig. 4). The mutated allele harbored an additional amino acid change, Ile490Leu; this rather conservative alteration may be a simple polymorphism, but given the absence of any functional studies on pendrin there is no formal proof for this hypothesis.

Although the function of pendrin is not known, it is thought to be a sulfate transporter based on its high homology to members of this family found in yeast, plants, and animals (10). The closest relatives of the *PDS* gene are the human *DRA* (down regulated in adenoma) and the *DTD* (diastrophic dystrophia) genes (23, 24). Notably, *DRA* is telomerically oriented in a head-to-tail arrangement in close vicinity to *PDS*, suggesting an ancient gene duplication. *DRA* encodes an intestine-specific sulfate transporter which is mutated in congenital chloride diarrhea (23, 25). Mutations in the *DTD* gene can result in various subtypes of chondrodysplasias caused by impaired development and stability of connective tissues (24, 26).

The function of pendrin and its role in thyroid follicular cells, as well as inner ear development and physiology, remain to be defined. As demonstrated by Northern analysis, *PDS* gene expression is almost exclusively found in the thyroid gland (10). Only weak signals were demonstrated in adult and fetal kidney, as well as in fetal brain, and *PDS* expression was also found to be positive by probing a human cochlear cDNA library (10). Thyroglobulin is known to contain sulfate in complex carbohydrates (27–29), and the impaired organification of iodide commonly observed in patients with Pendred's syndrome could point to a role of pendrin in the sulfation of thyroglobulin (30). Of particular interest is the functional role of pendrin in the inner ear, as *PDS* defines a new class of deafness genes, a category that is rapidly expanding (31). Pendrin may play a role in inner ear development since one of the most striking features of Pendred's syndrome is the Mondini cochlea. Based on the fact that mutations in sulfate transporters like *DTD* can cause chondrodysplasias, it is tempting to speculate that mutations in *PDS* may cause a local form of chondrodysplasia resulting in malformation of the cochlea. Alternatively, pendrin may also play a role as transporter of sulfate or other anions in the inner ear.

In conclusion, a subset of individuals in this complex kindred with the characteristic signs for Pendred's syndrome, deafness and goiter, are homozygous for a novel mutation in the *PDS* gene. This report illustrates, however, that clinical evaluation may be confounded by the presence of phenocopies caused by distinct environmental or genetic factors. This observation, together with the findings of Li *et al.* (12) on a family with non-syndromic deafness without cochlear malformations, indicates that analysis of the *PDS* gene may be crucial for establishing an accurate diagnosis. This study gives further support to the notion that the non-syndromic deafness linked to the DFNB4 locus on chromosome 7q31 is in fact an allelic form of the syndromic hearing loss in Pendred's syndrome (32). Further studies on patients with Pendred's syndrome are particularly important because this disorder may be the cause of both syndromic and non-syndromic deafness in a large number of patients. Molecular analysis of the *PDS* gene is useful in making a definite diagnosis and will be helpful in determining the true prevalence of this disorder.

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