

Molecular and Functional Characterization of Human Pendrin and its Allelic Variants

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Key Words

Pendrin • Pendred syndrome • Deafness • Mutations
• Functional test • Ion transport

Abstract

Pendrin (SLC26A4, PDS) is an electroneutral anion exchanger transporting I⁻, Cl⁻, HCO₃⁻, OH⁻, SCN⁻ and formate. In the thyroid, pendrin is expressed at the apical membrane of the follicular epithelium and may be involved in mediating apical iodide efflux into the follicle; in the inner ear, it plays a crucial role in the conditioning of the pH and ion composition of the endolymph; in the kidney, it may exert a role in pH homeostasis and regulation of blood pressure. Mutations of the pendrin gene can lead to syndromic and non-syndromic hearing loss with EVA (enlarged vestibular aqueduct). Functional tests of mutated pendrin allelic variants found in patients with Pendred syndrome or non-syndromic EVA (ns-EVA) revealed that the pathological phenotype is due to the reduction or loss of function of the ion transport activity. The diagnosis of Pendred syndrome and ns-EVA can be difficult because of the presence of phenocopies of Pendred syndrome and benign polymorphisms occurring in the general population. As a conse-

quence, defining whether or not an allelic variant is pathogenic is crucial. Recently, we found that the two parameters used so far to assess the pathogenic potential of a mutation, i.e. low incidence in the control population, and substitution of evolutionary conserved amino acids, are not always reliable for predicting the functionality of pendrin allelic variants; actually, we identified mutations occurring with the same frequency in the cohort of hearing impaired patients and in the control group of normal hearing individuals. Moreover, we identified functional polymorphisms affecting highly conserved amino acids. As a general rule however, we observed a complete loss of function for all truncations and amino acid substitutions involving a proline. In this view, clinical and radiological studies should be combined with genetic and molecular studies for a definitive diagnosis. In performing genetic studies, the possibility that the mutation could affect regions other than the pendrin coding region, such as its promoter region and/or the coding regions of functionally related genes (*FOX11*, *KCNJ10*), should be taken into account. The presence of benign polymorphisms in the population suggests that genetic studies should be corroborated by functional studies; in this context, the existence of hypo-functional variants and possible differences between the I⁻/Cl⁻

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and $\text{Cl}^-/\text{HCO}_3^-$ exchange activities should be carefully evaluated.

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Tissue expression and physiological role of pendrin

The pendrin protein was initially described as a gene product that, if mutated, is responsible for the Pendred syndrome (OMIM#274600) [1]. Human pendrin (SCL26A4, PDS) is a 780 amino acid membrane protein with transport function, expressed in tissues as diverse as the thyroid gland [2], kidney [3], inner ear [4, 5], airways [6], mammary gland [7], testis [8], placenta [9], endometrium [10] and liver [11]. Particularly well described is the localization and respective function of pendrin in the thyroid gland, inner ear and kidney. In the thyroid gland, pendrin is expressed exclusively at the apical membrane of thyroid follicular cells [2]. Several points of evidence indicate that the pendrin transporter is crucially involved in follicular iodide transport, i.e. (i) its localization, (ii) the iodide organification defect presented by patients with Pendred syndrome [12] and (iii) a number of functional studies in heterologous expression systems (that will be reviewed here). In the inner ear, pendrin is expressed in the epithelium of the endolymphatic sac and duct [5, 13], on the apical membrane of transitional cells in the saccule, utricle, ampulla [5], and in a variety of diverse cell types in the cochlea (inner and outer hair cells, Deiter's cells, Claudius cells, spiral ligament, spiral ganglion, spiral prominence, external sulcus cells) [5, 14-17]. In these compartments, pendrin plays a crucial role in conditioning endolymph pH and ion composition [18]. In the kidney, pendrin is expressed on the apical membrane of β and non- α , non- β intercalated cells of the distal convoluted tubule (DCT), cortical collecting duct (CCD) and connecting tubule (CNT) [3, 19, 20] where it exerts a role in pH homeostasis [21] and blood pressure regulation [22, 23]. The physiological role of pendrin in other tissues is less well understood and deserves further investigation.

Transport properties of wild type pendrin

The thyroid

Pendrin was described as a member of the multifunctional transporters SLC26 [24] family, and after its discovery, it was assumed that pendrin transports

sulphate. However, later studies in over-expression systems failed to demonstrate that pendrin can transport sulphate or other divalent anions [25, 26]. In addition, Kraiem et al. found that sulphate transport in thyrocytes obtained from Pendred syndrome patients was not defective [27]. Thereafter, it was shown that pendrin can transport iodide [25], and could therefore be involved in mediating apical iodide efflux from the thyroid cell into the follicular lumen [28, 29]. As time progressed, it became increasingly evident that pendrin acts as an electroneutral, i.e. non-rheogenic [30] iodide/chloride (I^-/Cl^-) exchanger with a 1:1 stoichiometry [31] and preference for iodide over other anions [32, 33].

It is noteworthy that some investigators assume that pendrin might also secrete bicarbonate into the thyroidal follicle, since thyroid follicular transepithelial potential and pH are reduced in pendrin knock-out mice [34]. For the supporting evidence as well as arguments questioning the role of pendrin in mediating iodide efflux in thyrocytes, see the reviews by Twyffels et al. [35] and Bizhanova et al. [36] in this Special Issue.

The kidney

Heterologous overexpression studies also demonstrated that pendrin can function as a chloride/hydroxide (Cl^-/OH^-) or chloride/bicarbonate ($\text{Cl}^-/\text{HCO}_3^-$) exchanger [37]. Studies using pendrin knock-out mice led to the conclusion that one role of pendrin in the kidney is bicarbonate secretion [3, 21]. Accordingly, pendrin expression was significantly increased following oral bicarbonate loading in mouse [38] and rat [39], and downregulated during metabolic acidosis in rat [39-41] and rabbit [42]. Patients with Pendred syndrome have normal renal function and do not display abnormalities in acid-base metabolism under basal conditions. This indicates that other chloride-base exchangers compensate for the loss of function of pendrin [43]. Recently however, two interesting case reports of severe metabolic alkalosis in patients with Pendred syndrome indicated that pendrin may play a role in protecting against metabolic alkalosis in the context of intercurrent illness [44] or pharmacological therapy [45].

Pendrin also plays a role in chloride reabsorption, since it is downregulated in response to high chloride intake and upregulated in response to chloride depletion induced by furosemide [46] or sodium chloride (NaCl) restriction [47, 48]. Accordingly, major changes in pendrin protein expression were found in experimental models that are associated with altered renal chloride transport [49].

Recently, it was proposed that pendrin in the kidney also plays a role in the maintaining of iodide balance, particularly under high water intake [50]; however, the mechanism by which pendrin drives renal iodide reabsorption in cases of increased water intake remains to be elucidated.

