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Identification of Allelic Variants of Pendrin (SLC26A4) with Loss and Gain of Function

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Key Words

Pendrin • Inherited hearing loss • Pendred syndrome • Hypertension • Asthma • Functional tests

Abstract

Background: Pendrin is a multifunctional anion transporter that exchanges chloride and iodide in the thyroid, as well as chloride and bicarbonate in the inner ear, kidney and airways. Loss or reduction in the function of pendrin results in both syndromic (Pendred syndrome) and non-syndromic (**n**on-**s**yndromic **e**nlarged **v**estibular **a**queduct (ns-EVA)) hearing loss. Factors inducing an up-regulation of pendrin in the kidney and the lung may have an impact on the pathogenesis of hypertension, **c**hronic **o**bstructive **p**ulmonary **d**isease (COPD) and asthma. Here we characterize the ion transport activity of **w**ild-**t**ype (WT) pendrin and seven of its allelic variants selected among those reported in the **s**ingle **n**ucleotide **p**olymorphisms **d**ata **b**ase (dbSNPs), some of which were previously identified in a cohort of individuals with normal hearing or deaf patients belonging to the Spanish population. Methods: WT and mutated pendrin allelic variants were functionally characterized in a heterologous over-expression system by means of fluorometric methods evaluating the I^-/Cl^- and Cl^-/OH^- exchange and an assay evaluating the efflux of radiolabeled iodide. Results: The transport activity of pendrin P70L, P301L and F667C is completely abolished; pendrin V609G and D687Y allelic variants are functionally impaired but retain significant transport. Pendrin F354S activity is indistinguishable from WT, while pendrin V88I and G740S exhibit a gain of function. Conclusion: Amino acid substitutions involving a proline always result in a severe loss of function of pendrin. Two hyperfunctional allelic variants (V88I, G740S) have been identified, and they may have a contributing role in the pathogenesis of hypertension, COPD and asthma.

Introduction

Pendrin (PDS, SLC26A4) is a multifunctional anion exchanger [1, 2] expressed in tissues as diverse as the thyroid gland, inner ear, kidney, and airways. Pendrin plays different roles in these various tissues and human

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pathological conditions are well established (Pendred syndrome, **e**nlarged **v**estibular **a**queduct (EVA)) or hypothesized (hypertension, **c**hronic **o**bstructive **p**ulmonary **d**isease (COPD), asthma) to be associated with loss of function of pendrin or its upregulation, respectively.

Pendrin malfunction mainly affects the thyroid gland and the inner ear. In the thyroid gland, pendrin is expressed on the apical membrane of thyrocytes [3] and most likely exchanges iodide for chloride [4, 5], thereby mediating iodide flux into the follicular lumen [6]. In the inner ear, pendrin is expressed in the endolymphatic sac and duct, saccule, utricle, ampulla and cochlea [7] and is responsible for bicarbonate secretion into the endolymph [8, 9], thereby controlling and conditioning its pH and ion composition. Pendrin reduction or loss of function causes both syndromic (Pendred syndrome) and non syndromic hearing loss. Pendred syndrome (OMIM#274600) [10] is an autosomal recessive disease characterized by the association of sensorineural hearing loss and a partial iodide organification defect, clinically revealed by a positive perchlorate discharge test, with or without goiter and hypothyroidism [11, 12]. Deafness is due to malformations of the inner ear, typically an EVA or a dysplastic (Mondini) cochlea [13], as well as alterations of the endocochlear potential [8]. Moreover, monoallelic [14-16] or biallelic [16] mutations of the pendrin gene can result in **n**on-**s**yndromic **e**nlarged **v**estibular **a**queduct (ns-EVA) without an obvious thyroid phenotype.

The pathological conditions potentially linked to hyperfunction and/or overexpression of pendrin are related to the role of the transporter in the kidney and airways. In the kidney, pendrin is expressed on the apical membrane of β and non-α, non-β intercalated cells within the cortical collecting duct of the nephron [17, 18] and mediates bicarbonate secretion [17, 19] via exchange of bicarbonate for chloride [1]. Recently it has been proposed that pendrin could contribute to the pathogenesis of hypertension [20]. Indeed, pendrin expression and activity are upregulated by aldosterone analogues [21] and angiotensin II [22] and, interestingly, pendrin knock-out mice are protected against hypertension induced by aldosterone analogues [21, 23]. As a consequence, it has been suggested that pendrin-mediated chloride transport through intercalated cells might represent a potential target for blood pressure control [24]. In the airways, an increase in pendrin mRNA expression has been described in murine asthma models [25] and has been associated with mucus overproduction in murine models of bronchial asthma and COPD [26]. Moreover, chloride reabsorption via pendrin could participate in the control of airway surface liquid thickness [27]. An excess of chloride reabsorption could be paralleled by water reabsorption, leading to the production of a compact layer of dehydrated mucus, with a possible role in exacerbations of asthma and COPD. This phenomenon is particularly important during inflammation, when pro-inflammatory cytokines lead to increases in pendrin expression [25-30]. These findings collectively suggest that pendrin may be a therapeutic target candidate for bronchial asthma and COPD.

