

CASES SERIES AND REPORTS

Further characterisation of the recently described *SLC26A4* c.918+2T>C mutation and reporting of a novel variant predicted to be damaging

Caratterizzazione della mutazione SLC26A4 c.918+2T>C e report di una nuova variante potenzialmente a rischio

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SUMMARY

Pendred syndrome (PS) is the second most common type of autosomal recessive syndromic hearing loss (HL). It is characterised by sensorineural HL and goiter with occasional hypothyroidism. These features are generally accompanied by malformations of the inner ear, as enlarged vestibular aqueduct (EVA). In about 50% of probands, mutations in the *SLC26A4* gene are the cause of the disease. Here we report the case of a Portuguese female, aged 47, presenting with severe to profound HL and hypothyroidism. Her mother and sister, both deceased, had suffered from HL and goiter. By MRI and CT, an enlarged vestibular aqueduct and endolymphatic sac were observed. Molecular study of the patient included screening for *GJB2* coding mutations and *GJB6* common deletions followed by screening of all *SLC26A4* exons, as well as intronic regions 8 and 14. Mutation c.918+2T>C was found for the first time in homozygosity in the intronic region 7 of the *SLC26A4* gene. Whilst sequencing the control samples, a novel mutation c.821C>G was found in heterozygosity in the exon 7 of *SLC26A4* gene and was predicted to be damaging. This study thus led to the finding of two novel *SLC26A4* genotypes and provides new insight on the phenotypic features associated with PS.

KEY WORDS: Pendred syndrome (PS) • Hearing loss (HL) • Enlarged vestibular aqueduct (EVA) • Magnetic resonance imaging (MRI) • Computerised tomography (CT) • Videonystagmography (VNG) • Berkeley Drosophila Genome Project (BDGP)

RIASSUNTO

La sindrome di Pendred è, in ordine di frequenza, la seconda causa di ipoacusia su base genetica autosomica recessiva. Si manifesta con un'ipoacusia accompagnata dalla presenza di un gozzo tiroideo con eventuale ipotiroidismo. Tali caratteristiche si accompagnano a malformazioni dell'orecchio interno, quali l'acquedotto vestibolare largo. Nel 50% dei casi vi è una mutazione del gene SLC26A4. Riportiamo nel presente lavoro il caso di una paziente portoghese di 47 anni affetta da ipoacusia di grado severo/profondo e ipotiroidismo. La madre e la sorella della paziente, entrambe decedute, erano a loro volta affette da ipoacusia associata a gozzo tiroideo. La risonanza magnetica e la TC hanno entrambe evidenziato un allargamento dell'acquedotto vestibolare e del sacco endolinfatico. La paziente è stata sottoposta a uno studio di GJB2 e GJB6 seguiti da uno screening di tutti gli esoni di SLC26A4 e delle regioni introniche 8 e 14. È stata rilevata, per la prima volta in omozigosi, una mutazione c.918 + 2T>C nella regione intronica 7 del gene SLC26A4. Sequenziando i campioni di controllo è stata rilevata una nuova mutazione c.821C>G presente in eterozigosi nell'estone 7 del gene SLC26A4, per la quale si è ipotizzato un ruolo dannoso. Il presente studio ha condotto alla scoperta di due nuovi genotipi di SLC26A4, e alla miglior definizione degli aspetti fenotipici associati alla sindrome di Pendred.

PAROLE CHIAVE: *Sindrome di Pendred • Ipoacusia • Sindrome dell'acquedotto vestibolare largo • Risonanza magnetica • Tac • Videonistagmografia • Berkeley Drosophila Genome Project*

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Introduction

Hereditary syndromic hearing loss (HL) includes about 400 syndromes, such as Pendred syndrome (PS). This

syndrome is the second most common type of autosomal recessive syndromic HL worldwide ¹, with an incidence estimated to be as high as 7.5 to 10 in 100,000 individuals ^{2,3}.