The inner ear

Similarly to the kidney, pendrin acts as a $\text{Cl}^-/\text{HCO}_3^-$ exchanger in the inner ear, controlling the pH of the endolymphatic fluid. Earlier studies in pendrin knock-out mice, which are completely deaf and also display signs of vestibular dysfunction, revealed a severe endolymphatic dilatation after embryonic day 15, reminiscent of that seen radiologically in deaf individuals with pendrin mutations and EVA. Additionally, in the second postnatal week, severe degeneration of sensory cells in the Corti organ and vestibular maculae, and malformation of otoconia and otoconial membrane occurred. These observations provided important clues for understanding the cause of deafness and vestibular dysfunction in these mice and, possibly, in patients with Pendred syndrome [51]. Further studies revealed that pendrin is crucial in maintaining the endocochlear potential, albeit without being directly involved in potassium secretion [5]. Later, Wangemann et al. clarified that the loss of the endocochlear potential observed in the Pendred syndrome mouse model is due to a loss of expression of the potassium channel KCNJ10 [15]. The same group successively published a series of detailed studies on the role of pendrin in the different inner ear compartments using pendrin knock-out mice. These studies revealed a reduction in pH and utricular endolymphatic potential, and an increase in the endolymph calcium concentration, possibly due to the inhibition of pH-sensitive transient receptor potential vanilloid (TRPV) 5 and TRPV6 cation channels [52]. These findings might explain the vestibular dysfunction observed in Pendred syndrome patients. A similar mechanism could induce the observed cochlear sensory hair cell degeneration, which, as a consequence, leads to deafness [18]. It was also hypothesized that a loss of bicarbonate secretion could hamper fluid reabsorption in the endolymphatic sac, an event eventually leading to cochlear enlargement and again, deafness [53]. All of the described functional derangements observed in the inner ear of pendrin knock-out mice would hence be associated with a decrease in endolymphatic bicarbonate secretion. Beside pendrin knock-out mice, also the use of genetically modified mice bearing pendrin mutations is a powerful tool in understanding the etiology of the pathological conditions

related to pendrin malfunction. The *Slc26a4^{loop}* mouse, that was generated by N-ethyl N-nitrosourea (ENU) mutagenesis bearing the homozygous loss of function mutation S408F, led to the discovery of new inner ear pathology that has complemented the work on the *Slc26a4* knock-out mouse with its novel phenotypic variation. Recently, we found dramatic changes in the composition, size, and shape of otoliths within the utricle and saccule of *Slc26a4^{loop}* mouse, possibly as a consequence of the deregulation of the endolymphatic pH [54].

The airways

Predominant pendrin expression in the airways was not discovered until 2005, when Kuperman et al. described upregulation of the transporter mRNA in the lungs of 3 separate murine asthma models [55]. Since the initial observation of pendrin expression in the bronchial epithelium, a multitude of studies have followed in which the transporter has been associated with increased antimicrobial activity in the airway surface liquid (ASL) [6], mucus production [56] and regulation of the ASL thickness [57]. These recent data underscore a role for pendrin in respiratory distresses including allergy, rhinovirus infection, bronchial asthma and chronic obstructive pulmonary disease (COPD). For more detailed information regarding pendrin and the airways, see the Review by Nofziger et al. in this Special Issue [58].

The role of pendrin in human pathology

Diseases linked to pendrin malfunction

Mutation of the pendrin gene can lead to syndromic and non-syndromic hearing loss with enlarged vestibular aqueduct (EVA) [59]. Pendred syndrome, the most common form of syndromic deafness [60], was originally described more than one century ago as a combination of deafness and goiter unrelated to environmental factors [61]. Pendred syndrome is an autosomal recessive disease characterized by bilateral sensorineural deafness and a partial iodide organification defect disclosed by a positive perchlorate discharge test [62], even in the absence of overt goiter. Deafness in Pendred syndrome is usually severe to profound with an early onset, or fluctuating and progressive, and seldom occurring later in life or after head trauma [63]. Deafness is associated with a malformation of the inner ear, i.e. an EVA, accompanied by an enlarged endolymphatic sac and duct, or Mondini

cochlea, and can be diagnosed by computed tomography (CT) or magnetic resonance imaging (MRI) of the temporal bone [64]. By definition, ns-EVA is a condition where deafness due to inner ear malformations is not associated with thyroid dysfunction (the perchlorate discharge test in these patients is negative), and can be found in patients with zero, one [65, 66] or two mutations in the pendrin gene [67-69]. Whether or not ns-EVA can be associated with two mutations of the pendrin gene is a matter of debate. Pryor et al. found a strong correlation between Pendred syndrome and two mutant *SLC26A4* alleles, while ns-EVA correlated with zero or one mutant *SLC26A4* alleles. [65]. This study therefore suggests that biallelic *SLC26A4* mutations are consistently associated with a positive perchlorate test, and hence, with Pendred syndrome. As mentioned earlier, other studies indeed suggested that ns-EVA can be associated with two mutations of the pendrin gene [67-69]. Nevertheless, it is noteworthy that in the study of Azaiez et al. there was no definitive assessment of the thyroid phenotype, that was defined as “palpable goiter or abnormal perchlorate discharge test” [67]. Albert et al. reported several cases of biallelic *SLC26A4* mutations with a normal perchlorate discharge test [68]. Unfortunately, these Authors did not specify the exact cut-off value for considering the test as positive. Tsukamoto et al. reported several cases of ns-EVA with biallelic *SLC26A4* mutations; once again, the criterion for the assessment of the thyroid phenotype was not precisely described. These Authors defined patients with Pendred syndrome as “those having either a palpable goiter or abnormal perchlorate discharge test” [69]. In conclusion, these studies seem to indicate that although biallelic *SLC26A4* mutations are often associated with Pendred syndrome, cases of ns-EVA associated with biallelic *SLC26A4* mutations can also occasionally be found.

Thyroid dysfunction in Pendred syndrome is variable, as is the presence and dimension of goiter [70]. Patients are usually euthyroid, or may present modestly elevated serum levels of thyroid stimulating hormone (TSH); often thyroglobulin (TG) is significantly increased [71] and occasionally hypothyroidism may develop [72]. Pendred syndrome is typically linked to biallelic mutations (as homozygosity in inbred families, or as compound heterozygosity, Fig. 1) occurring in the pendrin coding region. In addition, cases of Pendred syndrome/non-syndromic EVA have been reported with mutations occurring in the consensus binding region for the transcription factor forkhead box (FOX) II (present in the promoter region of pendrin) [73], and double

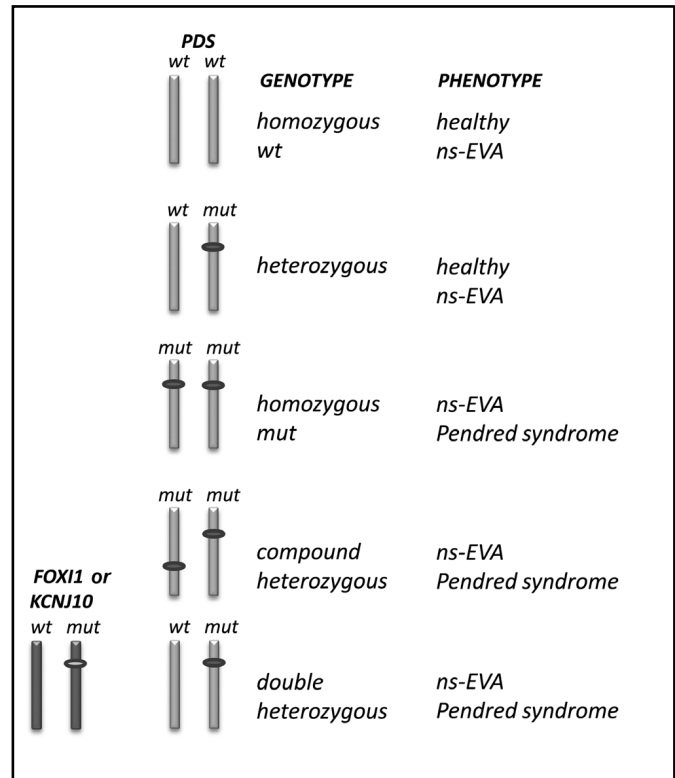


Fig. 1. Nomenclature used in this review; ns-EVA could be found in individuals with zero, one, or two (as homozygous or compound heterozygous) mutations of the pendrin gene, or in individuals bearing mutations in the pendrin gene and another functionally related gene (double heterozygous). Pendred syndrome could only be found in individuals with two (as homozygous or compound heterozygous) mutations of the pendrin gene or in individuals bearing mutations in the pendrin gene and another functionally related gene (double heterozygous).

heterozygous mutations in the *FOX11* coding region or in the potassium channel *KCNJ10*, which participates in the generation of the endocochlear potential [74] (Fig. 1).