Functional tests of mutated pendrin allelic variants found in patients with Pendred syndrome or ns-EVA revealed that the pathology is linked to a reduction or a loss of function in pendrin activity [31]. More than 170 mutations within the pendrin gene have been identified so far (http://www.healthcare.uiowa.edu/labs/pendredandbor/slcMutations.htm), but the functional characterization for only a minority of them is available (for more detailed information regarding the functional characterization of pendrin and its allelic variants, see [32]). Besides clinical and radiological assessments, sequencing of the pendrin gene is essential for a definitive diagnosis of Pendred syndrome and ns-EVA [13]. In addition, in cases where mutations within the pendrin coding region are detected, the diagnosis could be corroborated by a functional test of the respective allelic variant. Indeed, the clinical condition of pseudo-Pendred syndromes [33-36] and the high incidence of benign functional pendrin polymorphisms in some populations [37] could lead to an incorrect definition of the genetic cause of the disease. Single nucleotide pendrin polymorphisms are continuously detected and reported in the **s**ingle **n**ucleotide **p**olymorphisms **d**ata**b**ase (dbSNPs) of the National Center for Biotechnology Information. Some of them (Table 1) have also been found in a group of normal-hearing Spanish controls (F354S, F667C), as well as in a cohort of deaf Spanish patients and controls (V609G, G740S) [37].

Here we set out to determine the function of seven selected allelic variants of pendrin (P70L, P301L, F354S, V609G, F667C, D687Y, G740S, Table 1), all of which are reported in the dbSNPs, with the aim to (i) discriminate between benign polymorphisms and potentially pathological mutations, (ii) identify amino acids substitutions with functional impact and (iii) compare and validate results obtained with two different methods (fluorometric and radiometric) for evaluating the iodide transport ability of pendrin. In addition, the transport activity of the previously characterized pendrin allelic

variant V88I [37] was further explored.

Materials and Methods

Cloning procedures and plasmid constructs

Standard procedures were used for DNA preparation, cloning, purification, and sequencing. The pTARGET (Promega Corporation) vector, containing the **o**pen **r**eading **f**rame (ORF) of full length human pendrin cloned from normal thyroid tissue, was originally provided by Prof. P. Beck-Peccoz, University of Milan (Italy). The pendrin mutants were made using the QuikChange® site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol, using the following primers: for pendrin P70L, forward: 5'GAC TCT TGT GCT CAT CTT GGA GTG3' and reverse: 5'CAC TCC AAG ATG AGC ACA AGA GTC3'; for pendrin V88I, forward: 5'GGC TGC TTA GTG ACA TCA TTT CGG GAG TTA G3' and reverse: 5'CTA ACT CCC GAA ATG ATG TCA CTA AGC AGC C3'; for pendrin P301L, forward: 5'CCC AGT CCC TAT TCT TAT AGA AGT AAT TG3' and reverse: 5'CAA TTA CTT CTA TAA GAA TAG GGA CTG GG3'; for pendrin F354S, forward: 5'GCT GGC TGC ATC ATC TTC CAT CGC TGT GG3' and reverse: 5'CCA CAG CGA TGG AAG ATG ATG CAG CCA GC3'; for pendrin V609G, forward: 5'CAT CAT AAG TGA TGC TGG TTC AAC AAA TAA TGC3' and reverse: 5'GCA TTA TTT GTT GAA CCA GCA TCA CTT ATG ATG3'; for pendrin F667C, forward: 5'GGA GCT ATA TCT TGC CTG GAC GTT GTT GG3' and reverse: 5'CCA ACA ACG TCC AGG CAA GAT ATA GCT CC3'; for pendrin D687Y, forward: 5'CCA AAG AAT TTA TGT GAA TGT G3' and reverse: 5'CAC ATT CAC ATA AAT TCT TTG G3'; for pendrin G740S, forward: 5'CTC AAG AGG GTC AAA GTT CCA TTT TAG AAA CG3' and reverse: 5'CGT TTC TAA AAT GGA ACT TTG ACC CTC TTG AG3'.

