

A Novel *SLC26A4* (*PDS*) Deafness Mutation Retained in the Endoplasmic Reticulum

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Objectives: To identify mutations in the *SLC26A4* gene in individuals with nonsyndromic hearing loss and enlarged vestibular aqueduct, to design a predicted model of the pendrin protein, and to characterize novel mutations by means of localization in mammalian cells and effect of the mutation on the predicted model.

Design: Validation of the mutation by its exclusion in more than 300 individuals with normal hearing.

Setting: A laboratory of genetics of hearing loss research, clinical genetics laboratories, an otolaryngology department at Tel Aviv University, and medical centers in Israel.

Patients: A patient with nonsyndromic hearing loss and enlarged vestibular aqueduct, 203 deaf probands, and 310 controls with normal hearing.

Interventions: Sequencing the *SLC26A4* gene in the patient with nonsyndromic hearing loss and enlarged vestibular aqueduct. Transfection of yellow fluorescent protein (YFP) constructs into mammalian COS7 cells.

Designing a computational model of the human *SLC26A4* protein.

Main Outcome Measure: Detection of a novel c.1458_1459insT *SLC26A4* mutation.

Results: A computational model of the human pendrin protein suggests that the novel c.1458_1459insT mutation leads to a prematurely truncated protein, p.Ile487TyrfsX39. Mammalian COS7 cells transfected with the YFP-1458_1459insT construct showed mislocalization of the mutant protein.

Conclusions: A novel *SLC26A4* mutation was detected in Israel. Because current estimates demonstrate that *SLC26A4* mutations are involved in up to 4% of nonsyndromic deafness, our findings emphasize the importance of adding a molecular test for the *SLC26A4* gene in the diagnosis of deafness, particularly when bone abnormalities are involved, to the list of genes screened in Israel and elsewhere in the world.

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OVER 100 MUTATIONS IN THE *SLC26A4* (*PDS*) gene (OMIM 605646) are involved in both Pendred syndrome¹ (PS) (OMIM 274600) and nonsyndromic sensorineural hearing loss² (NSHL) *DFNB4* (OMIM 600791) associated with temporal bone abnormalities.³ The *SLC26A4* gene, located on the long arm of chromosome 7, encodes pendrin, a protein expressed in various tissues, including the inner ear, thyroid, and kidney. Pendrin is a transmembrane anion exchanger, with 11 or 12 predicted transmembrane domains,^{2,4} that belongs to the solute carrier 26 family. It functions as a transporter exchanging chloride, iodine,⁵ bicarbonate,⁶ and formate.⁷

Mutations in *SLC26A4* are associated with congenital NSHL beginning at high frequencies, with fluctuating and some-

times progressive hearing loss (HL) with malformations of the temporal bone. The temporal bone abnormalities vary, ranging from isolated enlarged vestibular aqueduct (EVA) to Mondini dysplasia, an abnormality in which the normal cochlear spiral of 2.5 turns is replaced by a hypoplastic coil of 1.5 turns. In many populations, mutations in the *SLC26A4* gene are the major genetic cause of these temporal bone inner ear malformations, accounting for up to 90% of the typical population with PS, 80% of individuals with EVA, and the same rate among patients with Mondini dysplasia.^{2,8,9} The same combination of mutations resulting in variable phenotypic expression, ranging from isolated nonsyndromic EVA to Mondini dysplasia to PS, have been described, suggesting that the same etiology underlies all conditions.⁹ In contrast, Pryor et al¹⁰ re-

ported 2 mutant *SLC26A4* alleles in all patients with PS in their study, whereas the nonsyndromic patients with EVA had either 1 or no *SLC26A4* mutant alleles. They concluded that PS and nonsyndromic EVA are distinct clinical and genetic phenomena, with PS being caused by biallelic *SLC26A4* mutations, and at least some cases of nonsyndromic EVA being associated with a single *SLC26A4* mutation. In an additional study among white French families with NSHL and EVA,³ patients with biallelic mutations had more severe deafness, an earlier age at onset, and a more fluctuating course of hearing levels than patients in whom no mutation was identified.

Current estimates demonstrate that *SLC26A4* mutations are involved in up to 4% of NSHL,³ which emphasizes the importance of the molecular confirmation of the *SLC26A4* gene in the diagnosis of deafness, particularly when bone abnormalities are involved.

METHODS

PATIENTS

The project was approved by the Helsinki (institutional review board) Committees of the Israel Ministry of Health, Tel Aviv University, Tel Aviv; the Rabin Medical Center, Petach Tikva; and the Haim Sheba Medical Center, Tel Hashomer. Blood samples were obtained following informed consent from each individual and, in case of individuals younger than 18 years, from their parents.