Pendrin hyper-function and blockers

As previously mentioned, chloride reabsorption via pendrin at the level of the kidney could contribute to the pathogenesis of hypertension. Indeed, expression and activity of the transporter are upregulated by aldosterone analogues [75] and angiotensin II [76]. Accordingly, pendrin knock-out mice are protected against aldosterone analogue -induced hypertension [75]. Pendrin may thus represent a potential target for blood pressure control [22]. In the airways, pendrin has been associated with mucus production [56] and is upregulated upon stimulation with pro-inflammatory cytokines [6, 56, 57, 77, 78]. The chloride reabsorption via pendrin could reduce the airway surface liquid thickness [57], exacerbating, together with

mucus overproduction, the symptoms of asthma and COPD [77]. In such pathological conditions, blocking or reducing pendrin activity with the use of selective drugs could be beneficial. Moreover, very recently pendrin allelic variants with a modest, but significant, gain of function have been identified [79]. These hyperfunctional mutants could be genetic modifiers contributing to the severity of the phenotype of hypertension, asthma and COPD. A considerable effort has been devoted in characterizing the pharmacological profile of this transporter; nevertheless, no selective, potent, non-toxic inhibitors have been identified so far. Pendrin shows an unusual inhibitor profile when compared to other anion exchangers. In some heterologous overexpression systems, pendrin seems to be scarcely sensitive [25] or even resistant [32] to the addition of DIDS (4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid), a well-known blocker of Cl⁻/HCO₃⁻ exchangers [80]. Similarly, other inhibitors, such as furosemide and probenecid, only showed a partial effect, even at high concentrations [25]. In contrast, pendrin mediated chloride transport is sensitive to the chloride channel blocker NPPB (5-Nitro-2-(3-phenylpropylamino)benzoic acid) [32]. Interestingly, the most potent pendrin inhibitor at present seems to be the nonsteroidal anti-inflammatory drug niflumic acid, which, at the concentration of 10⁻⁴ M, was able to reduce pendrin associated chloride uptake by ~70% [32]. Accordingly, Pedemonte et al. screened a multiple compounds library and found that only niflumic acid blocked pendrin associated anion transport [6]. The identification of potent and selective inhibitors of pendrin deserves further investigation.

Molecular and functional characterization of pendrin allelic variants

Functional tests

More than 170 mutations within the pendrin gene have been identified so far (<http://www.healthcare.uiowa.edu/labs/pendredandbor/slcMutations.htm>). The majority (~64%) of these mutations are single nucleotide changes leading to amino acid substitutions, followed by ~16% leading to amino acid insertions or deletions, ~13% affecting splicing sites, and ~6% leading to premature truncations of the protein. Functional tests of pendrin mutations identified as allelic variants in patients with Pendred syndrome or ns-EVA revealed that the pathological phenotype is the consequence of a reduction or loss of function of pendrin-

driven ion transport [81]. In the first study aimed at determining the transport activity of pendrin mutants, Scott et al. measured the uptake of radiolabeled iodide and chloride in *Xenopus laevis* oocytes injected with wild-type (WT) or mutated pendrin cRNA [81]. Taylor et al. successively measured the activity of mutated pendrin using radiolabeled iodide efflux assays in human cells transfected with WT and mutated pendrin [82]. The use of human cells instead of a non-mammalian system for chloride uptake [32] and iodide efflux [83-85] studies was a considerable improvement, since they provided a more physiologically relevant environment to determine pendrin activity. A further improvement was the use of polarized mammalian cells by Gillam et al. These authors developed a functional test with polarized Madin-Darby canine kidney (MDCK) cells loaded with radiolabeled iodide by means of the Na⁺/I⁻ symporter (NIS) in a double-chamber system, allowing the measurement of iodide efflux *via* WT pendrin [29]. Determination of pendrin transport activity using radioisotopes is advantageous since radiolabeled iodide can be used at relatively low concentrations (close to the micromolar concentration present in the cytoplasm of the thyrocyte); however, these studies are simultaneously burdened by the use of radioactivity. In 2006, we described a non-radioactive, fluorescence-based assay suitable for the measurement of pendrin function [31, 54, 70, 86]. A further improvement of this technique was acquired with the use of an enhanced yellow fluorescent protein (EYFP) isoform (EYFP H148Q/I152L) that is more sensitive to the intracellular iodide concentration when compared to chloride, although not specific (pKa for iodide and chloride 3 and 88 mM respectively, at pH 7.5) [87]. In addition, the Cl⁻/HCO₃⁻ and Cl⁻/OH⁻ exchange activity of WT pendrin and its mutants can be measured by means of the pH_i-sensitive dye BCECF [88]. It is worth to note that very few studies compared the I⁻/Cl⁻ and the Cl⁻/HCO₃⁻ exchange activity of the same mutants [79, 89-91]. Besides in heterologous overexpression systems, the activity (iodide efflux) of the mutated pendrin has been characterized in primary thyrocyte cultures from a patient with Pendred syndrome [92]. This model mimics the *in vivo* situation most adequately, however, this approach can only be seldom applied. Table 1 shows all pendrin mutations identified so far in patients with Pendred syndrome or ns-EVA for which a functional test or the determination of the subcellular localization has been reported. The topology of the mutations for which a functional characterization has been performed is also indicated on the putative model of pendrin (Fig. 2) that