The plasmid encoding for the **e**nhanced **y**ellow **f**luorescent protein H148Q/I152L (an EYFP mutant with substantially improved sensitivity for iodide [38] and hereafter referred to as EYFP*) was obtained by site-directed mutagenesis of the pEYFPN1 vector (Clontech) with the following primers: forward: 5'GGA GTA CAA CTA CAA CAG CCA GAA CGT CTA TTT GAT GGC CGA CAA GCA GAA G3' and reverse: 5'CTT CTG CTT GTC GGC CAT CAA ATA GAC GTT CTG GCT GTT GTA GTT GTA CTC C3'. All plasmid inserts were sequenced prior to use in experiments (Microsynth AG, Switzerland).

Cell culture and transient transfection

Human **e**mbryonic **k**idney (HEK) 293 Phoenix cells [39] were cultured in **M**inimum Essential **E**agle **M**edium (MEM, Sigma, Austria) supplemented with 10% **f**etal **b**ovine **s**erum (FBS, Cambrex Bio Science), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM pyruvic acid (sodium salt). TSA-201 cells, a transformed subclone of human embryonic kidney cell line 293, stably expressing simian virus 40 T-antigen, were cultured in **D**ulbecco's **M**odified **E**agle's **M**edium (DMEM) containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

The cells were maintained at 37°C, 5% CO_2, 95% air and 100% humidity. Subcultures were routinely established every second to third day by seeding the cells into 100 mm diameter Petri dishes following trypsin/EDTA treatment. For functional tests (fluorometric methods), HEK 293 Phoenix cells were seeded into poly-L-lysine-coated 96-multiwells, grown overnight and transfected with a total amount of 0.2 µg/well of plasmid DNA by the calcium phosphate co-precipitation method [37]. Functional tests were performed 52 hours post-transfection.

Fluorometric method (iodide influx)

For measuring iodide influx, cells were co-transfected with 0.1 µg of pEYFP*N1 plasmid and 0.1 µg of pTARGET plasmid bearing the cDNA of **w**ild-**t**ype (WT) or mutated pendrin. Control cells were co-transfected with pEYFP*N1 and the empty pTARGET vectors. The functional test was performed as already described [37, 40-43], with adaptations allowing the use of a multiplate reader. Shortly, cells were washed from the culture medium and bathed in 70 µls of "high chloride" solution (in mM: KCl 2, NaCl 135, $CaCl_2$ 1, $MgCl_2$ 1, D-glucose 10, HEPES 20, 308 mOsm with mannitol, pH 7.4). After measuring the fluorescence intensity (1 measurement/sec for 3 sec), 140 µls of "high iodide" solution (in mM: KCl 2, NaI 135, $CaCl_2$ 1, $MgCl_2$ 1, D-glucose 10, HEPES 20, 308 mOsm with mannitol, pH 7.4) were injected in each well. Then, the fluorescence intensity was measured for 16 sec. with the VICTOR™ *X*3 Multilabel Plate Reader (Perkin Elmer) equipped with a liquid dispenser and the following filters: excitation: F485 (excitation **c**enter **w**ave**l**ength (CWL): 485 nm, bandwidth: 14 nm), emission: F535 (emission CWL: 535 nm, bandwidth: 25 nm). Experiments were performed at room temperature. The fluorescence measured in cells transfected with the pTARGET vector only (background signal) was subtracted from all other fluorescence measurements. Data were expressed as fluorescence variations in % (ΔF%).

Intracellular pH measurements

For intracellular pH measurements, the fluorescence indicator 2',7'-**b**is-(2- **c**arboxy**e**thyl)-5-(and-6)-**c**arboxy**f**luorescein **a**cetoxy**m**ethyl ester (BCECF-AM, Invitrogen Molecular Probes) was used. HEK 293 Phoenix cells were transiently transfected with 0.2 µg of pTARGET plasmid bearing the cDNA of WT or mutated pendrin or with the empty pTARGET plasmid (control). Before measurements, cells were loaded (30 min, 37°C) with BCECF-AM 1 µM in HBSS solution (Sigma). Then, cells were washed twice and bathed for 30 min at room temperature in 70 µls of bicarbonate-free, "low chloride" solution (in mM: KCl 2, NaGluconate 135, $CaCl_2$ 1, $MgCl_2$ 1, D-glucose 10, HEPES 20, 308 mOsm with mannitol, pH 7.4). After measuring the fluorescence intensity (3 measurements, one measurement every 5.6 sec), 140 µls of bicarbonate-free "high chloride" solution (in mM: KCl 2, NaCl 135, $CaCl_2$ 1, $MgCl_2$ 1, D-glucose 10, HEPES 20, 308 mOsm with mannitol, pH 7.4) were injected in each well. Then, the fluorescence intensity was measured again 16 times (one measurement every 5.6 sec) with the VICTOR™ *X*3 Multilabel Plate Reader equipped with a liquid dispenser and the following filters: excitation: P450 (excitation CWL: 450 nm, bandwidth: 8 nm) and P490 (excitation CWL: 492