PS is characterised by sensorineural HL, goiter and a partial defect in iodide organification. These features are generally accompanied by malformations of the inner ear, ranging from enlarged vestibular aqueduct (EVA) to Mondini dysplasia⁴. The clinical features observed in PS typically result from biallelic (homozygote/compound heterozygote) mutations in the *SLC26A4* gene. According to the Human Gene Mutation Database more than 260 mutations in the *SLC26A4* gene have been identified to date⁵, including splice site aberrations, frame shift and nonsense mutations, as well as large deletions (rare cases) and a relatively common mutation, c.-103 T > C, in a regulatory element of the promoter region of the *SLC26A4* gene^{6,7}. The mutation spectrum of *SLC26A4* varies widely among ethnic groups, with certain mutations demonstrating a higher prevalence in specific populations⁸⁻¹¹.

This gene, containing 21 exons, localises to chromosome 7 (7q22.3-q31.1) and encodes the multifunctional anion exchanger pendrin^{4,12}. Pendrin is a 73 kDa membrane protein that belongs to the SLC26 anion transporter family. It is comprised of 780 amino acids and is predicted to have 12 putative transmembrane domains, with both the amino- and carboxy-termini located on the cytosol^{13,14}. In the C-terminus region a STAS domain (Sulfate Transporter Antagonist of Anti-Sigma Factor) is located, which probably plays an important role in the biosynthesis, function and regulation of this transporter^{15,16}. The *SLC26A4* gene is expressed in specific areas of the endolymphatic compartment in the cochlea known to play a role in the endolymph reabsorption¹⁷. Moreover, in the absence of pendrin, profound prenatal endolymphatic hydrops are observed along with the destruction of many of the epithelial cells surrounding the scala media¹⁷. Regarding the thyroid organ, pendrin is involved in iodide metabolism as it transports intracellular iodide to the follicular lumen¹⁸ where the normal processes of iodide accumulation, oxidation and organification into thyroglobulin, leading to the production of the thyroid hormone, take place¹⁸. Patients with PS present a dysfunctional pendrin protein, and the thyroid gland is unable to accumulate and maintain iodide in the follicular lumen, place where thyroglobulin is kept and incorporates iodide to synthesise thyroid hormone¹⁹. Due to a defect in the synthesis of thyroid hormone, pathologies such as compensatory goiter and hypothyroidism may be present in these patients¹⁹. Herein, we report the case of a Portuguese female, aged 47, presenting with severe to profound HL and hypothyroidism.

Materials and methods

A Portuguese female presenting with severe to profound HL (Fig. 1) and hypothyroidism was referred for genetic analysis. This patient later reported that her mother and sister, both deceased, had also suffered from HL and goiter.

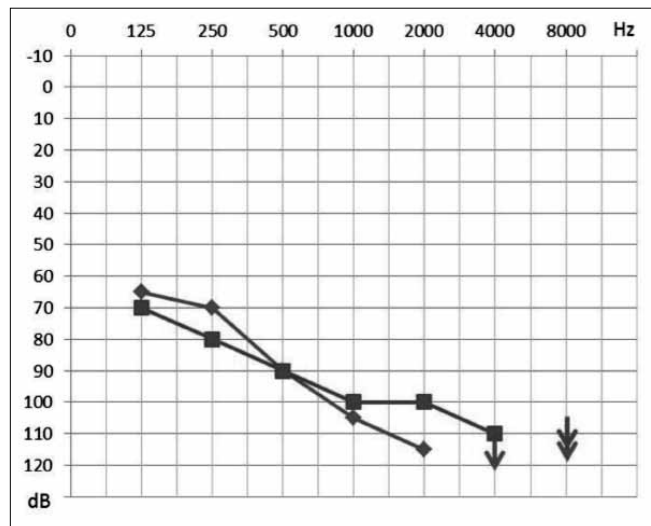


Fig. 1. Audiogram of the patient. Squares in red represent the right ear; diamonds in blue represent the left ear.