Mutation	Activity compared to WT	Nucleotidic substitution	Pathology	Localization	Function	Rescue	SNPs database ²⁰	Pathology ²¹	Reference #
S28R	-	84C>A	PS	PM ⁶	loss of chloride uptake ¹¹	Yes ¹⁷			[93]
		82A>C	EVA	intracellular ⁷	loss of chloride and iodide transport ¹² loss of Cl ⁻ /HCO ₃ ⁻ exchange activity ¹³				[31] [88]
E29Q	-	85G>C	EVA		reduction of the chloride and iodide transport ¹²		Yes	EVA	[86]
P70L	-	209C>T			loss of chloride and iodide transport ¹²		Yes		[79]
V88I	+	262G>A	EVA (+R409H)		increased chloride and iodide transport ¹² Cl ⁻ /OH ⁻ exchange activity not reduced ¹³ increased iodide efflux ¹⁵				[86] [79]
FS93>96X ¹ , C416-1G_A ²	-	279delT	PS	intracellular ^{6,8}	intracellular iodide retention (primary thyrocytes culture) ^{14,15}				[92]
G102R	-	304G>A	PS	ER ⁹	loss of iodide efflux ¹⁵				[82]
L117F	=	349C>T	EVA	PM ⁹	normal iodide efflux ¹⁵		Yes		[82]
P123S	-	367C>T	PS	intracellular ⁸	loss of Cl ⁻ /I ⁻ exchange activity ¹⁵	Yes ¹⁸			[85]
V138F	-	412G>T	PS	ER ⁹	loss of iodide efflux ¹⁵		Yes	PS	[82]
P140H	-	419C>A	EVA		loss of chloride and iodide transport ¹²				[86]
P142R	-	425C>G	EVA	intracellular ⁷	loss of Cl ⁻ /HCO ₃ ⁻ exchange activity ¹³	+/- ¹⁷			[88]
M147V	-	439A>G	EVA	intracellular ⁷	loss of Cl ⁻ /HCO ₃ ⁻ exchange activity ¹³		Yes ¹⁷		[88]
			EVA	intracellular ⁸	loss of Cl ⁻ /I ⁻ exchange activity ¹⁵		Yes ¹⁸	[85]	
M147T		441T>C		intracellular ^{6,8}					[104]
S166N	=	497G>A	EVA	PM ⁷	normal Cl ⁻ /HCO ₃ ⁻ exchange activity ¹³				[88]
G209V	-	626G>T	PS, EVA	PM ⁹	severe reduction of iodide efflux ¹⁵		Yes	EVA	[82]
L236P	-	707T>C	PS	ER ⁹	loss of iodide efflux ¹⁵		Yes	PS	[82]
			PS	ER ⁹	loss of chloride and iodide uptake ^{11,14}				[81] [96]
			PS, EVA	intracellular ⁷	loss of Cl ⁻ /HCO ₃ ⁻ exchange activity ¹³ loss of Cl ⁻ /I ⁻ and Cl ⁻ /HCO ₃ ⁻ exchange activity ¹⁶	No ^{17, 19}		[88] [89]	
V239D	-	716T>A	EVA	ER ¹⁰	severe reduction of chloride and iodide transport ¹² loss of Cl ⁻ /OH ⁻ exchange activity ¹³		Yes	pathogenic	[95] [91]
V250A	=	749T>C	EVA		normal Cl ⁻ /HCO ₃ ⁻ and Cl ⁻ /I ⁻ exchange activity ¹⁶				[90]
D266N	=	796G>A	EVA		normal Cl ⁻ /HCO ₃ ⁻ and Cl ⁻ /I ⁻ exchange activity ¹⁶		Yes		[90]
FS297>302X	-	890delC	PS		loss of iodide efflux ¹⁵				[84]
P301L	-	902C>T			loss of chloride and iodide transport ¹²		Yes		[79]
E303Q	-	907G>C	EVA	PM ⁸	loss of Cl ⁻ /I ⁻ and Cl ⁻ /HCO ₃ ⁻ exchange activity ¹⁶				[90]
FS306>309X	-	916insG	PS	intracellular ⁹	loss of iodide efflux ¹⁵				[29]
G334V 335X	-	1001G>T	EVA		loss of chloride and iodide transport ¹² loss of Cl ⁻ /OH ⁻ exchange activity ¹³				[91]
F335L	-	1003T>C	EVA	PM ⁹	reduction of Cl ⁻ /I ⁻ and Cl ⁻ /HCO ₃ ⁻ exchange activity ¹⁶		Yes	EVA	[89]
F354S	=?	1061T>C	EVA		mild reduction of Cl ⁻ /HCO ₃ ⁻ exchange activity ¹⁶		Yes		[90]
					chloride and iodide transport not reduced ¹² Cl ⁻ /OH ⁻ exchange activity not reduced ¹³ iodide efflux not reduced ¹⁵			[79]	

Mutation	Activity compared to WT	Nucleotidic substitution	Pathology	Localization	Function	Rescue	SNPs database ²⁰	Pathology ²¹	Reference #
K369E	=	1105A>G	EVA	PM ⁸	normal Cl ⁻ /I ⁻ exchange ¹⁵		Yes	EVA	[85]
A372V	-	1115C>T	PS, EVA	intracellular ⁸	loss of Cl ⁻ /I ⁻ exchange activity ¹⁵	No ¹⁸	Yes	EVA	[85]
E384G	-	1151A>G	PS EVA	ER ⁹ intracellular ⁷	loss of chloride and iodide uptake ^{11,14} loss of Cl ⁻ /HCO ₃ ⁻ exchange activity ¹³ loss of Cl ⁻ /I ⁻ and Cl ⁻ /HCO ₃ ⁻ exchange activity ¹⁶	No ¹⁷	Yes	PS	[81] [96] [88] [89]
N392Y	-	1174A>T	PS	intracellular ⁸	loss of Cl ⁻ /I ⁻ exchange activity ¹⁵	No ¹⁸			[85]
V402M	-	1204G>A	EVA	intracellular ⁹	loss of Cl ⁻ /HCO ₃ ⁻ and Cl ⁻ /I ⁻ exchange activity ¹⁶				[89]
R409H	-	1226G>A	PS	partially PM ⁶	loss of iodide efflux ¹⁵ reduction of iodide and chloride transport ¹²		Yes	probably pathogenic	[84] (unpublished data)
R409H/V88I ³	-	1226G>A 262G>A	EVA		reduction of the chloride and iodide transport ¹²				[86]
T410M	-	1229C>T	EVA	ER ⁹	loss of iodide efflux ¹⁵		Yes	Pathogenic	[82]
Q413P	-	1238A>C	PS		loss of chloride and iodide transport ¹²				[86]
T416P	-	1246A>C	PS EVA	ER ⁹ intracellular ⁷	loss of chloride and iodide uptake ^{11,14} loss of Cl ⁻ /HCO ₃ ⁻ exchange activity ¹³ loss of Cl ⁻ /HCO ₃ ⁻ and Cl ⁻ /I ⁻ exchange activity ¹⁶	+/- ¹⁷	Yes	PS	[81] [96] [88] [89]
G424D	-	1271G>A	PS		reduction of the chloride and iodide transport ¹²				[86]
L445W		1334T>G	EVA/PS	intracellular ⁹ intracellular ^{6,8}			Yes	PS	[89] [104]
Q446R	-	1337A>G	EVA	ER ⁹	loss of iodide efflux ¹⁵				[82]
V480D	-	1440T>A	EVA		reduction of chloride and iodide uptake ^{11,14}				[81]
T485R	-	1454C>G	PS		reduction of the chloride and iodide transport ¹²				[86]
I487YFSX39 (526X)	-	1458_1459insT	EVA	ER ¹⁰	loss of chloride and iodide transport ¹² loss of Cl ⁻ /OH ⁻ exchange activity ¹³				[94] [91]
I490L	-	1468A>C	EVA		mild reduction of chloride and iodide uptake ^{11,14}				[81]
G497S	-	1489G>A	EVA EVA	intracellular ⁷	strong reduction of chloride and iodide uptake ^{11,14} loss of Cl ⁻ /HCO ₃ ⁻ exchange activity ¹³	+/- ¹⁷	Yes	pathogenic	[81] [88]
I490L G497S ⁵	-	1468A>C 1489G>A	EVA		strong reduction of chloride and iodide uptake ^{11,14}				[81]
Q514K	-	1541C>A	EVA, PS		loss of chloride and iodide transport ¹²		Yes	EVA	[86]
FS523>548X	-	1561_1571CTTGGAA TGGC	PS		loss of chloride and iodide transport ¹²				[70]
Y530H		1588T>C	PS	intracellular ⁹			Yes	PS	[89]
Y530S		1589A>C	EVA	intracellular ⁹					[89]
Y556C	-	1667A>G	PS	partially PM ⁹	loss of iodide efflux ¹⁵				[82]
C565Y	=?	1694G>A	EVA	PM ⁹ PM ⁸	reduction of Cl ⁻ /I ⁻ and Cl ⁻ /HCO ₃ ⁻ exchange activity ¹⁶ normal Cl ⁻ /I ⁻ exchange activity ¹⁵		Yes	probably pathogenic	[89] [85]
L597S	=?	1790T>C	controls only EVA	PM ⁹	normal chloride and iodide transport ¹² reduction of Cl ⁻ /I ⁻ and Cl ⁻ /HCO ₃ ⁻ exchange activity ¹⁶		Yes	probably non pathogenic	[86] [89]
V609G	-	1826T>C	NSHL		reduction of chloride and iodide transport ¹² reduction of iodide efflux ¹⁵		Yes	non pathogenic	[79]