Nucleotide change	Amino acid change	Frequency in controls (n=428)[a]	Frequency in patients (n=409)[a]	Patient Genotype[a]		Patient Phenotype[a]			Functionality (this study and [37])
				Allele 1	Allele 2	Deafness	CT	Goiter	
209C>T	P70L								loss
262G>A	V88I	1	1	E29Q	[V88I; R409H]	moderate/ mild	EVA	no	gain
902C>T	P301L								loss
1061T>C	F354S	3	0						not affected
1826T>G	V609G	2	2	V609G	-	moderate, bilateral	normal	no	reduction
				[V609G; D710D; G740S]	-	profound, bilateral	normal	no	
2000T>G	F667C	1	0						loss
2059G>T	D687Y								reduction
2218G>A	G740S	1	1	[V609G; D710D; G740S]	-	profound, bilateral	normal	no	gain
				G740S	-	moderate, bilateral	normal	no	

Table 1. Pendrin allelic variants characterized in the present study. [a]data are taken from [37]; n, number of alleles tested; the numbers indicate how often the mutant allele was identified in the group of normal-hearing controls or deaf patients. CT = computed tomography.

nm, bandwidth: 8 nm), emission: F535 (emission CWL: 535 nm, bandwidth: 25 nm). Experiments were performed at room temperature. All the measurements were subtracted from that detected in cells transfected with the pTARGET vector and not loaded with BCECF-AM (background signal). Intracellular pH was calculated following the standard ratiometric method (http://probes.invitrogen.com/media/pis/mp01150.pdf). Shortly, titration of the intracellular pH was performed in nigericin-treated (10 μM) HEK 293 Phoenix cells in the presence of 150 mM potassium (in mM: KCl 150, $MgCl_2.6H_2O$ 1, MES, HEPES or TRISMA base 20), at pHs of 5.0, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0. The resulting calibration curve was fitted with a sigmoidal dose-response equation:

$$Y = A + \frac{(B - A)}{1 + 10^{\log EC\,50 - X}}$$

In our conditions, the calculated pKa for BCECF-AM was 7.04. Results were expressed as fluorescence ratio ($\lambda_{490}/\lambda_{450}$) variations (ΔRatio%).

Radiolabeled iodide uptake studies (iodide efflux)

For radiolabeled iodide uptake assays, a pCMX plasmid containing **N**a^+/**I**$^-$ **s**ymporter (NIS) cDNA (500 ng/well), and pTARGET plasmids carrying WT pendrin cDNA or pendrin mutants (500 ng/well) were transfected using the calcium phosphate co-precipitation method. The empty pCMX and pTARGET vectors were used as negative controls. In co-transfection experiments of NIS and pendrin constructs, the total amount of DNA was kept constant by supplementation with empty vector. Radiolabeled iodide efflux studies were performed 48 hours following transfection of TSA-201 cells grown to 80% confluency in 12-well tissue culture plates. Cells were washed once with 1x PBS and incubated with HBSS containing 10 mM HEPES (pH 7.4), 1 mM methimazole, 1 mM DTT, and 10^{-5} M cold NaI labeled with $Na^{125}I$ (20 mCi/mmol) for 30 min at 37°C. The radiolabeled iodide solution was aspirated and cells were permeabilized using 1% Triton-X in 1x PBS. The intracellular iodide content was determined by quantifying the amount of radiolabeled iodide in the cell lysates using a gamma counter.

Salts, chemicals and drugs

All salts and chemicals used were of "pro analysis" grade.