Hearing levels were determined by pure-tone audiometry. Imaging study of the ear was performed by magnetic resonance imaging (MRI), computed tomography (CT) and videonystagmography (VNG). A complete clinical history was taken to exclude aetiologies for HL such as infection, acoustic trauma, or ototoxic drugs. The patient reported no familial consanguinity, although this possibility cannot be excluded.

Blood samples were collected after written informed consent was obtained. Total genomic DNA was extracted from peripheral blood using the JetQuick Blood and Cell Culture Kit (Genomed).

Molecular study of the proband included screening of *GJB2*, *GJB6* and *SLC26A4* genes. The most common *GJB6* deletions were screened by multiplex PCR, using the method described by del Castillo²⁰. Automated sequencing was performed for the coding exon of the *GJB2* gene²¹, and for all exons, as well as intronic regions 7 and 14, of the *SLC26A4* gene (Table I).

Two hundred control chromosomes, from 100 self-reported normal hearing individuals from the Portuguese population, were sequenced for intronic region 7 and exon 7 of the *SLC26A4* gene.

All PCR products were purified using a Jetquick PCR Product Purification Spin Kit (Genomed). The electrophoretograms from bidirectional sequencing were evaluated by visual inspection and pairwise alignment to reference sequences using NCBI's BLAST²².

The Berkeley Drosophila Genome Project (BDGP)²³ splice site prediction program was used to predict the effect of the splicing mutation found in the patient. The SIFT prediction software²⁴ was used to predict the effect of a new variant, c.821C > G (p.Ala274Gly), identified in an individual of the control sample.

Table 1. *SLC26A4* exons and intronic regions studied.

Primer name	Region	Primer sequence (5'-3')	Amplified region (bp)
<i>SLC26A4</i> 2F	Exon 2	GGCTGCAGCTAACAGGTGATC	432
<i>SLC26A4</i> 2R		GAGGACCGGAGACCGAAAGTC	
<i>SLC26A4</i> 3F	Exon 3	ACAGTTCTTGGCAAAAGCATGG	411
<i>SLC26A4</i> 3R		GAAGGGTAAGCAACCATCTGTCAC	
<i>SLC26A4</i> 4F	Exon 4	TTTGCATCATATAAAGGCAAAGTC	419
<i>SLC26A4</i> 4R		TGAAATCCCATTCCCTGACAA	
<i>SLC26A4</i> 5F	Exon 5	CTCAGCTTCTTTCGTGAACAAAC	439
<i>SLC26A4</i> 5R		TTTGGGTTCCAGGAAATTACTTTGT	
<i>SLC26A4</i> 6F	Exon 6	GTGCTATAGGCAGGCTACTAGTGTT	364
<i>SLC26A4</i> 6R		CCTGGCCAGACTCAGAGAAT	
<i>SLC26A4</i> 7/8F	Exons 7 and 8	TGGGAAGATTCATATGAGAATTGATTG	581
<i>SLC26A4</i> 7/8R		TGGTTGTTTCTCCAGATCACA	
<i>SLC26A4</i> IVS8F	Intron 8 (partial)	GTGTGCGTGTAGCAGCAGG	502
<i>SLC26A4</i> IVS8R		GGACTATTGAAGGAGTATCAGTG	
<i>SLC26A4</i> 9F	Exon 9	CATGTGAAATGGCATGGATGG	583
<i>SLC26A4</i> 9R		GGTCTGGTAAAAGAATCCAACC	
<i>SLC26A4</i> 10F	Exon 10	CGCAGAGTAGGCATGGGAGTTT	314
<i>SLC26A4</i> 10R		TTGTCTGCTAAGCTCGGTGC	
<i>SLC26A4</i> 11/12F	Exons 11 and 12	AGACAGGGAAAGTATGAAGTGTG	555
<i>SLC26A4</i> 11/12R		TTTCTCCTCTGGAGTTCCCAA	
<i>SLC26A4</i> 13F	Exon 13	AGGTAGTTATCACATGATGGTACCTG	501
<i>SLC26A4</i> 13R		GAGCACAGCAGTAGAGGACAT	
<i>SLC26A4</i> 14F	Exon 14	AAACACCAGAATGATGGGCTC	338
<i>SLC26A4</i> 14R		GTCAGAAGGTGCACTGGATC	
<i>SLC26A4</i> IVS14F	Intron 14 (partial)	GTTGAGTGCTGCTACCCAGCTCCTC	185
<i>SLC26A4</i> IVS14R		AGGTAGTAATAACTATGCCAGAC	
<i>SLC26A4</i> 15F	Exon 15	CTACCCAGCTCCTCTGACAA	329
<i>SLC26A4</i> 15R		GCCCTACACAAAGGGAAGAGGG	
<i>SLC26A4</i> 16F	Exon 16	ACCCTTTGAGAAATAGCCTTTCCAG	357
<i>SLC26A4</i> 16R		CCACTCCCGCTTGCCATATAA	
<i>SLC26A4</i> 17F	Exon 17	AGTTTGGGCTGAGGTGAAACC	486
<i>SLC26A4</i> 17R		CAAAGCCCATGTATTGCCCTG	
<i>SLC26A4</i> 18F	Exon 18	CGCTGGATGTTGCCTCTCT	357
<i>SLC26A4</i> 18R		GGCCTTCAGACATAATGTGCCA	
<i>SLC26A4</i> 19F	Exon 19	TTTCTTAGCTGGGCATGGTAGG	705
<i>SLC26A4</i> 19R		GGAATTATGTACACAAATCCCAGATCAC	
<i>SLC26A4</i> 20F	Exon 20	AGAAGCACCAGGAAAGCTTCA	283
<i>SLC26A4</i> 20R		GGGAATTATGTTCCCTGACAGTTC	
<i>SLC26A4</i> 21F	Exon 21	CCTAAGATGAGTAGCAGTAAGCA	354
<i>SLC26A4</i> 21R		GCTGCCAAATCGTCTGAATAATTC	

