

# SLC26A4 variations among Graves' hyper-functioning thyroid gland

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**Abstract.** Deleterious mutations of *SLC26A4* cause Pendred syndrome (PS), an autosomal recessive disorder comprising goitre and deafness with enlarged vestibular aqueducts (EVA), and nonsyndromic hearing loss (NSHL). However, the *SLC26A4* hyperactivity was recently associated with the emergence of autoimmune thyroid diseases (AITD) and asthma among human and mouse model. Here, by direct sequencing, we investigate the sequences of the 20 coding exons (2 to 21) of *SLC26A4* and their flanking intron-exon junctions among patients affected with Graves' disease (GD) hyperthyroidism. Ten mono-allelic variants were identified, seven of which are intronic and previously unreported. Two, c.898A>C (p.I300L) and c.1061T>C (p.F354S), of the three exonic variants are non synonymous. The p.F354S variant is already described to be involved in PS or NSHL inheritances. The exploration by PCR-RFLP of p.I300L and p.F354S variants among 132 GD patients, 105 Hashimoto thyroiditis (HT), 206 Healthy subjects and 102 families with NSHL have shown the presence of both variants. The p.F354S variation was identified both among patients (1 HT and 3 GD) and healthy subjects ( $n = 5$ ). Whereas, the p.I300L variant was identified only in GD patients ( $n = 3$ ). Our studies provide evidence of the importance of systematic analysis of *SLC26A4* gene sequences on models other than deafness. This approach allows the identification of new variants and the review of the pathogenic effects of certain mono-allelic variants reported responsible for PS and NSHL development.

Keywords: Pendred syndrome, autoimmune thyroid disease, graves' disease, *SLC26A4*

## 1. Introduction

Since 1997 the genomic sequence of PDS (*SLC26A4*) gene was investigated to explain the inheritance of autosomal syndromic or isolated hearing loss among family and sporadic patients [1]. Biallelic mutations in the *SLC26A4* gene lead to Pendred syndrome (PS), an autosomal recessive disorder characterized by sensorineural deafness, goiter, and impaired iodide organification [2]. The *SLC26A4* gene encodes the pendrin protein which is an anion transporter that is expressed

in the inner ear, the thyroid, the kidney, the placenta and the endometrium [3–7]. In the inner ear, pendrin has been found in the stria vascularis of the cochlea and in the endolymphatic duct and sac, where it functions as a  $\text{Cl}^-/\text{HCO}_3^-$  exchanger [8]. In the kidney, pendrin is localized in the apical membrane of type-B intercalated cells and non-A, non-B intercalated cells of the cortical collecting ducts and connecting tubules, where it again acts as a  $\text{Cl}^-/\text{HCO}_3^-$  exchanger regulating the acid-base status and chloride homeostasis [9]. In thyroid follicular cells, pendrin is expressed at the apical membrane [10]. Functional *in vitro* data and the impaired iodide organification observed in patients with PS support a role of pendrin as an apical iodide transporter.

In PS the thyroid symptoms are highly variable. Indeed, goiter is not a constant feature and can range

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from a slight increase in thyroid size to a large multinodular goiter [11]. Anyway, the *SLC26A4* mutation is often associated with hypo-functioning when thyroid affection is described.

In 2003, we reported the genetic involvement of *SLC26A4* in the susceptibility to autoimmune thyroid disease (AITD) [12]. The hyper expression of pendrin at the apical membrane of Graves' Disease (GD) thyrocytes was firstly described by Royaux et al. (2000). More recently, Yoshida A. et al. (2009) have shown the presence of anti pendrin auto-antibodies in the sera of patients affected with auto-immune thyroid diseases (AITD) [13]. In addition, recent studies in mouse models implicate *SLC26A4* hyperactivity in asthma and chronic obstructive pulmonary disease (COPD) development [14–16]. Expression of pendrin in the lung increases in response to allergens and high concentrations of IL-13 [17,18]. Recently, in order to evaluate the association between *SLC26A4* variants and asthma emergence, the investigation of asthma prevalence among PS patients (two mutant alleles) or subjects with zero and one mutant alleles of *SLC26A4* gene was described [19].