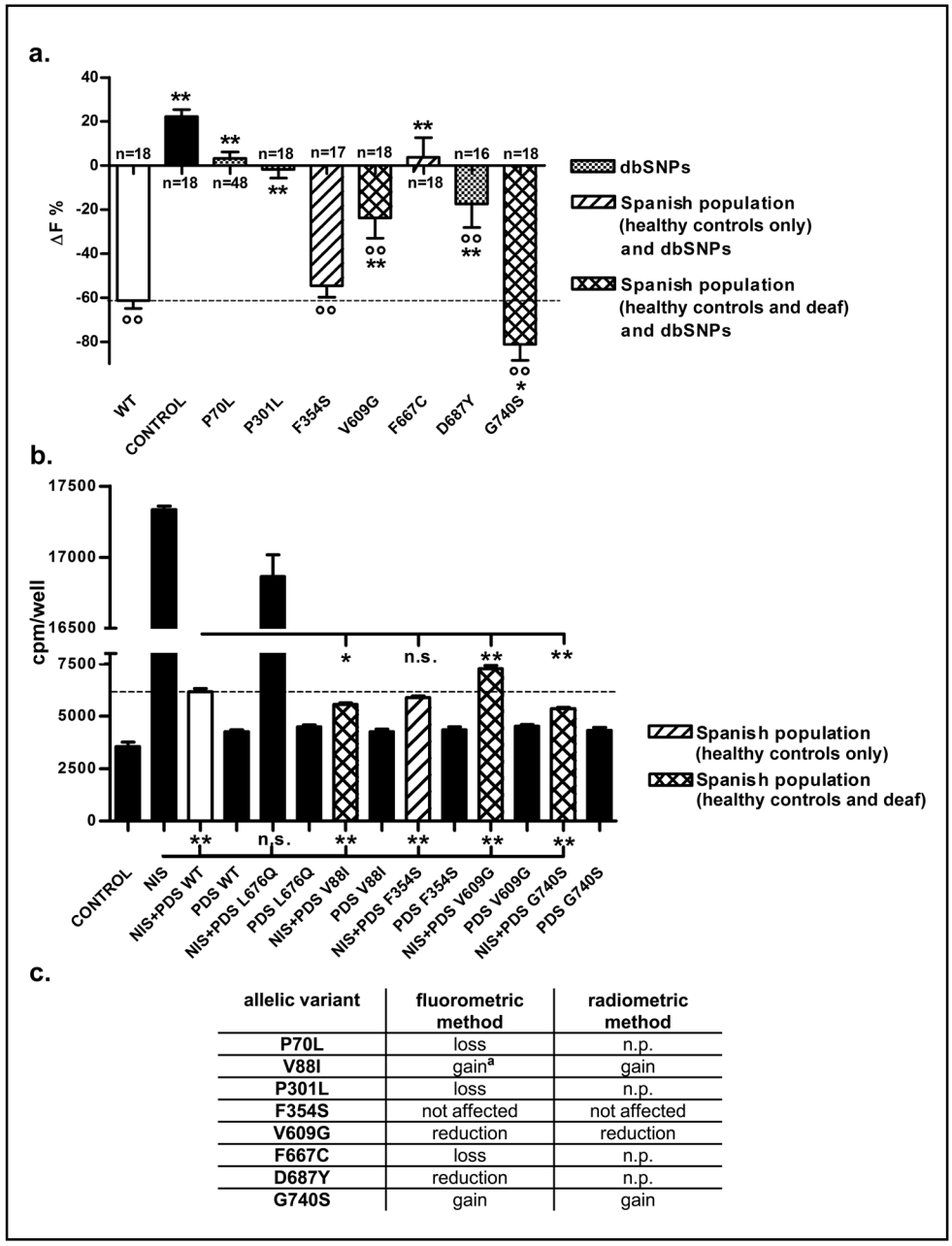
Statistical Analysis

All data are expressed as arithmetic means ± S.E.M. For statistical analysis, GraphPad Prism software (version 4.00 for Windows, GraphPad Software, San Diego, California, USA) was used. Significant differences between means were tested by Student's t test or one way **An**alysis **o**f **Va**riance (ANOVA) with Dunnet's post-test, as appropriate. Statistically significant differences were assumed at $p<0.05$ (* $p<0.05$; ** $p<0.01$); (n) corresponds to the number of independent measurements.

Results

Iodide influx

In order to test the iodide influx of WT pendrin and its allelic variants (Table 1), we measured the EYFP* fluorescence in cells over-expressing WT or mutated pendrin before and after addition of iodide to the



allelic variant	fluorometric method	radiometric method
P70L	loss	n.p.
V88I	gain[a]	gain
P301L	loss	n.p.
F354S	not affected	not affected
V609G	reduction	reduction
F667C	loss	n.p.
D687Y	reduction	n.p.
G740S	gain	gain

Fig. 1. Iodide transport determined in cells transfected with WT pendrin and its allelic variants. a, fluorometric method. The intracellular fluorescence intensity was measured in cells transfected with WT or mutated pendrin and EYFP* or EYFP* alone as a control. The percentage of fluorescence decrease (ΔF%) induced by the addition of iodide to the extracellular solution was determined over an experimental period of 19 sec. n indicates the number of independent samples collected over at least 3 experimental days. **: $p<0.01$, *: $p<0.05$ compared to WT, °°: $p<0.01$ compared to control, one way ANOVA with Dunnet's post-test. b, radiometric method. Non-polarized TSA-201 cells were co-transfected with the sodium iodide transporter NIS (to allow cells to accumulate iodide) and the pendrin allelic variants. The intracellular $^{125}I^-$ content in these cells was compared to that in cells co-transfected with WT pendrin and NIS or with NIS alone, as indicated. **: $p<0.01$, *: $p<0.05$, one way ANOVA with Dunnet's post-test, n=3. c, summary of the results of the experiments shown in a and b; loss, reduction, not affected and gain refer to the function of the corresponding allelic variant; n.p.= not performed. [a] taken from [37].