Results

Clinical and audiological evaluation

The patient had multinodular goiter at the time of diagnosis. Thyroid function was studied and revealed a slight increase in thyroid-stimulating hormone (TSH) levels, while serum thyroxine levels were below normal values. Thyroid microsomal antibodies were negative.

Hearing levels, determined by pure-tone audiometry, revealed severe to profound HL, as referred. After MRI and CT, enlargement of the vestibular aqueduct and the endolymphatic sac were observed (Fig. 2). VNG examination revealed bilateral hypoflexia.

Molecular analysis

The mutation c.918 + 2T > C (Fig. 3), previously reported

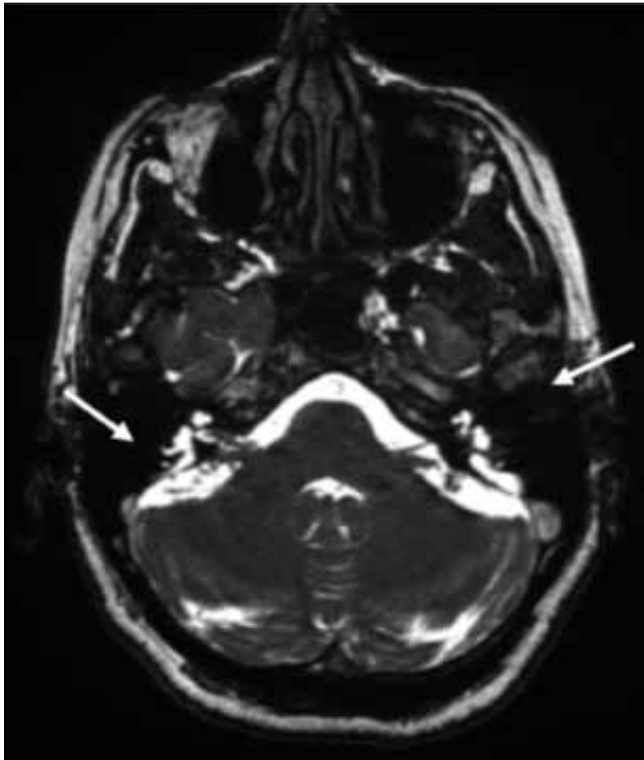


Fig. 2. Axial section MRI (FIESTA). Enlarged vestibular aqueduct (arrows).