Although the involvement of the *SLC26A4* gene has been reported with several pathological models, the systematic screening of the coding sequence of the gene is conducted only among deaf patients usually having a normal or hypo-functioning thyroid gland. Here, by direct sequencing we investigate the exonic sequence of *SLC26A4* among patient affected with Graves' hyper-functioning thyroid gland and we identified ten variants.

## 2. Subjects and methods

### 2.1. AITD patients and controls

One hundred thirty two unrelated patients (101 women and 31 men) with GD and 105 unrelated patients (94 women and 11 men) with Hashimoto Thyroiditis (HT) were included in this study. Data gathered from the patient group were compared with those obtained from a control population of 206 unrelated healthy subjects (103 women and 103 men) originating from the same area with no clinical evidence or family history of AITD and inflammatory joint disease.

GD was diagnosed on the basis of clinical and laboratory evidence of hyperthyroidism requiring treatment, palpable diffuse goiter, high thyroid hormonal rates, positive anti-TSHR, antithyroid peroxidase (anti-TPO),

and/or anti-thyroglobulin (anti-Tg) antibodies. HT was diagnosed by documented clinical and biochemical hypothyroidism requiring thyroid hormone replacement and presence of autoantibodies to thyroid peroxidase, with or without antibodies to thyroglobulin.

Twenty unrelated GD patients (gender: 16 women and 4 men, age at diagnosis:  $36.8 \pm 18$ , goitre: 95%, Graves' ophthalmopathy: 90%, anti-Tg: 70%, anti-TPO: 70%) were randomly selected from the pool of GD patients in order to perform the sequencing of *SLC26A4* gene exons and their flanking intron-exon junctions. Two patients (one man and one woman) extracted from the families already used in the linkage studies [12] were added to the sequencing project. All selected patients have no familial history of hearing loss.

### 2.2. Deaf patients

We analysed 102 Tunisian families in which severe to profound nonsyndromic (NSHL) is transmitted as an autosomal recessive trait. The medical history was obtained and all individuals were evaluated by clinical examination including otoscopic exploration and pure-tone audiometry. Syndromic forms and patients with suspected environmental cause of hearing impairment (HI) were excluded.

All experiments were undertaken with the understanding and written consent of each subject. This study conforms to The Code of Ethics of the World Medical Association and reviewed and approved by the local ethics committee.

### 2.3. PCR and direct sequencing

Genomic DNA was isolated from EDTA-containing whole blood using the standard phenol-chloroform extraction protocol. The 20 coding exons (numbered from 2 through 21) of the *SLC26A4* gene (and their flanking intron-exon junctions) were amplified by polymerase chain reaction (PCR) from peripheral leucocytes using primers listed in the Table 1. For all reactions, the 50- $\mu$ L reaction mixture contained about 200 ng of genomic DNA, 2.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L of each deoxynucleoside triphosphate (dNTP), 0.10  $\mu$ mol/L of each primer, 1  $\times$  reaction buffer, and 1 U of Taq DNA polymerase (Fermentas, EU). The PCR products were purified by enzymatic treatment using 10U of ExonucleaseI (Fermentas, EU) and 1U of Shrimp Alkaline Phosphatase (Fermentas, EU) and sequenced by automated DNA sequencing