extracellular solution. Since pendrin can act as a chloride/iodide (Cl^-/I^-) exchanger [2, 4, 41], the presence of extracellular iodide should induce an iodide flux into the cytoplasm. Iodide is a much better quencher of EYFP* fluorescence than chloride, therefore, an increase in intracellular iodide should lead to a decrease of EYFP* fluorescence [38]. Indeed, as shown in Fig. 1a, addition of iodide to the extracellular solution led to a marked decrease (ΔF% = -61.3 ± 3.5%, n = 18) of EYFP* fluorescence in cells expressing WT pendrin. This value is significantly different from that measured in cells overexpressing only EYFP* (control) i.e. 22.2 ± 3.2%, n = 18, $p<0.01$. These results confirm that pendrin is an iodide transporter [44, 45] most likely acting as a Cl^-/I^- anion exchanger in this system [41].

The addition of iodide to the extracellular solution led to only a small change in the intracellular fluorescence of cells over-expressing the mutated pendrin isoforms P70L, P301L and F667C (ΔF% for P70L: 3.2 ± 2.9%, n = 48; for P301L: -1.8 ± 3.8%, n = 18; for F667C: 3.8 ± 8.9%, n = 18). These values were significantly reduced compared to WT ($p<0.01$) and not significantly different from control, indicating that the corresponding mutants display a loss of function. In contrast, the changes

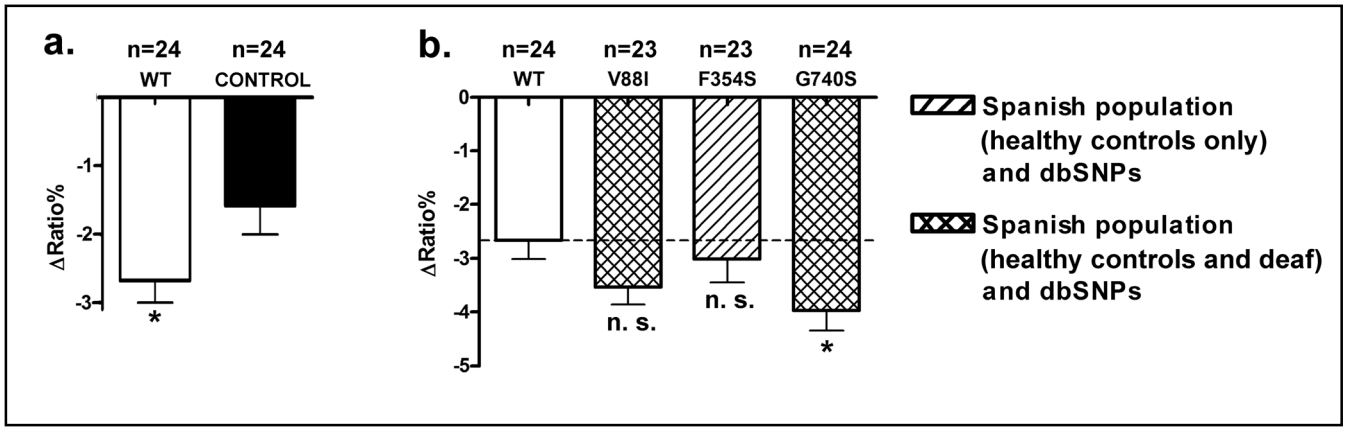


Fig. 2. Cl^-/OH^- exchange activity of WT pendrin and its allelic variants. Cells transfected with WT pendrin and the pendrin allelic variants, or with the pTARGET empty vector as a control were loaded with BCECF-AM 1 μM and bathed for 30 min in a bicarbonate-free, low chloride solution and then exposed to a high chloride solution. Fluorescence emissions at 535 nm following excitation at 450 and 490 nm were measured over time, subtracted for their respective backgrounds and expressed as ratios. The percentage of fluorescence decrease (Δ Ratio%) determined over the experimental period (107 sec) is reported in the graphs. a, *: $p<0.05$, unpaired t Student's test. b, *: $p<0.05$, n. s.: not statistically significant compared to WT, one way ANOVA with Dunnet's post-test. n indicates the number of independent samples, collected over at least three independent experiments.

in intracellular fluorescence of cells over-expressing pendrin V609G (ΔF% = -23.9 ± 9.0, n = 18) and D687Y (ΔF% = -17.5 ± 10.6, n = 16) were significantly different from both the WT and control ($p<0.01$), indicating that these mutants retain residual transport activity, even if reduced with respect to the WT. The transport activity of pendrin F354S (ΔF% = -54.5 ± 5.3, n = 17) was indistinguishable from WT. Surprisingly, the iodide influx mediated by pendrin G740S (ΔF% = -81.2 ± 7.2, n = 18) was significantly higher (~32%) compared to WT ($p<0.05$).