by Chai et al. (2013)²⁵, was found in homozygosity in the intronic region between exons 7 and 8 of the *SLC26A4* gene. We sequenced 200 Portuguese control chromosomes to determine the allelic frequency of this mutation in the Portuguese population. The variant was not found in any of the control samples. No mutations were found in *GJB2* or *GJB6* genes. Regarding its functional effect, c.918 + 2T > C abolishes a donor splicing site, since the first two nucleotides of the intron 7 in the wild-type sequence, a guanine (G) and a thymine (T), respectively, are predicted to be a donor splicing site, with a cut-off of 0.9 and a score of 0.99 (according to the BDGP splice site prediction program). Thus, the presence of the transition T > C leads to the loss of this donor splicing site, thus skipping exon 8 and forming a non-functional protein product.

Whilst checking whether the mutation c.918 + 2T > C found in the PS patient was present in any of the 100 normal hearing control individuals, a new variant, c.821C > G (p.Ala274Gly), was found in heterozygosity in the exon 7 of *SLC26A4* gene (Fig. 4). This mutation changes alanine to glycine at position 274 and is predicted to impair protein function by SIFT software, with a score of 0.04 and a median conservation of 2.24. This variant was not found in any of the other Portuguese controls in the study and is not reported in 1000 Genomes, HGDM, ClinVar, or Pendred/BOR databases from Hereditary Hearing Loss Homepage²⁶. Since this individual was a random control from the Portuguese population, no information concerning phenotype was available.

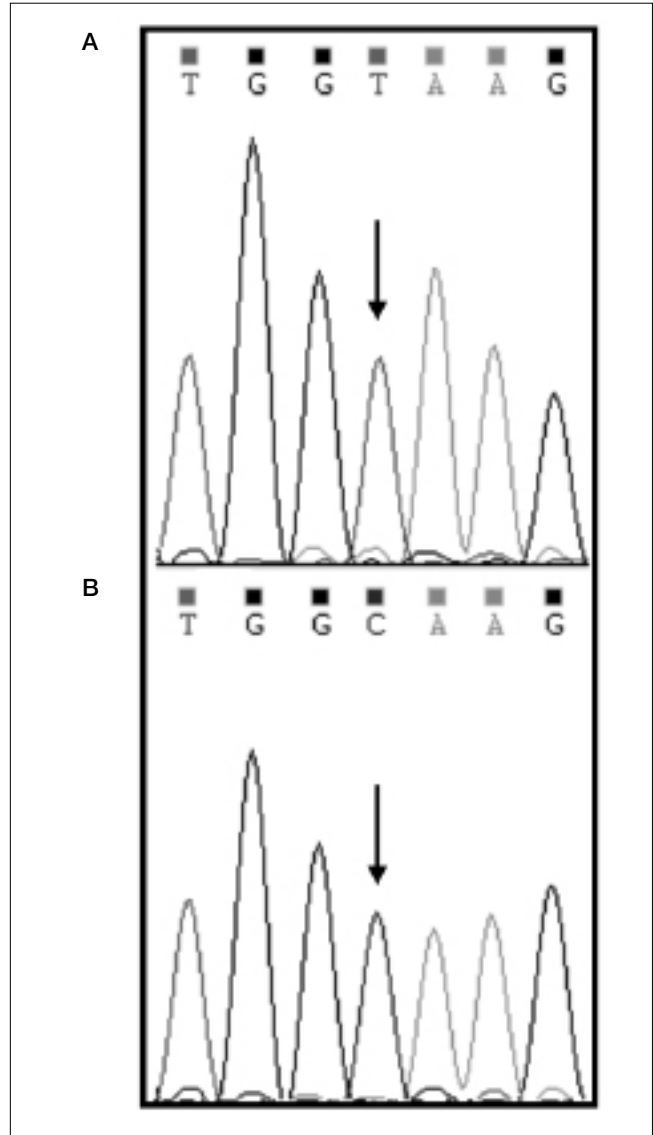


Fig. 3. Electrophoretograms showing: A - wild-type sequence; B - *SLC26A4* novel mutation c.918 + 2T > C in homozygosity.