One deaf individual residing in Israel (who was of Iranian ethnic origin) was referred to the study because he was diagnosed as having NSHL and EVA. Palpation of the thyroid gland did not reveal any enlargement or mass. His HL began at age 6 years and progressed to profound deafness. In his mid 20s he underwent cochlear implantation. In his preoperative computed tomographic scan, EVA was observed, suggesting involvement of the *SLC26A4* gene. Therefore, the *SLC26A4* gene was sequenced.

On detecting the c.1458_1459insT mutation (p.Ile487TyrfsX39) (**Figure 1**), 203 Jewish deaf probands of mixed ethnic origin, including 3 with Mondini dysplasia, were screened for the mutation by the restriction enzyme assay described in the subsection titled "Detection of c.1458_1459insT."

Anonymous DNA samples from 310 Jews with hearing levels within the reference range, 21 of whom were of Iranian ethnic origin, were obtained from the Haim Sheba Medical Center and the Rabin Medical Center and used as controls.

MUTATION EXCLUSION OF CONNEXIN 26

The open reading frame of connexin 26 (*GJB2*) (OMIM 121011) was sequenced for mutations as described previously.¹¹ Briefly, primers *GJB2-1F*, 5'-TCT TTT CCA GAG CAA ACC GC-3' and *GJB2-2R*, 5'-GGG CAA TGC GTT AAA CTG GC-3' amplified a 722-bp fragment that was sequenced and checked for mutations using the connexin-deafness database.

SEQUENCING THE *SLC26A4* GENE

Genomic primers were designed for all the coding exons (2-21) of the *SLC26A4* gene (NM_000441.1; <http://genome.ucsc.edu>), using the Primer 3 Input (version 0.2; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Polymerase chain reactions (PCRs) were performed in a total of 50 μ L, using PCR Red-

dyMix (ABgene; Epsom, Surrey, England), including 1 μ L of genomic DNA with a mean concentration of 200 ng/ μ L. The PCR cycling conditions were 95°C for 5 minutes, followed by a touch-down program of 60°C to 55°C and 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Two final steps followed at 72°C and 4°C for 10 minutes each. After verification of the PCR products by separation of 3 μ L of reaction by electrophoresis on a 1% gel, the PCR products were purified by Wizard SV Gel and the PCR Clean-Up System (Promega, Madison, Wisconsin). Sequencing was performed using the same primers as those used for PCR: the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California) and a DNA sequencer (model 377; ABI). Mutations were confirmed by sequencing in both directions.

DETECTION OF c.1458_1459insT

A restriction enzyme assay was designed. This method was based on the principle of PCR-mediated, site-directed mutagenesis, followed by *Bst*Z171 digestion. The primer set consisted of a wild type (WT) forward primer, *SLC26A4*-13F: 5'-TGTAATTTGTTTGTGGATCATTG-3', and a modified reverse primer, *SLC26A4*-*Bst*Z171-R: 5'-GAGATCCAGCCCCAGAATGATGGACAGTAT-3', that was modified at nucleotide 1662 (G→C). This modification is located 4 nucleotides downstream of the insertion site. The amplified WT fragment of 155 bp has a newly created restriction site for the *Bst*Z171 enzyme; that is, the digestion occurs only in the modified WT fragment. The insertion of thymine 4 nucleotides upstream of the modification eliminates the restriction site. The PCR reactions were performed as described in the previous subsection in a total of 30 μ L; 10 μ L of the reaction mix was separated by electrophoresis on a 1% gel. The PCR product was digested by *Bst*Z171 (37°C for 8 hours) and run on a 2% gel electrophoresis. Because a *Bst*Z171 site is inserted in the modified primer, a *Bst*Z171 digest of WT DNA produces 2 fragments of 126 bp and 29 bp; digestion of heterozygous DNA produces 3 fragments of 156 bp, 126 bp, and 29 bp; and c.1458_1459insT homozygous DNA results in 1 fragment of the 156-bp original PCR product size.

COMPUTATIONAL ANALYSIS OF THE p.Ile487TyrfsX39 MUTATION

Two independent predictions of a hypothetical model for the human pendrin protein sequence (UniProtKB/Swiss-Prot: O43511) were performed. The first prediction was provided by the UniProtKB/Swiss-Prot protein sequence database (entry version 34, release 7.0/49.0; February 7, 2006; <http://www.expasy.org/sprot>) and is presented in Figure 1B. A second prediction was performed by the TMHMM program¹² (version 1; February 2006; <http://www.cbs.dtu.dk/services/TMHMM-2.0/>) (data not shown).