Mutation	Activity compared to WT	Nucleotidic substitution	Pathology	Localization	Function	Rescue	SNPs database ²⁰	Pathology ²¹	Reference #
E625X	-	1873G>T	EVA	intracellular ⁷	loss of Cl ⁻ /HCO ₃ ⁻ exchange activity ¹³	No ¹⁷			[88]
V653A	-	1958T>C	EVA		reduction of chloride and iodide uptake ^{11,14}				[81]
S657N	-	1970G>A	EVA	intracellular ⁸	loss of Cl ⁻ /I ⁻ exchange activity ¹⁵	Yes ¹⁸			[85]
S666F	-	1997C>T	EVA	intracellular ⁸	loss of Cl ⁻ /I ⁻ exchange activity ¹⁵	No ¹⁸			[85]
F667C	-	2000T>C			loss of chloride and iodide transport ¹²	Yes		PS	[79]
G672E	-	2015G>A	PS	partially PM ⁹	loss of iodide efflux ¹⁵	Yes		pathogenic	[82]
L676Q	-	2027T>A	PS EVA	intracellular ⁹ intracellular ⁷	loss of iodide efflux ¹⁵ loss of Cl ⁻ /HCO ₃ ⁻ exchange activity ¹³	+/- ¹⁷	Yes	pathogenic	[29] [88]
D687Y	-	2059G>T			reduction of chloride and iodide transport ¹²	Yes			[79]
D697A	-	2090A>C	EVA	intracellular ⁸	reduction of Cl ⁻ /I ⁻ exchange activity ¹⁶	Yes			[90]
K715N	-	2145G>T	EVA	intracellular ⁸	reduction of Cl ⁻ /I ⁻ exchange activity ¹⁶				[90]
T721M	-	2162C>T	EVA	intracellular ⁸	loss of Cl ⁻ /I ⁻ exchange activity ¹⁵	No ¹⁸	Yes	PS, EVA	[85]
H723R	-	2168A>G	PS	intracellular ⁷ intracellular ⁸	loss of Cl ⁻ /HCO ₃ ⁻ exchange activity ¹³ loss of Cl ⁻ /I ⁻ exchange activity ¹⁵	Yes ^{17,19} Yes ¹⁸	Yes	PS	[88] [85]
D724G	-	2171A>G	EVA		loss of chloride and iodide transport ¹²				[86]
E737D	-	2211G>C	EVA	intracellular ⁸	reduction of Cl ⁻ /I ⁻ and Cl ⁻ /HCO ₃ ⁻ exchange activity ¹⁶				[90]
G740S	+	2218G>A	NSHL		increased chloride and iodide transport ¹² increased Cl ⁻ /OH ⁻ exchange activity ¹³ increased iodide efflux ¹⁵		Yes		[79]
M775T	-	2324T>C	EVA	PM ⁹	reduction of Cl ⁻ /I ⁻ and Cl ⁻ /HCO ₃ ⁻ exchange activity ¹⁶				[89]
R776C	=?	2326C>T	TPO mut. EVA	PM ⁹	normal iodide efflux ¹⁵ reduction of Cl ⁻ /I ⁻ and Cl ⁻ /HCO ₃ ⁻ exchange activity ¹⁶		Yes	probably non pathogenic	[83] [89]

Table 1. Summary of all the pendrin allelic variants for which the functionality or the subcellular localization is known as of now. For some mutants, the possibility to rescue the function with chemical or physical chaperones is reported. If the allelic variant is reported in the single nucleotide polymorphisms (SNPs) database is also indicated. +, gain of function; -, reduction or loss of function; =, benign polymorphisms; =?, controversial; the corresponding allelic variant may be hypofunctional; ¹originally reported as: Ser93ArgfsX3; ²acceptor splice site mutation; ³both mutations are present on the same chromosome; ⁴originally reported as: Ile487TyrfsX39; ⁵mutations are present on different chromosomes; the respective mutated proteins were co-expressed; ⁶western blot; ⁷N-glycosylation; ⁸confocal microscopy, immunofluorescence; ⁹GFP-fusion protein; ¹⁰YFP-fusion protein; ¹¹³⁶Cl⁻ uptake; ¹²fluorometric method; ¹³measure of the pH_i (BCECF); ¹⁴¹²⁵I uptake; ¹⁵¹²⁵I efflux; ¹⁶³⁶Cl⁻ efflux (rate constant evaluation); ¹⁷low temperature; ¹⁸10 mM salicylate; ¹⁹Na-butyrate; ²⁰http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?showRare=on&chooseRs=all&locusId=5172&mrna=NM_000441.1&ctg=NT_007933.15&prot=NP_000432.1&orien=forward&refresh=refresh, accessed on the 14th of September 2011; ²¹<http://omim.org/entry/605646>; del: deletion; ins: insertion; PS: Pendred syndrome; EVA: enlarged vestibular aqueduct; NSHL: non-syndromic hearing loss; TPO: thyroperoxidase; PM: plasma membrane; ER: endoplasmic reticulum; GFP: green fluorescent protein; YFP: yellow fluorescent protein; BCECF: 2',7'-bis-(2- carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester.

we recently suggested [93]. It is noteworthy that this model is very different from other predictions and that there are no experimental data proving any of the suggested models aside from findings suggesting that both the amino and the carboxyl termini are intracellular [93]. The functional tests allowed the identification of pendrin allelic variants with a reduction of loss of function respect

to WT; for these allelic variants a pathogenic potential can likely be assumed, and they are thereafter referred as “mutations”. In addition, allelic variants whose function is not significantly different from WT were found, and are thereafter referred as “benign polymorphisms”. Moreover, very recently, allelic variants showing a moderate gain of function were identified [79].