Discussion

Since its discovery, many studies have been performed to better understand the genetics of PS, possible genotype-phenotype correlations and the pathologies associated with this syndromic condition²⁷⁻³⁰.

Previously, we found a novel splice site mutation in the *SLC26A4* gene, in a consanguineous Portuguese family³¹. Herein, we report the case of a Portuguese female diagnosed with PS and found to be homozygous for the donor splice site c.918 + 2T > C mutation in the *SLC26A4* gene. This mutation was recently reported by Chai et al. (2013)²⁵ in a Chinese child. The authors found this mutation in compound heterozygosity with another *SLC26A4* variant, c.919 - 2A > G, and described the patient as a non-syndromic severe to profound HL individual, presenting bilateral enlargement of the vestibular aqueduct²⁵.

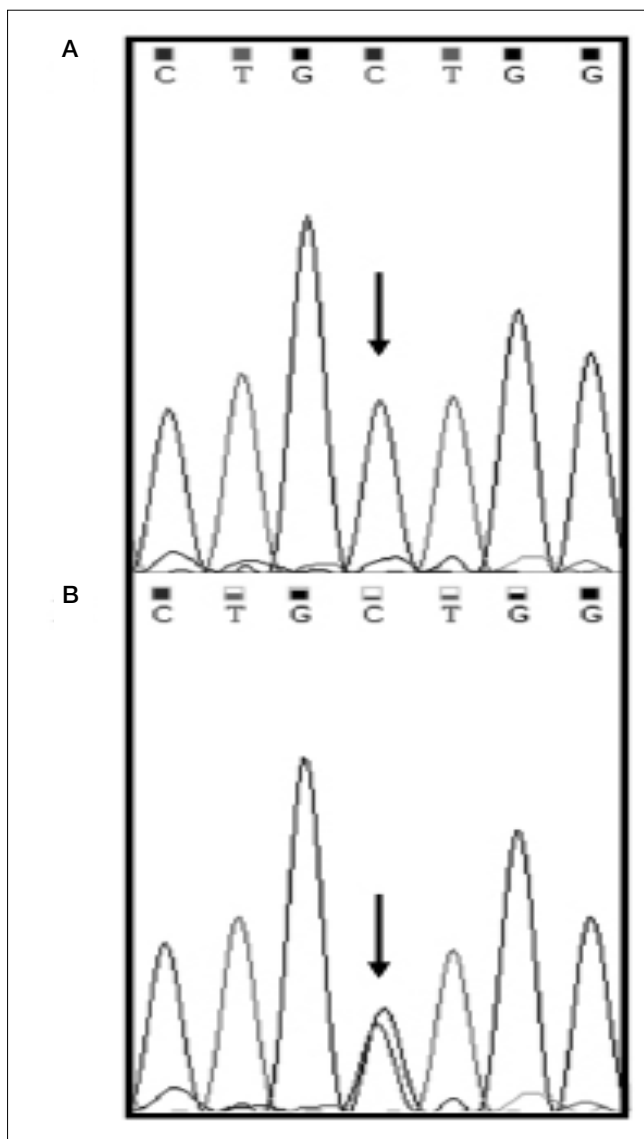


Fig. 4. Electrophoretograms showing: A - wild-type sequence; B - *SLC26A4* novel mutation c.918 + 2T > C in homozygosity.

In the present study, we describe for the first time the c.918 + 2T > C mutation in homozygosity in a PS individual and also provide new insight on its phenotypic characterisation. The severe to profound HL phenotype, enlargement of the vestibular aqueduct and endolymphatic sac along with goiter and hyporeflexia are all compatible with features affecting PS patients.