A list of current known human mutations in the pendrin protein, demonstrated by colored circles in Figure 1B, was made based on 2 resources, the UniProtKB/Swiss-Prot database and the study by Tsukamoto et al.⁹

LOCALIZATION OF YFP CHIMERAS

The *SLC26A4* c.1458_1459insT mutation was created by PCR mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California) and subcloned into the yellow fluorescent protein (YFP) expression vector pEYFP-C1 ("p" indicates plasmid) (Clontech, Mountain View, California). Cell cultures and expression of chimeras were performed as previously described.¹³

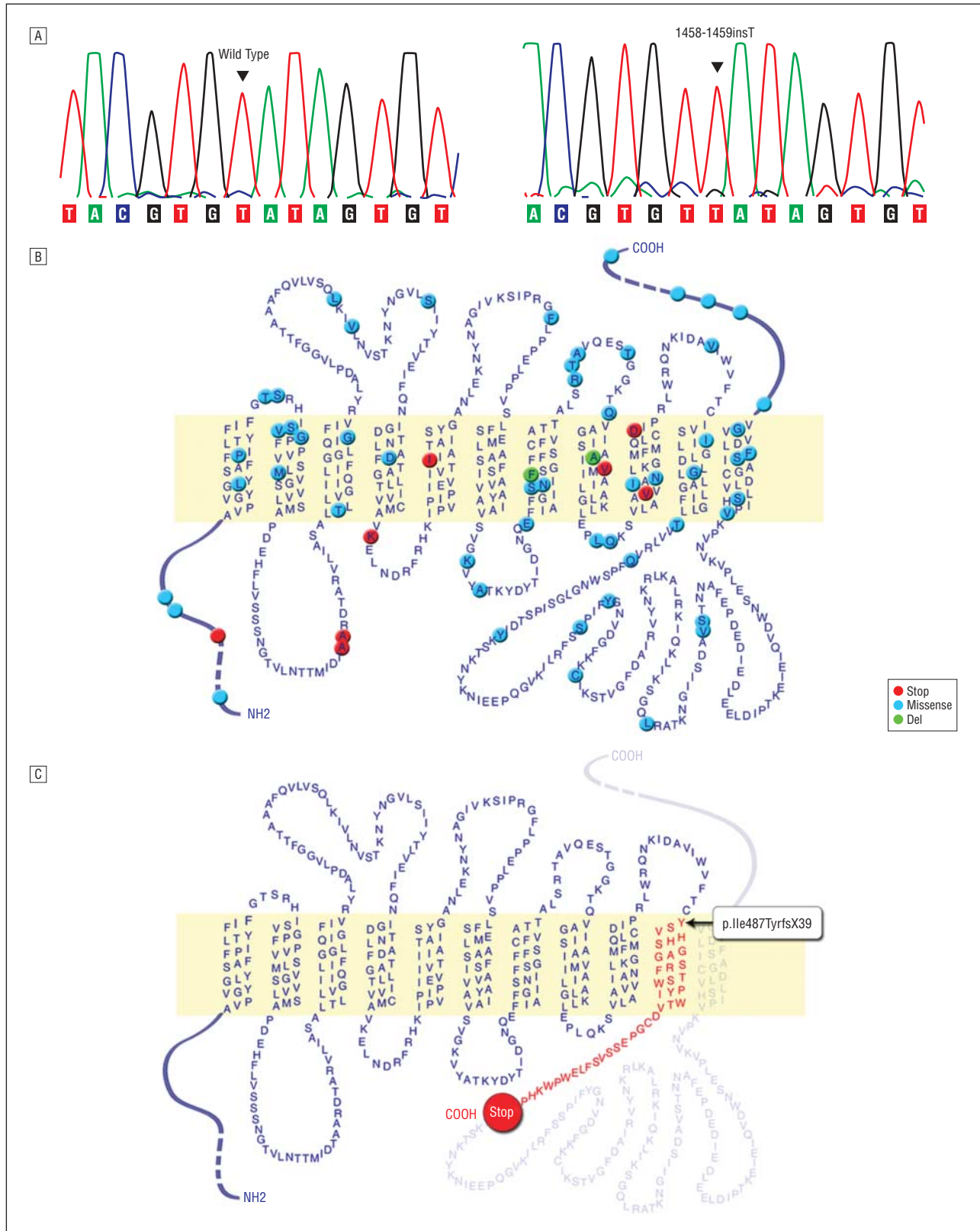


Figure 1. A novel mutation in *SLC26A4* (*PDS*) is responsible for nonsyndromic sensorineural hearing loss in a Jewish Israeli patient of Iranian origin. **A**, The sequencing of *SLC26A4* revealed a 1458-1459insT mutation in exon 13, leading to a predicted stop codon at position 526 (p.Ile487TyrfsX39) in the affected individual. **B**, A potential model of the human pendrin amino acid sequence and its membrane arrangement is predicted by the UniProtKB/Swiss-Prot database (<http://www.expasy.org/sprot>). Known