Iodide efflux

In order to analyze the ability of pendrin allelic variants to mediate iodide efflux, we co-transfected non-polarized TSA-201 cells, lacking endogenous pendrin expression, with the sodium iodide transporter NIS to allow cells to accumulate iodide. TSA-201 cells transiently expressing NIS alone demonstrate a significant increase (488%) in iodide uptake, compared with cells transfected with the empty vector (control, Fig. 1b). Co-expression of NIS and WT pendrin (intracellular content of iodide in cpm/well: 6162 ± 163, n = 3, Fig. 1b) led to a significant decrease (~64%) in intracellular iodide concentration compared with cells transfected only with NIS (17334 ± 26 cpm/well, n = 3, $p<0.01$), thereby confirming that pendrin can mediate iodide efflux in this system [45, 46]. Co-transfection of cells with NIS and the previously characterized pendrin loss-of-function mutant L676Q, found in a patient with Pendred syndrome [45], did not result in a significant decrease in intracellular iodide (16863 ± 153 cpm/well, n= 3), and served as a negative control. Cells co-expressing pendrin V88I and NIS, as well as those co-transfected with pendrin G740S and NIS, showed a slightly larger decrease (68% and 69% respectively) in intracellular iodide amount (in cpm/well: 5572 ± 70, n = 3 and 5364 ± 69, n= 3 respectively, Fig. 1b) than cells co-transfected with WT pendrin and NIS. These findings suggest that the pendrin allelic variants V88I and G740S may have a minor, but significant ($p<0.05$ and $p<0.01$ respectively), gain of function. Co-transfection of pendrin F354S and NIS led to a 66% decrease in intracellular iodide content (4352 ± 143, n = 3, Fig. 1b), not statistically different from the decrease obtained following transfection with WT pendrin and NIS, indicating that this mutation does not affect the ability of the protein to mediate iodide transport. The intracellular iodide concentration of cells co-expressing pendrin V609G and NIS (7275 ± 162, n = 3, Fig. 1b) was significantly reduced with respect to cells co-expressing NIS alone ($p<0.01$); nevertheless, the cells co-expressing pendrin V609G and NIS showed a smaller decrease in intracellular iodide concentration if compared to cells co-expressing WT pendrin and NIS (58% vs. 64% respectively, $p<0.01$). These results show that the transport ability of pendrin V609G is reduced, but not completely annihilated.

Cl^-/OH^- exchange

In order to test the Cl^-/OH^- exchange activity of WT pendrin and its allelic variants, BCECF-AM fluorescence ratio variations (ΔRatio%) were measured in chloride-depleted, pendrin-transfected cells before and after addition of chloride to the extracellular solution. As pendrin acts as an Cl^-/OH^-, Cl^-/HCO_3^- exchanger [1], the presence of extracellular chloride should induce a chloride flux into the cytoplasm in exchange for OH^-, leading, as a consequence, to a decrease (acidification) of the intracellular pH. A decrease in BCECF-AM fluorescence ratio is indicative of such a pH change. Indeed, as shown in Fig. 2a, the addition of chloride to the extracellular solution led to a significant decrease in fluorescence ratio (corresponding to an intracellular acidification) in cells transfected with WT pendrin compared to the control cells ($p<0.05$). Similarly, the fluorescence ratio decrease measured in cells transfected with pendrin G740S (Fig. 2b) was significantly higher ($p<0.05$) with respect to WT, indicating a moderate gain of function of this allelic variant in mediating the Cl^-/OH^- exchange.

Discussion

Mutations within the coding region that reduce or annihilate pendrin transport activity are the most common cause of syndromic deafness [47] and, in addition, are found in up to 4% of patients with non-syndromic hearing loss [16]. Moreover, the up-regulation of pendrin activity and/or expression by pro-inflammatory cytokines [25-30] or pharmacological treatments [21, 22] may be involved in the pathogenesis of hypertension and contribute to exacerbations of asthma and COPD.

Here we determined the function of seven selected allelic variants of pendrin (P70L, P301L, F354S, V609G, F667C, D687Y, G740S, Table 1), all of which are reported in the dbSNPs and some of which were previously identified in a cohort of deaf Spanish patients and/or normal-hearing controls. In addition, the transport activity of the previously characterized pendrin allelic variant V88I was further explored [37].

The allelic variants P70L, P301L and D687Y are reported in the dbSNPs, but their functional and clinical significance was unknown. The fluorometric functional test (Fig. 1a) showed that iodide transport (I^-/Cl^- exchange) of D687Y is significantly reduced compared to WT, but not completely blunted; in contrast, P70L and P301L displayed a complete loss of function. We conclude that P70L, P301L and D687Y could all be pathogenic. In addition, we confirmed that amino acid substitutions involving a proline always have a negative impact on pendrin function [37, 40].