Table 1  
Primer sequences of the 20 coding exon of *SLC26A4* gene

| Exon | Primer sequence                                       | Fragment size |
|------|---|---------------|
| 2    | cagtgatcccgttcttct<br>aggaccggagaccgaaagt             | 418bp         |
| 3    | catcagcagaatccagttca<br>tacagccctgaaggtaag            | 338bp         |
| 4    | ggcaaaagtcataagtgaacc<br>tgcaccaacctaataagaggtataa    | 295bp         |
| 5    | aaaccctatgcagacacattg<br>tttgggtccaggaatta            | 375bp         |
| 6    | aggcaggctactagtgtttca<br>ggcccagactcagagaatga         | 353bp         |
| 7    | atggttttcatgtggaag<br>tggcagtagcaattatcgtc            | 385bp         |
| 8    | ttcagcattattgtgtgaca<br>tttctccagatcacacaaa           | 296bp         |
| 9    | gactagcagatagcatttga<br>tccaaccctcttttagctg           | 332bp         |
| 10   | ctgaaatactcagcgaaggtct<br>gccttctctgttgcatt           | 250bp         |
| 11   | ggctgtctatcacacatcca<br>aaatctctgtttattatctgtagc      | 240bp         |
| 12   | aacaggagatttaacaatcacaca<br>atagtataatggcaggaagca     | 241bp         |
| 13   | ttttatagtagttatcacatgatgg<br>aacgaaagaaagtggtctca     | 281bp         |
| 14   | caccagaatgatggctctt<br>cgtaaaatggagctgctgaa           | 240bp         |
| 15   | ctgctaccagctctctga<br>cctgattggaccagtaaa              | 296bp         |
| 16   | gccttccagataacagttgc<br>titaatttagacccttaactgctc      | 250bp         |
| 17   | ggaacagtgtgtagtcttttgg<br>gcaatactggacaaccaca         | 400bp         |
| 18   | tcctgagcaagtaactgaatgc<br>aatgtgaccacagtcccaga        | 215bp         |
| 19   | agtgaacatgatgccactg<br>gacttgtaattttgccatt            | 341bp         |
| 20   | cattttcagtgagcatcagg<br>tgttccctgacagttcttaatac       | 250bp         |
| 21   | aaaagataatgcagacttaaggagaa<br>aaagcctagaagcagcttagtgc | 235bp         |

analysis with fluorescence-labeled dideoxyterminators (BigDye Terminator V3.1 Cycle Sequencing Kits, Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations (ABI 3100-4 Genetic Analyser, Applied Biosystems, Foster City, CA).

#### 2.4. PCR-RFLP

The -103 T/C (rs60284988), c.898A>C and c.1061T>C variants were explored by the polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP) using the appropriate primers (Table 1). The -103 T/C transition on the untranslated exon1 sequence of *SLC26A4* gene was explored using the following primers: forward CCTTCGACCAAG-

GTGTCTGC and reverse TTAAGAGTCCCATCCG-CAAC with introducing mutation in the forward sequence in order to create a *BsrBI* specific restriction site containing the "C" allele.

PCR amplification was performed as described above. Genotypes discriminations were achieved by fragments restrictions using *BsrBI*, *MboII* and *MnII* (fermentas, EU) to explore -103 T/C, 1061T>C and 898A>C variants respectively. Digestion reactions were performed according to the provider protocols. To verify digestion reactions, the same procedure was performed on a commercial plasmid cloning vector (pUC19: New England Biolabs) containing 3 *BsrBI*, 8 *MboII* and 13 *MnII* restrictions sites and three different PCR fragments containing a single restriction site for each used restriction enzyme. Amplified products were resolved on 4% agarose gels. Subjects with the rare allele were sequenced as described above.

### 3. Results and discussion

Pendrin is a member of the anion transporter family *SLC26*, which mediates the exchange of anions including  $\text{Cl}^-$ ,  $\text{HCO}_3^-$ ,  $\text{OH}^-$ ,  $\text{I}^-$ , or formate [20], and is encoded by the *SLC26A4* gene. Apically located pendrin [10] seems to be responsible for the efflux of iodide into the follicular lumen [21-23].

