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Original Article

Compound Heterozygosity for Two Novel *SLC26A4* Mutations in a Large Iranian Pedigree with Pendred Syndrome

Nasrin Yazdanpanahi[1] · Mohammad Amin Tabatabaiefar[2,3] · Effat Farrokhi[2] · Narges Abdian[2] · Nader Bagheri[2]
Shirin Shahbazi[2] · Zahra Noormohammadi[4] · Morteza Hashemzadeh Chaleshtori[2]

[1]Department of Biochemistry, Falavarjan Branch, Islamic Azad University, Isfahan, Iran; [2]Cellular and Molecular Research Center, School of Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran; [3]Department of Medical Genetics, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; [4]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

Objectives. The aim of this study was to detect the genetic cause of deafness in a large Iranian family. Due to the importance of *SLC26A4* in causing hearing loss, information about the gene mutations can be beneficial in molecular detection and management of deaf patients.

Methods. We investigated the genetic etiology in a large consanguineous family with 9 deaf patients from Fars province of Iran with no *GJB2* mutations. Initially, linkage analysis was performed by four DFNB4 short tandem repeat markers. The result showed linkage to DFNB4 locus. Following that, DNA sequencing of all 21 exons, their adjacent intronic sequences and the promoter of *SLC26A4* was carried out for mutation detection.

Results. Two novel mutations (c.863-864insT and c.881-882delAC) were identified in exon 7 of the gene, in both homozygous and compound heterozygous state in patients.

Conclusion. Our results supported the importance of the *SLC26A4* mutations in the etiology of hearing loss among the Iranian patients and therefore its mutation screening should be considered after *GJB2* in the molecular diagnostics of hearing loss, especially when enlarged vestibular aqueduct or goiter is detected.

Keywords. *Novel mutation, Compound heterozygosity, SLC26A4, Pendred syndrome, Hearing loss, Linkage analysis, Iran*

INTRODUCTION

Up to now over 400 syndromic forms of hearing loss (HL) have been described [1]. Pendred syndrome (PS) (MIM#274600) is one of the most frequent types of syndromic HL, although it may remain underdiagnosed due to the late onset (usually after the second decade of life) and reduced penetrance of some of its common clinical features, especially the goiter. Over 90% of PS is caused by *SLC26A4* mutations in different populations [2].

SLC26A4 has been known by Everett et al. [3] using positional cloning and mapped on 7q22-31.1. This gene produces a 5 Kb transcript and a 86 kDa protein with 780 amino acids, called pendrin, which is a member of solute carrier family 26A (SLC26A).

SLC26A4 mutations result in both PS and non syndromic HL (DFNB4; MIM#600791). These disorders have autosomal recessive inheritance [2,4]. Similar HL severity and inner ear malformations ranging from enlarged vestibular aqueduct (EVA), the most frequent radiological abnormality in sensorineural HL (SNHL), to Mondini dysplasia (MD), a condition that normal cochlear spiral is 1.5 turns instead of 2.5 turns, are seen in both of them. EVA and MD are detectable with computed tomography (CT) [5]. The major difference between the two disorders is thy-

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• Corresponding author: **Morteza Hashemzadeh Chaleshtori**
Cellular and Molecular Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran
Tel: +98-381-3346692, Fax: +98-381-3330709
E-mail: mchalesh@yahoo.com

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roid abnormality (goiter) which is observed to be segregating with PS [2]. To date, more than 200 mutations have been identified for *SLC26A4* (http://www.healthcare.uiowa.edu/labs/pendredandbor/slcMutations.htm).

While *GJB2* is responsible for up to half of autosomal recessive nonsyndromic HL (ARNSHL) in Mediterranean, most European and north American populations [6-8], this gene accounts for only 18.29% and/or 16.7% of Iranian families with HL [9, 10]. In contrast, *SLC26A4* appears to have a more crucial role in the etiology of hearing impairment (HI) in Asian than non-Asian populations. About 7.2% of prelingual HL in Pakistan and 5% of this disease in east and south Asia happens due to *SLC26A4* mutations [11,12]. Results of a former study suggest that *SLC26A4* could be considered as the second cause of HI, after *GJB2*, in Iran [13].

In addition to frequency, the type of *SLC26A4* mutations is also different among populations. While IVS7-2A>G and H723R are the most frequent *SLC26A4* mutations in east Asia, R79X and R409H are more common in the Iranian HL population [13-15]. Epidemiological data regarding ethnic and population specificity of *SLC26A4* mutations could be beneficial for diagnosis, clinical decision-making, family planning as well as designing cost-effective strategy for molecular testing.

In the present investigation, a large deaf Iranian kindred with 9 patients was subject to genetic linkage analysis and two novel mutations were identified.

MATERIALS AND METHODS

Subjects

The research was approved by the Institutional Review Boards of Shahrekord University of Medical Sciences. We studied a large consanguineous family Iranian 3 (IR3), including 9 patients with hereditary HL from Fars province of Iran, with no *GJB2* mutation in our previous research [9]. There were four consanguinity loops in the pedigree: subjects III-1 and III-2, IV-3 and IV-4, IV-18 and IV-19, and individuals III-7 and III-8 had first cousin marriages (Fig. 1). We also recruited 100 healthy matched controls from the same ethnic background. Written informed consent was obtained from all the subjects or their parents.

Phenotype evaluation

Audiological testing

For measurement of hearing, all patients underwent pure tone audiometry (PTA) test for air conduction at a range of frequencies from 250 to 8,000 Hz. Audiogram results of all patients were defined using a PTA average at three frequencies (500, 1,000, 2,000 Hz): mild for 21-40 dB, moderate for 41-70 dB, severe for 71-95 dB and profound for >95 dB.