The allelic variants F354S and F667C were detected in a group of normal-hearing controls belonging to the Spanish population, but not in a cohort of deaf patients (Table 1 and [37]); both of them are also reported in the dbSNPs. The allelic variant F354S was previously identified in compound heterozygosity in a patient with Pendred syndrome [48] and in heterozygosity with the WT allele in a patient with ns-EVA [16] and exhibited a moderate reduction in Cl^-/HCO_3^- exchange activity [49]. Nevertheless, the functional characterization of pendrin F354S by the fluorometric method (Fig. 1a), corroborated by the radiometric method (Fig. 1b), demonstrate that its iodide transport activity is indistinguishable from the WT. In addition, the Cl^-/OH^- exchange activity of this variant was not affected (Fig. 2b). These observations, along with the high incidence of this mutation in the control population (Table 1 and [37]), indicate that F354S should be considered as a benign polymorphism and, if identified in patients with goiter and deafness, other genetic causes should be considered as determinants of the clinical phenotype. Pendrin F667C was previously identified in a family with Pendred syndrome [10]. This allelic variant displayed a complete lack of function (Fig. 1a), which indicates that F667C is a pathogenic loss of function mutation.

The allelic variants V88I, V609G and G740S where found with approximately the same frequency in the group of normal-hearing controls belonging to the Spanish population and in the cohort of deaf patients (Table 1 and [37]); for this reason, it is impossible to unambiguously define their pathogenic potential without performing a functional test. Pendrin V88I was previously characterized by us [37] using the fluorometric method and displayed a significant gain of function; here we confirmed this finding by the use of a radiometric method as an alternative approach (Fig. 1b). Pendrin V609G was previously identified in compound heterozygosity or in heterozygosity with the WT allele in patients with Pendred syndrome and ns-EVA [15, 16, 50]. The assays in this study (Fig. 1a and b) show that the function of this allelic variant is impaired but not completely annihilated, thereby indicating that pendrin V609G could have a pathogenic potential,

especially if associated with other genetic and/or environmental factors. The allelic variant G740S was previously identified in a screening of the pendrin gene in a cohort of patients with the clinical diagnosis of EVA or Mondini dysplasia [51]. The functional studies reported here (Fig. 1a,b and 2b) show that pendrin G740S has a modest, but significant gain of function compared to WT, thereby excluding this allelic variant as a genetic determinant for syndromic or non-syndromic deafness. Interestingly, the gain of function was observed for both the iodide transport (Fig. 1a and b) and Cl^-/OH^- exchange activity (Fig. 2b).

In summary, this work shows the functionality of eight (six of which were previously uncharacterized) pendrin allelic variants in which the results of a fluorometric method [41] suitable for measuring the iodide transport of pendrin were validated (Fig. 1c) with a radiometric method [45]. Pendrin P70L, P301L and F667C displayed a loss of function, whereas V609G and D687Y caused a significant reduction of function. Therefore, these allelic variants can be assumed to be pathogenic. The function of pendrin V88I, F354S, G740S was not reduced compared to WT; consequently these allelic variants should not be considered as genetic determinants for Pendred syndrome/ns-EVA. F354S is most likely a benign polymorphism. Surprisingly, pendrin V88I and G740S showed a modest, but significant, gain of function; these isoforms could potentially participate in the pathogenesis of hypertension and/or exacerbations of asthma and COPD [26].

Conclusion

Functional characterization of pendrin allelic variants is essential for the identification of substitutions whose function is not reduced and, as a consequence, should not be considered as genetic determinants of Pendred syndrome or ns-EVA. Amino acid substitutions involving a proline have a detrimental effect on the function of pendrin. The identification of allelic variants with gain of function is of particular interest. Given the possible role of pendrin in the pathogenesis of hypertension, COPD and asthma, these variants could be genetic modifiers contributing to the severity of the phenotype and/or exacerbations of compromised airway function.

Abbreviations

ns-EVA (non-syndromic enlarged vestibular aqueduct); COPD (chronic obstructive pulmonary disease); dbSNPs (single nucleotide polymorphisms data base); NIS (Na^+/I^- symporter); ORF (open reading frame); EYFP (enhanced yellow fluorescent protein); FBS (fetal bovine serum); EDTA (ethylenediaminetetraacetic acid); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); BCECF-AM (2',7'-bis-(2- carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester); MES (2-(N-morpholino)ethanesulfonic acid); TRISMA base (2-amino-2-(hydroxymethyl) propane-1,3-diol); HBSS (Hanks' Balanced Salt Solution); PBS (phosphate buffered saline); DTT (1,4-Dithiothreitol).

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