missense, deletion (del), and stop mutations are demonstrated with colored circles. The current list of human mutations was made based on the study by Tsukamoto et al⁹ and the UniProtKB/Swiss-Prot database. **C**, The reported mutation, p.Ile487TyrfsX39, leads to a 40 amino acid change in the pendrin protein sequence beginning at position 487 and terminating with a stop codon at position 526. A hypothetical model of the disrupted pendrin protein with the subsequent premature truncation is demonstrated, showing the mutation within a transmembrane domain and the stop codon within an intracellular domain. COOH and NH2 indicate the carboxyl group and amino group of amino acids, respectively.

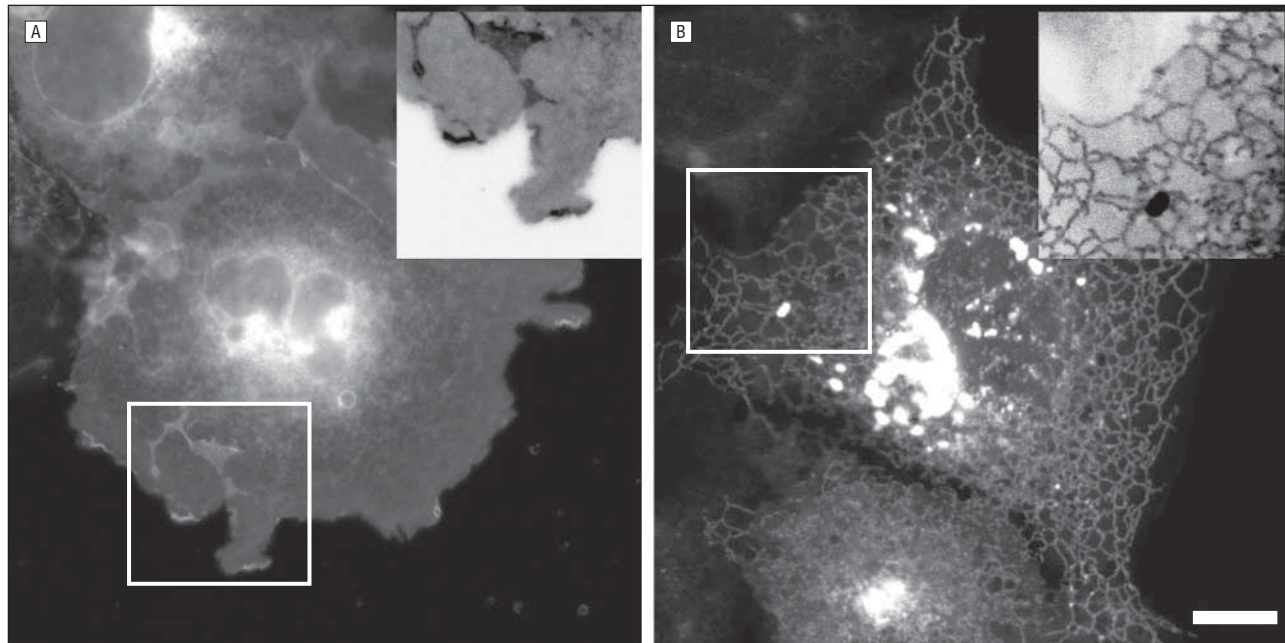


Figure 2. The 1458-1459insT pendrin mutation is retained in the endoplasmic reticulum (ER). A, Confocal images of living cells co-expressing yellow fluorescent protein (YFP) PDS. B, The YFP-PDS 1458-1459insT mutation is seen as an elaborated reticulum extending off the nuclear envelope. Inverted inserts are magnified $\times 1.5$ to demonstrate the plasma membrane and ER membrane. The bar indicates 10 μm .

