High phenotypic intrafamilial variability in patients with Pendred syndrome and a novel duplication in the SLC26A4 gene: clinical characterization and functional studies of the mutated SLC26A4 protein

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Abstract

Objective: Pendred syndrome (PS) is characterized by the association of sensorineural hearing loss (SNHL) and a partial iodide organification defect at the thyroid level. It is caused by mutations in the SLC26A4 gene. The encoded transmembrane protein, called pendrin, has been found to be able to transport chloride and other anions.

Design: The aim of the present study was to characterize a family with PS, which shows a strong intrafamilial phenotypic variability, including kidney atrophy in one member. The age of disease-onset was significantly different in all three affected siblings, ranging from 2 to 21 years for thyroid alterations and from 1.5 to 11 years for SNHL.

Methods: Clinical and genetic studies were carried out in affected siblings. The functional activity of the novel duplication found was studied by a fluorimetric method in a human renal cell line (HEK293 Phoenix) in which the protein was overexpressed.

Results: All three siblings were found to be compound heterozygotes for the missense mutation (1226G>A, R409H) and for a novel 11 bp duplication (1561_1571CTTGGAATGGC, S523fsX548). The latter mutation creates a frameshift leading to the loss of the entire carboxy-terminus domain. Functional studies of this mutant demonstrated impaired transport of chloride and iodide when expressed in HEK 293 Phoenix cells, when compared with wild type pendrin.

Conclusions: A novel 11 bp duplication was found in a family with Pendred syndrome, showing a high intrafamilial phenotypic variability. An impaired transmembrane anionic transport of the mutated SLC26A4 protein was demonstrated in functional studies using a heterologous cell system.

Introduction

In 1997, the SLC26A4 (or PDS, OMIM 605646) gene was cloned and mapped on chromosome 7q22-31.1 (1). It codes for a protein called pendrin, which is mainly expressed not only in the thyroid, inner ear, and kidney, but also in the breast, endometrium, placenta, and testis (2–7). Pendrin is thought to be composed of 12 transmembrane domains with an intracellular N-terminus and C-terminus (8, 9). Mutations of this gene have been associated, in compound heterozygosity or in homozygosity, with Pendred syndrome (PS), an autosomal recessive disorder first described in 1896 and characterized by the association of sensorineural hearing loss (SNHL) and goiter (10). The phenotype of PS is highly variable with regard to deafness, goiter, and thyroid function (12–15). Sensorineural deafness is congenital, and in most cases has a progressive course. However, in some patients it is already severe at birth. Deafness is associated with radiologically detectable structural malformations of the inner ear. In particular, typical malformations are both an enlarged vestibular aqueduct (EVA) and an enlarged endolympathic duct and sac (EED and EES) (16, 17). SNHL is a constant feature of PS, whereas goiter is observed in about 50% of the affected individuals and can vary from a slight enlargement to a large multinodular goiter, with the time of onset ranging from the first months of life until puberty. The goiter is the consequence of a partial iodide organification defect, as demonstrated by the positive perchlorate discharge test. However, the defect is only partial and most patients are euthyroid or subclinically...
hypothesis that patients affected with PS may have disturbances of renal function, especially in the regulation of electrolytes and acid–base balance. However, no abnormalities of systemic pH or serum chloride levels have been reported in PS patients, possibly due to the redundancy of compensatory mechanisms in the kidney.

After cloning of the SLC26A4 gene, more than 100 different mutations have been described (http://www.medicine.uiowa.edu/pendredandbor) in patients with classical PS or affected with a non-syndromic hearing loss named familial EVA syndrome or DFNB4 and characterized by the typical inner ear malformations without thyroid abnormalities (24). The vast majority of PDS mutations involve a single nucleotide (either missense, non-sense, or splice-site). In the present study, a novel 11 bp duplication of the SLC26A4 gene is described and functionally characterized. Interestingly, this mutation was found in compound heterozygosity in a family with an extremely variable phenotype.

Materials and methods

Patients

Patients MM, MA, and MC were born to apparently non-related parents who have normal hearing, no signs of hypothyroidism and no goiter. Patient MC came to our attention for pre-conceptional genetic counseling.