Mutations in *SLC26A4* cause nonsyndromic hearing loss associated with an enlarged vestibular aqueduct (ns-EVA, also known as DFNB4) and PS, the most common type of autosomal-recessive syndromic deafness (up to 10%) [24,25]. PS seems to be linked to bi-allelic mutations of the *SLC26A4* genes. ns-EVA is genetically more heterogeneous relative to PS, and although some cases show mono- or bi-allelic *SLC26A4* mutations (on cis- or trans-position), other cases are not linked to the *SLC26A4* locus. Since its characterization [1], about 200 allelic variants of the *SLC26A4* gene have been described (www.healthcare.uiowa.edu/labs/pendredandbor and literature), all of which are located along the coding sequence, the splice sites, and in the noncoding exon 1 within the *FOXII* binding transcriptional regulatory elements [26]. In PS patients, pendrin impaired function at the thyroid level can result in goiter, defects in iodide organification, and hypothyroidism [11,27,28]. The variability of thyroid symptoms in PS is well described [24,29]. Moreover, *SLC26A4* mutations associated with congenital hypothyroidism (CH), multinodular dysshormonogenetic goitre or goitrous CH are reported, even though they

Table 2  
*SLC26A4* gene identified variants by direct sequencing among 20 GD patients

| Nucleotide change | Exon/Intron | aa change | Genotype | Patient number |
|-------------------|-------------|-----------|----------|----------------|
| c.164+11C>G       | In 2        | –         | CG       | 1              |
| c.164+48A>T       | In 2        | –         | AT       | 1              |
| c.164+62G>C       | In 2        | –         | GC       | 1              |
| c.304+24T>C       | In 3        | –         | TC       | 2              |
| c.415+54C>A       | In 4        | –         | CA       | 2              |
| c.898A>C          | Ex 7        | I300L     | AC       | 1              |
| c.1061T>C         | Ex 9        | F354S     | TC       | 1              |
| c.1368C>G         | Ex 12       | A456A     | CG       | 1              |
| c.1614+8C>T       | In 14       | –         | CT       | 1              |
| c.1614+14T>G      | In 14       | –         | TG       | 1              |

are rare [30–32]. Unfortunately, independently to PS clinical evidence, the analysis of *SLC26A4* genomic sequence among patients with thyroid disease is poor documented.

Here, with the aim to explain our previous genetic study [12] and in order to bring more clarity on the variability of thyroid affection under *SLC26A4* mutations, we examine the variability of the *SLC26A4* gene sequence among AITD patients. We started our project by direct sequencing of the exonic sequences of gene and their flanking intron-exon junctions among GD patients. The choice of GD was not random. Indeed, all the mutations described until now are responsible for a hypothyroidism state. Thus, from a functional standpoint, the identification of allelic variants in patients suffering from hyper function of the thyroid gland is also attractive.

Exploration of *SLC26A4* gene sequence in 20 Tunisian unrelated GD patients reveals 10 mono-allelic variants, seven of which are intronic and previously unreported (Table 2). However, no polymorphisms have been identified in patients extracted from AITD families already used in the genetic study [12]. Besides, the exploration of the –103 T/C transition (rs60284988) in the 5'UTR (exon1) of the *SLC26A4* gene showed the absence of the "C" allele in all tested subjects. Three different exonic variants; two transversion in exon 7 (c.898A>C) and 12 (c.1368C>G) and one transition in exon 9 (c.1061T>C) were identified. The positive patient for c.1061T>C also displays a heterozygosis for c.304+24T>C and c.164+62G>C variants.

In this work, we focus on the non synonymous variants. In fact, the c.898A>C and c.1061T>C led to an amino acids substitution respectively at the positions 300 (p.I300L) and 354 (p.F354S) of the pendrin sequence (Fig. 1). The analysis of both variations by PCR-RFLP among patients (GD ( $n = 132$ ) and HT ( $n = 105$ )) and healthy subjects ( $n = 206$ ) identify 9 heterozygous subjects for the p.F354S variant, 3 GD,

1 HT and 5 healthy subjects (3 males and 2 females). Nevertheless, the p.I300L variation was detected only in 3 GD heterozygous patients. For the two variants, all identified patients were female with goitre, no Graves' ophthalmopathy and no hearing loss family history. The absence of homozygous subjects for both variations reflects the weak frequencies of the rare alleles in the general population (around 1%). The exploration of both variants among 102 NSHL Tunisian families confirms once again their rarities. Indeed, both variants are absent.