CONFOCAL LASER-SCANNING MICROSCOPY

The COS7 cells were imaged in Dulbecco's Modified Eagle's Medium (Biological Industries, Kibbutz Beit Haemek, Israel) without phenol red but supplemented with 20 mM of Hepes (pH, 7.4). Transfection and imaging were performed in Labtek chambers (Nunc, Naperville, Illinois). A laser scanning microscope (PASCAL; Zeiss, Jena, Germany) equipped with an Axiovert 200 inverted microscope (Zeiss) and Ar 514 nm laser line was used for EYFP. The confocal images were captured using a 63 1.4 numerical aperture objective with a pinhole diameter equivalent to 1 to 2 Airy units.

RESULTS

SLC26A4 GENE c.1458-1459InsT MUTATION DETECTION

The *SLC26A4* gene was sequenced in a proband with NSHL and EVA after excluding mutations in *GJB2* by sequencing. The c.1458_1459insT mutation was detected on both alleles (Figure 1A). The *SLC26A4* gene contains 21 exons that encodes a predicted protein of 780 amino acids. The mutation c.1458_1459insT in exon 13 causes a frameshift at position 487, leading to a stop codon at position 526 of the pendrin protein, p.Ile487TyrfsX39.

COMPUTATIONAL ANALYSIS OF THE PREDICTED TRUNCATED PENDRIN PROTEIN

Two methods were employed to make a prediction of the 3-dimensional structure of the pendrin protein, the UniProtKB/Swiss-Prot protein sequence database (<http://www.expasy.org/sprot>) and the TMHMM program (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>).¹² According to both, our reported mutation, p.Ile487TyrfsX39, leads to an amino acid change within a potential transmem-

brane domain and a stop codon within a potential intracellular domain (Figure 1B).

PROBABILITY OF THE c.1458_1459insT MUTATION IN THE ISRAELI JEWISH POPULATION

Deaf probands (n=203), in whom mutations in the exon 2 of the connexin 26 were excluded, were screened for the c.1458_1459insT mutation by the restriction enzyme assay described in the subsection titled "Detection of c.1458_1459insT." Three individuals with Mondini dysplasia were included in this population. The 203 probands were WT for *SLC26A4*. In total, the approximated probability of the c.1458_1459insT mutation in the *SLC26A4* gene among the Jewish deaf population in Israel is 0.5% (1 in 204).

In addition, 310 controls with normal hearing were screened, including 21 who were of Iranian ethnic origin, and they were WT for *SLC26A4* as well. No carriers of the mutation were detected.

LOCALIZATION OF YFP-WT VS YFP-MUTATED PENDRIN

We observed YFP-WT PDS in the plasma membrane and in the endoplasmic reticulum (ER) (Figure 2A), whereas the YFP-1458_1459insT was completely retained in the ER in transfected COS7 cells, seen as an elaborated reticulum extending off the nuclear envelope (Figure 2B).

COMMENT

The mutation c.1458_1459insT leads to a predicted stop codon at position 526 of the pendrin protein (p.Ile487TyrfsX39). Although the putative truncated protein produced is only two-thirds the length of the WT

pendrin and is missing 1 or 2 transmembrane domains, the affected individual has NSHL with EVA, but not PS. Localization experiments of the mutated protein show that the YFP-1458_1459insT is retained in the ER, whereas the WT pendrin targets the PM. Retention of pendrin in the ER is a major mechanism for PS and was observed for other mutations involved in PS, including L236P, T416P, G384E, and V239D.^{13,14} In another study,¹⁵ mutations in pendrin associated with NSHL were also shown to cause retention of the mutated protein in the ER, as was observed for mutations causing PS. These findings suggest involvement of modifier genes, and/or nutritional factors, including iodine uptake, which determine the thyroid phenotype and determine the difference between PS and NSHL. This hypothesis is supported by mutations found to be involved both in PS and in NSHL in different individuals, as is reported for the L445W,¹⁶ H723R,⁹ and the IVS7-2A>G¹⁷ mutations. Furthermore, intrafamilial variability of the thyroid phenotype has been reported in 1 large consanguineous family in which the L445W mutation was identified in all affected individuals, either with PS or with NSHL.¹⁶

In our study, the affected individual homozygous for the mutation has NSHL associated with EVA, but no goiter is present. However, the thyroid phenotype usually does not develop until the second decade of life or even at a later age, so PS cannot be ruled out in the proband in this study because he may develop goiter in the future.

The novel c.1458_1459insT mutation in the *SLC26A4* gene was detected in 1 family only, and no carriers were identified. The low probability obtained in Israel might be due to the small population of hearing-impaired patients with EVA and to the small control group of Jews originating in Iran. However, because the *SLC26A4* gene is involved in up to 4% of NSHL in some populations,³ our study strongly suggests that it would be beneficial to test for this novel mutation in the Israeli deaf population with associated temporal bone malformations.

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