Patient MM, female aged 45 years, had a diagnosis of SNHL at the age of 5 years. At that time, she had normal speech. At the age of 5 years and 4 months, the diagnosis of subclinical hypothyroidism with low free thyroid hormones levels and elevated TSH (8.1 mU/l) was made and L-T4 replacement treatment was immediately started. Thyroid ultrasound showed a slightly enlarged gland. Patient MC had a normal IQ. None of the three patients reported symptoms suggestive of vestibular dysfunction.

Clinical analyses

Serum TSH, free T4, free T3, thyroglobulin, and anti-thyroglobulin/anti-thyroperoxidase antibodies levels were measured by an immunometric assay (AutoDelfia; Wallac, Turku, Finland). Normal value ranges are 0.25–4.2 mU/l for TSH, 9–20 pmol/l for FT4, and 3.8–8.0 pmol/l for FT3. In all patients, ultrasound (US) of the neck was carried out, and the thyroid volume (TV) was calculated by the ellipsoid formula.

A thyroid scintigraphy (TS) with 123I and a perchlorate discharge test were performed. In particular, 123I was administered intravenously and counts were obtained at 1 and 2 h. For the discharge test, 1 g of potassium perchlorate (KClO4) was administered orally and the uptake was calculated after 1, 2, and 3 h respectively.

In the three patients, the presence of alterations of the cochlea and of the VA was tested by high-resolution computed tomography (CT) of temporal bones in the 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 (16). High-resolution fast-spin echo (FSE) T2-weighted magnetic resonance imaging (MRI) was carried out (in axial...
and coronal planes) to study the membranous labyrinth and in particular the endolymphatic duct and sac (ED and ES). ES is rarely seen in normal subjects, despite high-quality FSE MR images, and it is considered enlarged when the diameter exceeds 2.5 mm (17).

**Molecular studies**

DNA was extracted by standard methods from the whole blood of the three patients and their parents. In patient MC, all 20 coding SLC26A4 exons were amplified using primers flanking each exon, as previously described (1). The experimental studies were approved by the ethics committees of each Institution. Informed consent was obtained for all screened subjects.

**Functional analysis**

In order to evaluate the functional activity of the novel mutation found in the present family, the properties of pendrin transport were studied by a fluorimetric method in a human renal cell line (HEK293 Phoenix) in which the protein was overexpressed (21). To this purpose, a plasmid containing the mutated SLC26A4 sequence was generated by site-directed mutagenesis (Quik Change site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA) starting from the SLC26A4WT cDNA previously cloned and characterized (25). The following primers were used: forward: 5'-CCTTCTTGGATG-GACTTGGAATGGCCTTGGAAGCATCCCTAGC-3', and reverse: 5'-GCTAGGGATGGCCTTTCCAAGGCCATTCCAAGTCCATTCCAAGAAGG-3'.

**Cell culture and transient transfection**

HEK 293 Phoenix cells were grown as previously described (21). For in vivo fluorometric chloride/iodide measurements, HEK 293 Phoenix cells were transiently transfected with wild type pendrin (pendrinWT) or mutated pendrin showing the duplication (pendrinS523fsX548), cloned into the pIRES2-EYFP vectors; cells transfected with the empty plasmid (pIRES2-EYFP) were used as negative control. The vector used in this study allows to coexpress at the same time pendrin or its mutant as well as EYFP, by transfecting only one plasmid. The bicistronic mRNA will produce two separated proteins in the same cell; in this way, the functional integrity of the protein under study will be maintained, and the transfection efficiency will be easily determined, as the transfected cells will show yellow-green fluorescence. The day before transfection, cells were seeded in poly-L-lysine-treated glass coverslip (Ø 40 mm), placed into Petri dishes (Ø 60 mm), and grown to 60–80% confluency. HEK 293 Phoenix cells were transfected by calcium–phosphate coprecipitation method: for each coverslip, 6 μg plasmid dissolved in 162 μl water were mixed with 18 μl buffer A (2.5 M CaCl₂, pH 5.8) and 180 μl buffer B (140 mM NaCl, 1.5 mM Na₂HPO₄, 50 mM HEPES, pH 7.05 adjusted with NaOH). After incubation for 15–20 min at room temperature, the transfection mixture was spread over the cells. The experiments were performed 48 h after transfection.