The patient here considered reported that mother and sister, both deceased, had suffered from HL and goiter. This feature does not fit with the recessive pattern of PS inheritance. Due to the lack of additional familial information, the apparently dominant HL and goiter within this family remains to be explained. Since Chai et al. (2013)²⁵ reported the c.918 + 2T > C mutation in compound heterozygosity with another *SLC26A4* mutation in a child presenting features compatible with PS, we may also consider

the hypothesis that the mother could have harboured this mutation in compound heterozygosity, thus giving rise to the HL and goiter phenotype. Although excluded by the patient, we cannot exclude consanguinity in this family, which would better explain the homozygous genotype observed in the patient and the HL and goiter phenotype of her deceased sister. Unfortunately, no information was provided regarding the father.

Conclusions

Having into account that: no alteration was found in all other exons of the *SLC26A4* gene or in the *GJB2* and *GJB6* genes; the c.918 + 2T > C mutation abolishes a donor splicing site and occurs in homozygosity, affecting both alleles; this mutation was not present in any of the 200 Portuguese control chromosomes analysed (allelic frequency < 0.99%), the *SLC26A4* genotype [c.918 + 2T > C + c.918 + 2T > C] could be pointed as the likely cause for the PS phenotype presented by the patient.

Considering the novel variant, c.821C > G (p.Ala274Gly), found in heterozygosity in a control individual, it is predicted to be probably damaging and it was not found in any of the remaining Portuguese control individuals. Further genotyping of Portuguese PS patients might eventually lead to the identification of this allele in a compound heterozygous patient. Moreover, since the mutation spectrum of *SLC26A4* has been shown to vary widely among ethnic groups, future determination of the mutation spectrum of *SLC26A4* gene in the Portuguese population might reveal some interesting specificities.

References

- Hilgert N, Smith RJ, Van Camp G. *Forty-six genes causing nonsyndromic hearing impairment: which ones should be analyzed in DNA diagnostics?* Mutation Research 2009; 681:189-96.
- Fraser, GR. *Association of congenital deafness with goitre (Pendred's syndrome)*. Ann Hum Genet 1965; 28:201-49.
- Reardon W, Coffey R, Phelps PD, et al. *Pendred syndrome – 100 years of underascertainment?* Q J Med 1997;90:443-7.
- Pera A, Dossena S, Rodighiero S, et al. *Functional assessment of allelic variants in the SLC26A4 gene involved in Pendred syndrome and nonsyndromic EVA*. PNAS 2008;105:18608-13.
- Stenson PD, Ball EV, Mort M, et al. *Human Gene Mutation Database (HGMD): 2003 update*. Hum Mutat 2003;21:577-81.
- Anwar S, Riazuddin S, Ahmed ZM, et al. *SLC26A4 mutation spectrum associated with DFNB4 deafness and Pendred's syndrome in Pakistanis*. J Hum Genet 2009;54:266-70.
- Pique LM, Brennan ML, Davidson CJ, et al. *Mutation analysis of the SLC26A4, FOXI1 and KCNJ10 genes in individuals with congenital hearing loss*. Peer J 2014;8:2:e384.
- Park H-J, Shaikat S, Liu X-Z, et al. *Origins and frequencies of SLC26A4(PDS) mutations in east and south Asians:*