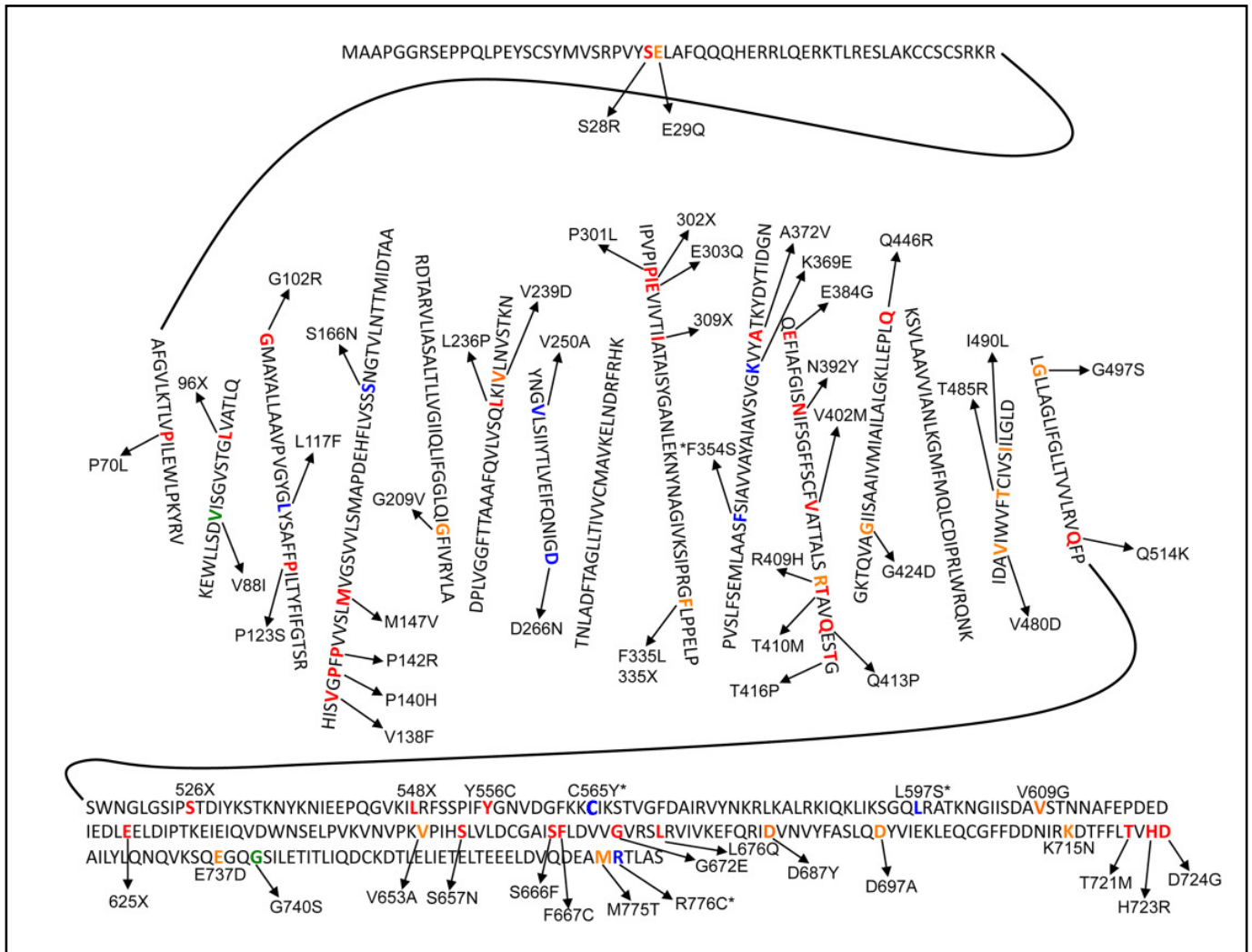


Fig. 2. Topology of pendrin allelic variants that have been functionally characterized as of now; the putative model of pendrin structure is taken from [87]. Amino acids that are affected by loss of function mutations are indicated in red, reduction of function are indicated in orange. Light blue indicates amino acid substitutions without functional impact, i.e. those mutations for which the activity is indistinguishable from WT (benign polymorphisms). Green indicates allelic variants with a modest gain of function. The extension ‘X’ indicates the mutations leading to premature truncation of the protein. Asterisks denote those allelic variants that are indicated as benign polymorphisms in this context, but with ambiguous function (they may be hypofunctional).

Intracellular localization

Localization studies revealed that mutated pendrin proteins are often retained in intracellular compartments and are unable to reach the plasma membrane [94-96]; as a consequence, the transport function is impaired [82, 85, 88-90, 92, 96]. These mutations most likely cause misfolding of the protein, with defective trafficking and subsequent degradation (“processing” defect). Some mutated proteins, however, reach the plasma membrane and show impaired transport function [32, 82, 89, 90]. These proteins are of particular interest, because the mutations possibly change ion binding or regulatory domains (Fig. 3). Several amino acid substitutions (F335L, C565Y, L597S, M775T, R776C), most of which are located

in the C terminus of the transporter, do not affect the targeting of the protein to the plasma membrane; their function is only slightly impaired compared to WT. They could, therefore, be classified as hypofunctional allelic variants [89]. Moreover, the function of C565Y, L597S, and R776C is controversial, as these allelic variants were classified as benign polymorphisms by other Authors [83, 85, 86] (Fig. 3). We conclude that these mutations probably do not affect important regions for ion binding or important regulatory domains of the transporter. The G209V [82] and E303Q [90] mutations also reach the plasma membrane, however, their function is severely impaired, indicating that these mutations do not impair trafficking, but might alter binding of the transported ions [90].