**Fluorometric analyses**

A fluorimetric method previously validated by our group was used to evaluate the chloride/iodide transport of SLC26A4 harboring the novel 11 bp duplication found in the family studied in the present work (26). Briefly, since the EYFP protein is a fluorescent dye sensitive to intracellular halide amount, fluorescence is measured before and after substituting extracellular chloride with iodide. Considering that iodide is a much better EYFP quencher than chloride, an increase of intracellular iodide should lead to a decrease of the EYFP fluorescence. In particular, to evaluate the pendrin-induced halide (chloride and iodide) transport, HEK 293 Phoenix cells transiently transfected with the vector containing either pIRES2-EYFP-pendrinWT or the 11 bp duplication or with the empty plasmid, were continuously perfused in a laminar-flow chamber (FCS2 System, Biophtechs Inc., Beck Road Butler, PA, USA) with ‘isotonic high Cl – K’ (2 mM KCl, 135 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM d-glucose, 20 mM HEPES, pH 7.4, 308 mM with mannitol) or ‘isotonic high I – K’ (2 mM KCl, 135 mM NaI, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM d-glucose, 20 mM HEPES, pH 7.4, 308 mM with mannitol) solutions. The used iodide concentrations, which are beyond physiological iodide concentrations, were chosen to enhance fluorescence sensitivity to ion exchange. The EYFP fluorescence measurements were performed using a Leica TCS SP2 AOBS confocal microscope (Leica Microsystems, Heidelberg, Germany) with a 515 nm Ar/ArKr laser beam for exciting the EYFP fluorescence. Traces representing fluorescence time course were obtained analyzing the regions of interest (ROI) within single cells. Measurements started after steady state conditions were reached. Maximal fluorescence variations (ΔF%) represent the maximal observed percentage difference with respect to the fluorescence intensity at the moment of the solution substitution. Statistical analysis was done by ANOVA–Bonferroni’s multiple comparison test.

**Results**

**Clinical data**

All patients had a normal thyroid function under L-T₄ replacement treatment (1.25, 1.5, and 1.6 μg/kg/day for
patient MM, MA, and MC, respectively). Anti-Tg and anti-TPO antibodies were negative in the three patients. Upon ultrasound examination, patient MM showed a small multinodular goiter (23 ml). Patient MA had a normal TV (7.2 ml), without echographic pattern alterations and nodules, while patient MC had a large multinodular goiter (65.7 ml) with tracheal deviation. The perchlorate (KClO₄) test, performed by measuring radiolabeled iodide thyroid uptake before and after the oral administration of 1 g of KClO₄, was positive in all patients with discharge rates of 40–60% of basal radiiodine uptake.

In the three siblings, malformations of the inner ear were documented. At CT scan, the VA was bilaterally enlarged, with a diameter ranging from 2 to 4 mm. The ED and ES resulted as enlarged in all patients at MRI examination, with a ES diameter ranging from 4 to 11 mm. Patient MA also had a dysplastic cochlea (Mondini malformation), which was not found in patients MM and MC (Fig. 1).

**Mutation analysis**

SLC26A4 sequence analyses showed a compound heterozygous pattern in the three patients: a known common missense mutation in exon 10 (1226 GA) and a novel 11 bp duplication in exon 14 (1561_1571dupCTTG-GAATGGC; Fig. 2). The first mutation leads to the substitution of arginine for histidine at codon 409 (R409H) and the second leads to a frameshift with the

**Figure 1** Radiological studies of the inner ear in patient MA. (A) CT scan of the petrous temporal bones (axial view). The vestibular aqueduct is bilaterally very enlarged (arrows). A cochlear dysplasia is also present. (B) MRI scan of the petrous temporal bones. The endolympathic duct and sac, which are usually not visible in normal subjects, are greatly enlarged in both ears (arrows).

**Figure 2** The sequences corresponding to a wild type and a mutated allele are reported. The nucleotide and aminoacidic sequences are also shown.
creation of a stop codon at 548. According to the nomenclature used, the latter mutation is denoted as S523fsX548 (27). Both mutations were inherited from the non-consanguineous parents. Whereas the mother is heterozygous for the 11 bp duplication mutation (S523fsX548), the father is heterozygous for the missense mutation (R409H; Fig. 3).