The analysis of the coding sequences of *SLC26A4* gene (protein and mRNA) of 12 animals showed the conservation of I300 and F354 among all used species at protein and mRNA levels (A898 and T1061) (Figs 1 and 2).

Two putative topologies of human pendrin were reported in the literature with 12 transmembrane model (described on the Pendred/BOR homepage (<http://www.healthcare.uiowa.edu/labs/pendredandbor/domains.htm>)) or 15 transmembrane-segment models [33]. p.I300L and p.F354S were localised respectively in the S7 and S8 transmembrane segments of the first model and in S9 and S10 transmembrane segments of the second model.

The p.F354S was already described by four different studies of *SLC26A4* sequence [34–37]. Blons et al. (2004) and Albert et al. (2006) attribute a pathogenic effect of this variant to explain syndromic or isolate hearing loss respectively among sporadic and familial appearances. Thyroid affection was described (hypothyroidism) when the compound heterozygosity effect with the p.Y530H mutation was detected [34]. Anyway, the F354S was never been described as a allelic mutation. Recently, Dai et al (2009) attribute a functional effect for p.F354S substitution. In fact, the mutant protein expressed in *Xenopus* oocytes displayed selective reduction in relative rate of  $\text{Cl}^-/\text{HCO}_3^-$  exchange compared with similarly measured rates of

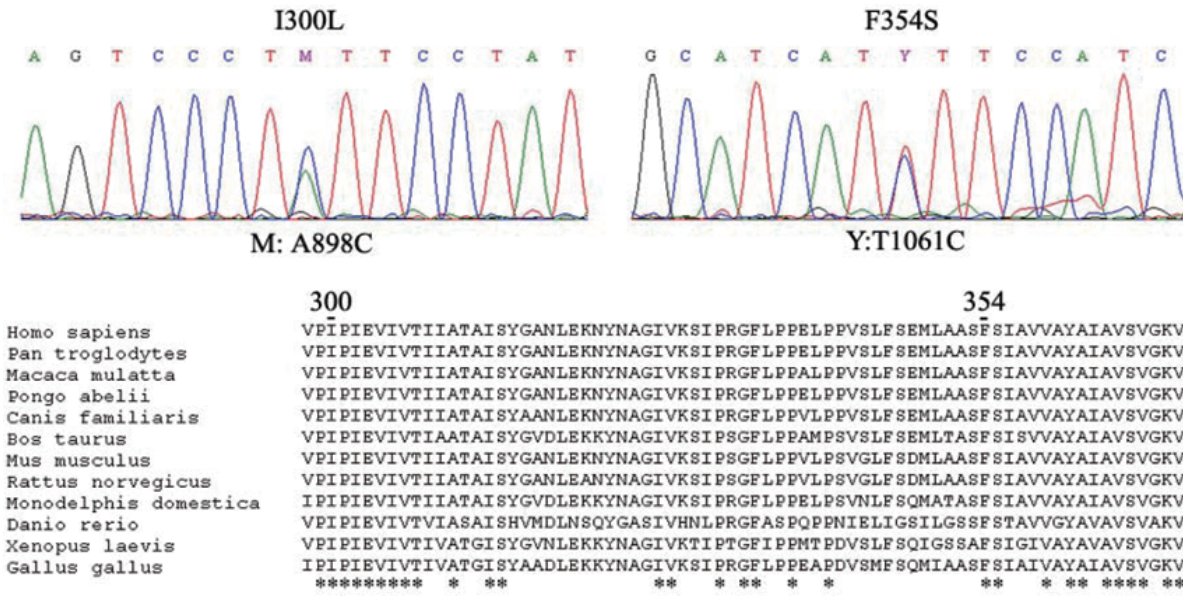


Fig. 1. The p.I300L and p.F354S variants: electrochromatogramme of heterozygous GD patients and the conservation of 300 and 354 position among 12 animal protein sequences. The protein alignment was carried out using respectively the following references: Homo sapiens NP\_000432.1, Pan troglodytes XP\_519308.2, Macaca mulatta XP\_001094049.1, Pongo abelii CAI30271.1, Canis Familiaris XP\_540382.2, Bos Taurus XP\_608706.3, Mus musculus NP\_035997.1, Rattus novogicus NP\_062087.1, Monodelphis domestica XP\_001363598.1, Danio rerio NP\_001159387.1, Xenopus laevis NP\_001089008.1, Gallus gallus XP\_425419.2.