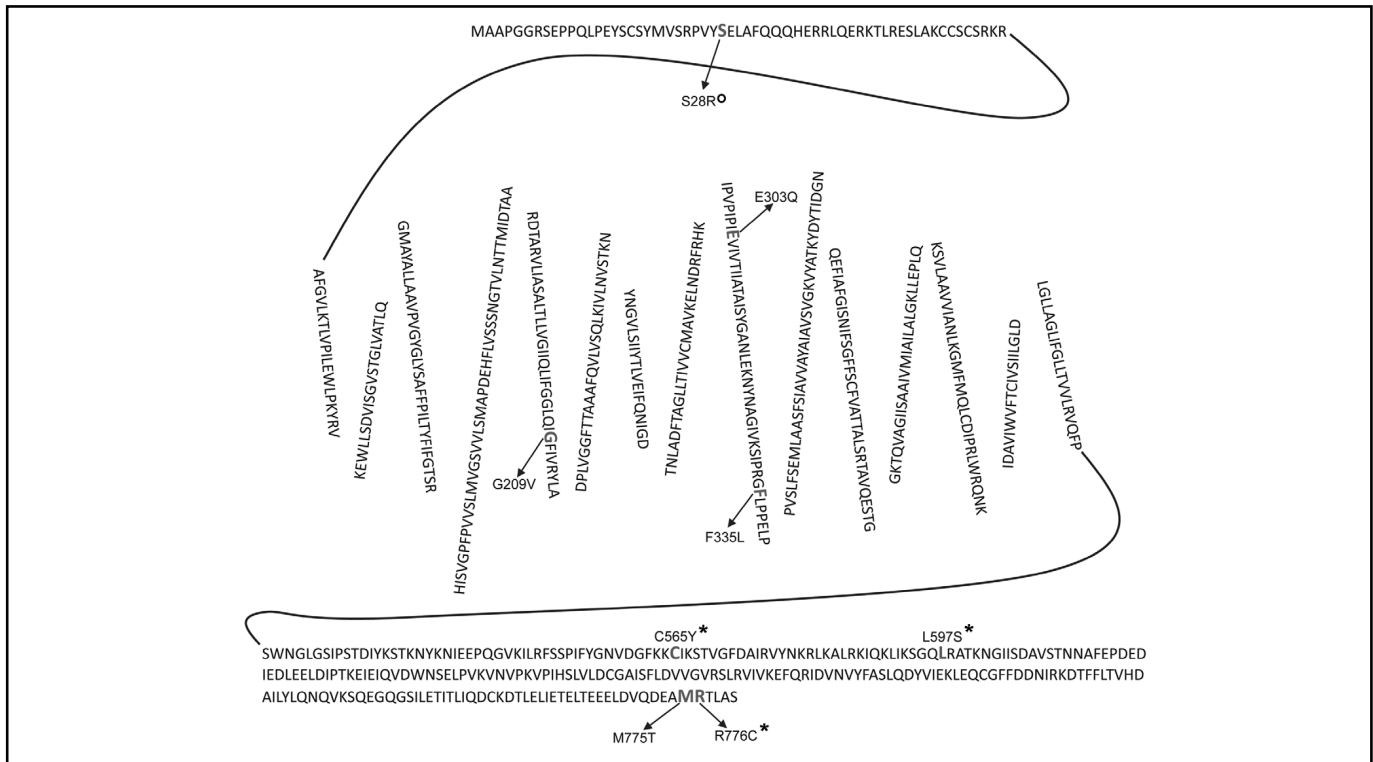


Fig. 3. Topology of the pendrin mutations with loss or reduction of function and normal trafficking to the plasma membrane; the putative model of pendrin structure is taken from [87]. Asterisks indicate those allelic variants whose functional alteration remains controversial. ° indicates a mutation for which the targeting to the plasma membrane is ambiguous.

Restoring transport function

Since the majority of pendrin mutants show a “processing” defect (see above), it is plausible that targeting of the mutants to the plasma membrane could be assisted by physical or pharmacological chaperones. Yoon et al. demonstrated that the plasma membrane targeting of some such mutants (S28R, M147V, H723R) (Table 1) could be rescued by growing the transfected cells at low temperature; interestingly, the Cl⁻/HCO₃⁻ exchange activity of the mutant H723R, which is the most common disease-associated mutation in East Asians [97], was also restored after low-temperature incubation or Na⁺-butyrate treatment [88]. Furthermore, Ishihara et al. demonstrated that 10 mM salicylate rescued the impaired trafficking of pendrin mutants P123S, M147V, S657N and H723R (Table 1) from the subcellular compartments to the plasma membrane, and restored transport activity (iodide efflux) [85]. Unfortunately, the response of the different pendrin mutants to chaperones is not identical; while some mutants are responsive to physical and chemical chaperones (such as M147V, H723R), others, such as L236P (the most common disease-associated mutation in Caucasians), A372V, E384G, N392Y, E625X, S666F and T712M, are not (Table 1).

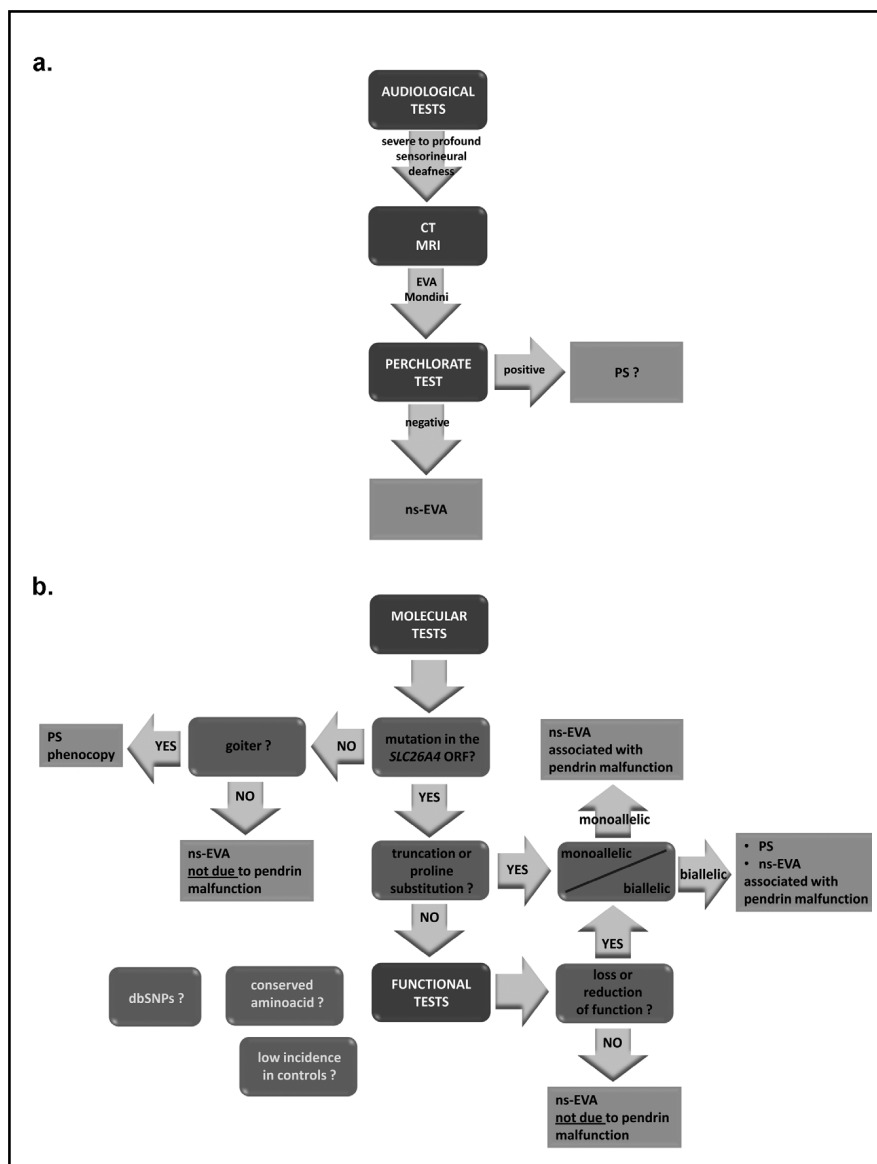
Benign polymorphisms

The functional characterization of pendrin mutants led to the identification of allelic variants for which the plasma membrane trafficking and transport function were not significantly impaired compared to WT (Table 1 and Fig. 2). At present, functional assays have shown that the transport activity of the following allelic variants is not significantly different compared to WT pendrin: L117F [82], S166N [88], V250A, D266N [90], F354S [79], K369E, C565Y [85], L597S [86] and R776C [83]. It is reasonable to assume that these mutations are benign polymorphisms, and that if they are found in patients with deafness and goiter, other genetic or environmental explanations should be considered in the diagnosis. However, others suggest that F354S [90], C565Y, L597S, R776C, despite retaining significant transport activity, should be considered hypo-functional and, if present in *trans* configuration (i.e. on a different allele of the pendrin gene) with a mutation, could display pathogenicity [89].