**Functional analyses**

After expressing pendrinS523fsX548, an impaired Cl⁻/I⁻ exchange can be documented in HEK 293 Phoenix cells (Fig. 4A). The intracellular fluorescence after iodide substituting for chloride in the bath solution (135 mM iodide instead of chloride) is decreased if wild type pendrin (pendrinWT) is expressed. Conversely, following the same protocol, after the expression of pendrinS523fsX548 no decrease in fluorescence can be observed. A similar result was obtained when the empty vector was used for transfection (in both latter cases a slight increase of fluorescence can be observed instead of a decrease; Fig. 4B).

Returning to the initial conditions (i.e. 135 mM chloride instead of iodide in the bathing solution) results in an increase in fluorescence (positive ΔF%) for pendrinWT as a consequence of the diminished quenching of EYFP that follows the iodide efflux in exchange with chloride. Control and pendrinS523fsX548 behave differently, continuing to show a slight increase in fluorescence as already observed when iodide substituted for chloride. The observed increase possibly reflects some osmotic perturbation induced by solution substitutions leading to an aspecific anion leakage and/or cell shrinkage.

**Discussion**

Mutations in SLC26A4 appear to be responsible for a number of overlapping clinical conditions ranging from nonsyndromic hearing loss (familial EVA syndrome or DFNB4) with widened VAs to typical PS with evident thyroid signs. The majority of SLC26A4 variants are missense substitutions, while a smaller subset consists of nonsense mutations with or without frameshift, and there are several mutations affecting splice donor or acceptor sites.

The present study reports the functional characterization of a novel 11 bp duplication (resulting in S523fsX548), which was found in compound heterozygosity with a missense mutation leading to substitution of R409H. This duplication creates a frameshift with stop codon at 548 and the deriving truncated protein lacks the entire carboxy-terminus...
maximal fluorescence variation of the pendrinWT is significantly different from the pendrinS523fsX548 and the empty vector (calculated by ANOVA–Bonferroni’s multiple comparison test). The respective changes of the extracellular solutions (isotonic high Cl⁻ column, the average of different ROI inside the cells (pendrinWT fluorescence % variation after the ionic substitution is calculated as K₈) are indicated. (B) Maximal fluorescence % variation after the ionic substitution is calculated as the average of different ROI inside the cells (pendrinWT n = 7; pendrinS523fsX548 n = 8, empty vector n = 16). In every panel, the maximal fluorescence variation of the pendrinWT is significantly different from the pendrinS523fsX548 and the empty vector (calculated by ANOVA–Bonferroni’s multiple comparison test).

Figure 4 HEK 293 Phoenix cells expressing pendrinS523fsX548 have an impaired I⁻ transport. (A) Relative fluorescence intensity values were obtained analyzing the ROI within single cells transfected with pIRE2-EYFP-pendrinWT (n = 7), pIRE2-EYFP-pendrinS523fsX548 (n = 8), or the empty (n = 16) plasmids and exposed to the ‘high Cl⁻’ or the ‘high I⁻’ solutions. (B) Maximal fluorescence % variation after the ionic substitution is calculated as the average of different ROI inside the cells (pendrinWT n = 7; pendrinS523fsX548 n = 8, empty vector n = 16). In every panel, the maximal fluorescence variation of the pendrinWT is significantly different from the pendrinS523fsX548 and the empty vector (calculated by ANOVA–Bonferroni’s multiple comparison test).

In conclusion, the identification of novel complex mutation expands the spectrum of mutations in the SLC26A4 gene. The impairment of chloride and iodide transport recorded for the mutant further supports the hypothesis that pendrin could play a crucial role in iodide transport from the thyroid cell to the colloid. The clinical studies on the present family confirm that the PS phenotype is extremely variable, both for the degree of impairment and the time of onset, the inner ear malformations being the only constant phenotypic feature.

References


3 Everett LA, Morsli H, Wu DK & Green ED. Expression pattern of the mouse ortholog of the Pendred’s syndrome gene (Pds) suggests a key role for pendrin in the inner ear. PNAS 1999 96 9727–9732.


8 Raux GM, Royaux IE, Mort-Aoki A, Everett LA, Kohn LD & Green ED. Pendrin, the protein encoded by the Pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells. Endocrinology 2000 141 839–845.


12 Wolff J. What is the role of Pendrin? Thyroid 2005 15 346–348.


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