Fig. 2. The conservation of A898 and T1061 positions among 12 animal SLC26A4 mRNA sequences. The alignment was carried out using respectively the following references: Homo sapiens NM\_000441.1, Pan troglodytes XM\_519308.2, Macaca mulatta XM\_001094049.1, Pongo abelii CR926479.1, Canis Familiaris XM\_540382.2, Bos Taurus XM\_608706.3, Mus musculus NM\_011867.2, Rattus novogicus NM\_019214.1, Monodelphis domestica XM\_001363561.1, Danio rerio NM\_001165915.1, Xenopus laevis NM\_001095539.1, Gallus gallus XM\_425419.2.

Cl<sup>-</sup>/Cl<sup>-</sup> and Cl<sup>-</sup>/I<sup>-</sup> exchange. This finding emphasised the role of p.F354S in the inner ear and reduce its pathogenic effect in the thyroid gland function. However, using a cohort of 214 normal-hearing individuals of Spanish–Caucasian population, Pera et al. [37] have reported the identification of p.F354S and the absence of this variant among patient group. Our work suggests that the p.F354S variant seems to be a rare polymorphism.

Different to p.F354S, the pathogenic effect of p.I300L was never been investigated. This variant was reported in one family by the Molecular Otolaryngology Research Laboratories (MORL) (<http://www.medicine.uiowa.edu/otolaryngology/MorlLab/SLC26A4>) without any clinical information and as a personal communication from Prasad S. The absence of p.I300L among patients with HT (n = 105), deafness (n = 102) and healthy subjects (n = 206) suggests a potential role of p.I300L in the GD genetic susceptibility. The comparison between the expected genotypic distribution (under Hardy Weinberg equilibrium) and the observed data showed a significant difference (Fisher exact test p < 0.01). However, even if p.I300L has a contribution in the development of GD, its role should be minor and involves only a reduced number of patients (3/132). Anyway, even if the genetic of AITD is complex, the

medicine.uiowa.edu/otolaryngology/MorlLab/SLC26A4) without any clinical information and as a personal communication from Prasad S. The absence of p.I300L among patients with HT (n = 105), deafness (n = 102) and healthy subjects (n = 206) suggests a potential role of p.I300L in the GD genetic susceptibility. The comparison between the expected genotypic distribution (under Hardy Weinberg equilibrium) and the observed data showed a significant difference (Fisher exact test p < 0.01). However, even if p.I300L has a contribution in the development of GD, its role should be minor and involves only a reduced number of patients (3/132). Anyway, even if the genetic of AITD is complex, the

involvement of *SLC26A4* in its development is not excluded. Interestingly, autoanti-body recognizing Pendrin protein has recently been described in HT and, to a lesser extent in GD, using immunoblotting with patient sera [13]. On the other hand, the concurrence of PS, autoimmune thyroiditis, and simple goiter in one family has been described [38]. More recently, the association between PS and autoimmune hyper-functioning thyroid gland was reported, when the pathogenic effect of compound heterozygosity (1001+1G>A and 2015G>A base changes) within the *SLC26A4* genomic sequence is suspect [39].

Although more than 20 exonic variants of the *SLC26A4* gene have already undergone functional exploration [33], the pathogenic effects of promoter (regulation) and intronic (splice) variants on the gene and pendrin expression remain unexplored. Further, such investigation could provide clarifications on the contribution of the *SLC26A4* gene in the development of certain complex diseases with population high prevalence such as AITD, hypertension and asthma. The considerable advances in sequencing techniques could enhance the *SLC26A4* large-scale systematic screening and validate/reject consequently the pathogenic effect of some reported mono-allelic variants responsible for PS or NSHL.

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