Gain of function mutations

V88I and G740S are allelic variants whose function is increased compared to WT [79, 86]. Given the aforementioned possible role of pendrin in the pathogenesis

Fig. 4. Suggested algorithm for the diagnosis of Pendred syndrome/ns-EVA related to pendrin dysfunction. (a) if in a patient with severe to profound sensorineural deafness the computed tomography (CT) or magnetic resonance imaging (MRI) of the temporal bone disclose malformations of the inner ear such as an EVA or Mondini cochlea, with or without a positive perchlorate discharge test, the molecular screening (sequencing) of the pendrin gene (b) is recommended. The detection of a mutation in the *SLC26A4* ORF leading to a truncated protein or an amino acid substitution involving a proline is a strong indication that the allelic variant is a pathogenic mutation. In all the other cases, only a functional test could discriminate between pathogenic mutations and allelic variants that cannot be considered as genetic determinants for Pendred syndrome/ns-EVA. Monoallelic mutations with reduction or loss of function are associated with ns-EVA. Biallelic (homozygous, compound heterozygous or double heterozygous, see Fig. 1) mutations with reduction or loss of function are associated with Pendred syndrome or, occasionally, with ns-EVA. The presence of a specific allelic variant in the dbSNPs should not imply a benign polymorphism; similarly, the low incidence in the control population or the involvement of a highly conserved amino acid should not be considered as indications of impaired function, and hence, pathogenicity. PS: Pendred syndrome; ns-EVA: non-syndromic enlarged vestibular aqueduct; ORF: open reading frame.



of hypertension, COPD and asthma, these variants may contribute to the severity of the phenotype and/or exacerbations of compromised airway function. The mechanism conferring a gain of function to the transporter is not known: as the putative anion binding site was postulated to be in a different region of the molecule [90], these amino acid substitutions likely do not increase the affinity of the transporter for its substrates. As WT pendrin is a slow-folding protein [98], with substantial retention in the intracellular compartments [32], there is the possibility that amino acid substitutions V88I and G740S aid in folding of the protein, consequently increasing its targeting to the plasma membrane. Alternatively, these amino acid substitutions could improve the affinity of the transporter for not yet identified regulatory partners that may increase pendrin activity.

Genotype-phenotype correlation

Considerable effort has been devoted to correlate the type of mutation with the phenotype found in the respective patient (age of onset and degree of deafness, presence of goiter, etc.). It was previously proposed that loss of function mutations could confer Pendred syndrome, while mutations with residual transport could be associated with ns-EVA [81]. However, the identification of mutations common to both pathological conditions led to the exclusion of this hypothesis [69]. The correlation between the specific pendrin mutation and the clinical phenotype of the patient is difficult for the following reasons: (i) for a significant number of patients described in the literature, the perchlorate discharge test has not been performed, so that no discrimination between Pendred syndrome and ns-EVA

in the absence of overt goiter was possible, (ii) if the mutation is found in compound heterozygosity, only rarely have both detected alleles been characterized functionally, and (iii) seldom have both the I/Cl⁻ and Cl/HCO₃⁻ exchange activities been determined. It is important to note that I/Cl⁻ exchange, which is more relevant at the level of the thyroid gland, may be less affected by mutations when compared to Cl/HCO₃⁻ exchange, that is more relevant at the level of the inner ear [89, 90]. This fact could explain, at least in part, the lower penetrance of thyroid abnormalities than of EVA and hearing loss [90]. In addition, nutritional iodide intake [99] and epigenetic factors, including individual variations in WT or mutant pendrin expression levels, or proteins that could partially substitute for pendrin function should be considered.

The functional characterization of pendrin allelic variants may be a valuable help in the diagnosis of Pendred syndrome and ns-EVA due to pendrin malfunction

The diagnosis of Pendred syndrome and ns-EVA due to pendrin malfunction is challenging and may rely on the following tools (Fig. 4a): (i) the audiological examination, that should reveal severe to profound sensorineural deafness; (ii) the imaging of the temporal bone, that should reveal malformations of the inner ear, such as an EVA or Mondini cochlea, and an enlarged endolymphatic sac when evaluated appropriately with MRI [64]; (iii) the perchlorate discharge test, that discloses a possible iodide organification defect and, in the absence of overt goiter, is essential for discriminating between Pendred syndrome and ns-EVA [65]. The existence of phenocopies of Pendred syndrome (i.e. patients displaying goiter and deafness unrelated to pendrin malfunction) [100-102], and the fact that ns-EVA could also be unrelated to pendrin mutations [65, 66], led to the conclusion that molecular tests (i.e. the sequencing of pendrin gene, Fig. 4b) are essential for the diagnosis of Pendred syndrome/ns-EVA due to pendrin malfunction [103]. However, the identification of one or two mutations in the pendrin gene is not sufficient to conclude that the detected mutation(s) is (are) the genetic cause of the phenotype of the patient. Indeed, the presence of functional, benign polymorphisms in some populations (Table 1 and Fig. 2) and the misclassification of these benign polymorphic variants as pathogenic alleles [89] can lead to erroneous classification. As a consequence, is essential that, whenever a mutation in the pendrin gene is found, its pathogenic potential is established.

Recently, we defined that the two parameters used so far to assess the pathogenic potential of a mutation, low incidence in the control population, and substitution of evolutionary conserved amino acids, are not always sufficient for defining the pathogenicity of pendrin allelic variants [86]. Indeed, a pathogenic mutation is expected to occur with lower frequency in the cohort of normal-hearing individuals with respect to the hearing-impaired cohort of patients. In contrast, in the Spanish population, we unexpectedly identified mutations with impaired function, hence most likely pathogenic, (E29Q, V609G, D724G) occurring with the same frequency in the cohort of hearing impaired patients and in the control group of normal hearing individuals [79, 86]. Similarly, the amino acid change F667C was identified in the control population only and not in deaf patients; despite that, the functional tests revealed that this allelic variant is a mutation with reduced function, and not a benign polymorphism, as expected from its incidence. In the same context, a pathogenic mutation is expected to affect conserved amino acids. However, we and other groups identified functional, benign polymorphisms affecting residues highly conserved among pendrin orthologs [93], such as F354S [79], K369E [85], and L597S [86]. Identifying new criteria for establishing the pathogenicity of an allelic variant is therefore crucial. As a general rule in this complex scenario, we previously observed a complete loss of function for all truncation mutations and mutations involving the substitution of a proline or a charged (acid or basic) amino acid [86]. As the effort of the functional characterization of pendrin allelic variants progressed, it became obvious that the involvement of a charged amino acid is not always sufficient to induce a detrimental effect on the ion transport. Indeed, the functionality of pendrin D266N and K369E is not reduced with respect to WT (Table 1 and Fig. 2). However, (i) truncation mutations and (ii) mutations involving the substitution of a proline always showed a complete loss of function (Table 1, Fig. 2 and 4b). In all the other cases, only a functional test allows for the discrimination between a pathogenic mutation and a benign polymorphism (Fig. 4b).

Conclusions

The diagnosis and the discrimination between Pendred syndrome and ns-EVA can be difficult because of the existence of Pendred syndrome phenocopies; in this view, clinical and radiological studies could be corroborated by genetic and molecular studies. In

performing genetic studies, the possibility that the mutation could affect the pendrin promoter, intronic regions or coding regions of functionally related genes (*FOXI1*, *KCNJ10*) should be taken into account. Of note, the high incidence of benign polymorphisms in the population could lead to false positive results. For this reason, genetic studies should be implemented together with functional studies, to unambiguously discriminate between pathogenic mutations and allelic variants that cannot be considered as genetic determinants for Pendred syndrome and ns-EVA. Assessing the functionality of pendrin allelic variants, the presence of hypo-functional variants and possible

differences between the I^-/Cl^- and Cl^-/HCO_3^- exchange activities should be carefully evaluated.

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