- global implications for the epidemiology of deafness. *J Med Genet* 2003;40:242-8.
- 9 Blons H, Feldmann D, Duval V, et al. *Screening of SLC26A4 (PDS) gene in Pendred's syndrome: a large spectrum of mutations in France and phenotypic heterogeneity.* *Clin Genet* 2004;66:333-40.
 - 10 Albert S, Blons H, Jonard L, et al. *SLC26A4 gene is frequently involved in nonsyndromic hearing impairment with enlarged vestibular aqueduct in Caucasian populations.* *Eur J Hum Genet* 2006;14:773-9.
 - 11 Wang QJ, Zhao YL, Rao SQ, et al. *A distinct spectrum of SLC26A4 mutations in patients with enlarged vestibular aqueduct in China.* *Clin Genet* 2007;72:245-54.
 - 12 Bizhanova A, Kopp P. *Genetics and phenomics of Pendred syndrome.* *Mol Cell Endocrinol* 2010;332:83-90.
 - 13 Royaux IE, Suzuki K, Mori A, et al. *Pendrin, the protein encoded by the Pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells.* *Endocrinology* 2000;141:839-45.
 - 14 Gillam MP, Sidhaye AR, Lee EJ, et al. *Functional characterization of pendrin in a polarized cell system. Evidence for pendrin-mediated apical iodide efflux.* *J Biol Chem* 2004;279:13004-10.
 - 15 Babu M, Greenblatt JF, Emili A, et al. *Structure of a SLC26 anion transporter STAS domain in complex with acyl carrier protein: implications for E. coli YchM in fatty acid metabolism.* *Structure* 2010;18:1450-62.
 - 16 Masindova I, Varga L, Stanik J, et al. *Molecular and hereditary mechanisms of sensorineural hearing loss with focus on selected endocrinopathies.* *Endocr Regul* 2012;46:167-86.
 - 17 Everett LA. *New insights into the role of pendrin (SLC26A4) in inner ear fluid homeostasis.* *Novartis Found Symp* 2006;273:213-25.
 - 18 Nilsson M. *Iodide handling by the thyroid epithelial cell.* *Exp Clin Endocrinol Diabetes* 2001;109:13-7.
 - 19 Xing M, Tokumaru Y, Wu G, et al. *Hypermethylation of the pendred syndrome gene SLC26A4 is an early event in thyroid tumorigenesis.* *Cancer Research* 2003;63:2312-5.
 - 20 Del Castillo FJ, Rodríguez-Ballesteros M, Álvarez A, et al. *A novel deletion involving the connexin-30 gene, del(GJB6-d13s1854), found in trans with mutations in the GJB2 gene (connexin-26) in subjects with DFNB1 non-syndromic hearing impairment.* *J Med Genet* 2005;42:588-94.
 - 21 Matos TD, Simões-Teixeira H, Caria H, et al. *The controversial p.Arg127His mutation in GJB2: report on three Portuguese hearing loss family cases.* *Genet Test Mol Biomarkers* 2010;14:141-4.
 - 22 Altschul SF, Madden TL, Schaffer AA, et al. *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.* *Nucleic Acids Res* 1997;25:3389-402.
 - 23 Reese MG, Eeckman FH, Kulp D. *Improved splice site detection in genie.* *J Comp Biol* 1997;4:311-23.
 - 24 Kumar P, Henikoff S, Ng PC. *Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm.* *Nat Protoc* 2009;4:1073-81.
 - 25 Chai Y, Huang Z, Tao Z, et al. *Molecular etiology of hearing impairment associated with nonsyndromic enlarged vestibular aqueduct in East China.* *Am J Med Genet A* 2013;161A:2226-3.
 - 26 van Camp G, Smith RJH. *Hereditary Hearing Loss Homepage 2014.* Accessed on 12th July 2014. URL: <http://hereditaryhearingloss.org/main.aspx?c=.HHH&n=86162>;
 - 27 Fraser GR, Morgans ME, Trotter WR. *The syndrome of sporadic goitre and congenital deafness.* *Q J Med* 1960;29:279-95.
 - 28 Everett L, Glaser B, Beck J, et al. *Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS).* *Nat Genet* 1997;17:411-22.
 - 29 Hauwe PV, Everett LA, Coucke P, et al. *Two frequent missense mutations in Pendred Syndrome.* *Hum Mol Genet* 1998;7:1099-104.
 - 30 Ito T, Choi BY, King KA, et al. *SLC26A4 genotypes and phenotypes associated with enlargement of the vestibular aqueduct.* *Cell Physiol Biochem* 2011;28:545-52.
 - 31 Simões Teixeira H, Matos TD, Marques MC, et al. *Novel splice-site mutation c.1615-2A > G (IVS14-2A > G) in the SLC26A4 gene causing Pendred Syndrome in a consanguineous Portuguese family.* *Am J Med Genet A* 2011;9999:1-4.

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