Thyroid phenotype testing

The phenotype of thyroid was investigated with evaluation of its structure, size and function, based on sex and age of patients.

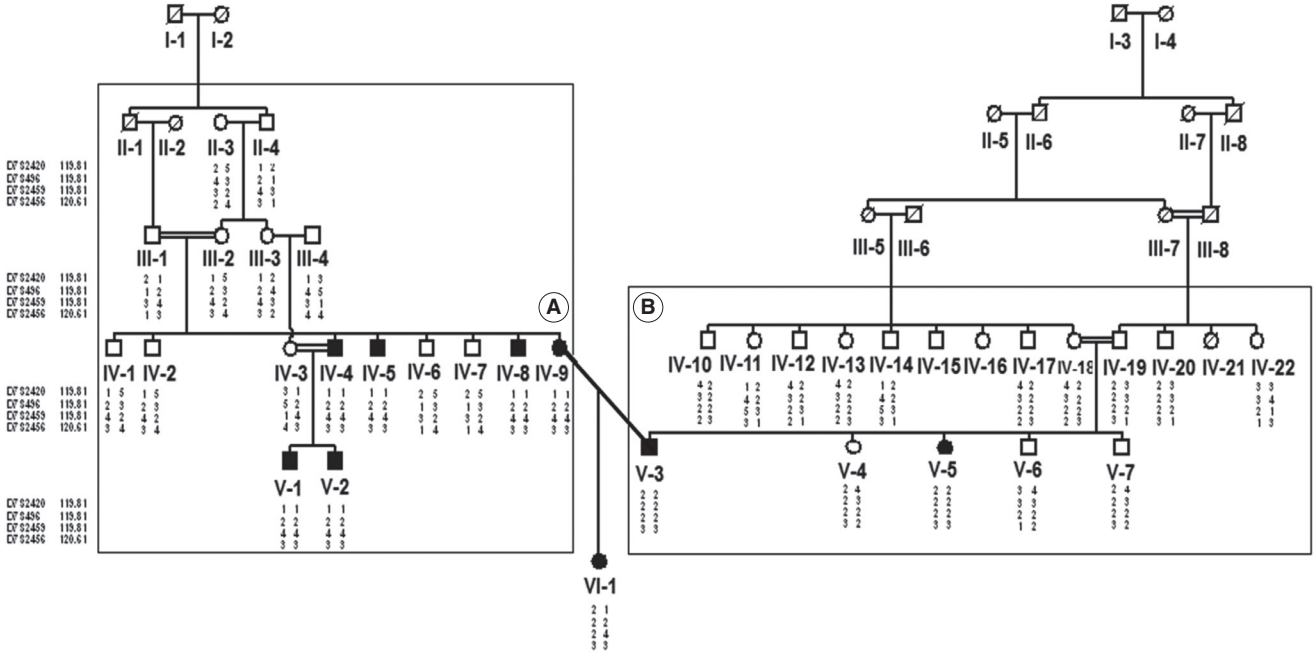

Fig. 1. Pedigree and haplotypes of the family Iranian 3 (IR3). The order of markers is based on the Marshfield map. The homozygous haplotype of part A was different from that of part B of the pedigree. Variants c.881-882delAC and c.863-864insT were found in part A and B, respectively. Patient VI-1 had both haplotypes simultaneously which were later shown to carry c.881-882delAC and c.863-864insT mutations (compound heterozygosity).

Ultrasonography with Sonoline G50 ultrasound system (Siemens Medical Solutions, Erlangen, Germany) was applied to identify thyroid size and structure for all patients. For functional analysis of thyroid in all patients, thyroid stimulated hormone (TSH), thyroxin (T_4), and triiodothyronine (T_3) levels were measured by means of a chemiluminescent immunoassay (Berthold Technology-CSA, Bad Wildbad, Germany).

CT scan of temporal bone

All patients except V-5 and V-2 underwent high resolution CT scan by Somatom Sensation Emotion 16-Slice Configuration (Siemens Medical Solutions) for examination of vestibular aqueduct. EVA was defined as the diameter at the midway between the common crus and the external aperture being equal or more than 1.5 mm [16].

Molecular studies

DNA extraction

Genomic DNA for all available members of the pedigree as well as 100 ethnically matched normal control persons was prepared from 500 μL of peripheral blood by a standard phenol chloroform protocol [17]. For evaluation of quantity and quality of extracted DNA, spectrophotometry (UNICO 2100, West Springfield, MA, USA) and agarose gel electrophoresis were carried out according to routine methods.

Slink calculation, DFNB4 STR marker genotyping and linkage analysis

Selection of four short tandem repeat (STR) markers (D7S2456, D7S2459, D7S496, and D7S2420) and their primers were based on their physical distance, available at NCBI UniSTS and NCBI map viewer (http: //www.ncbi.nlm.nih.gov/mapview).

Slink value was obtained by FastSlink ver. 2.51 option of EasyLinkage ver. 5.05 [18]. Polymerase chain reaction (PCR) amplification of STR markers was performed as follow: 1 μL of $MgCl_2$ (50 mM), 2.5 μL of Taq PCR buffer (10X), 0.4 μL of each of the primers (10 pM), 0.5 μL of dNTP mix (10 mM), 0.1 μL Taq DNA polymerase (5 U/μL), 2 μL DNA (50 ng), adjusted to 25 μL using ddH2O. For various primers some modifications were applied.

For amplification, the following touch-down PCR program was used: an initial denaturation at 95°C for 5 minutes, followed by six cycles of 95°C for 50 seconds (denaturation), 58°C for 50 seconds in the first cycle with 1°C reduction per cycle (annealing), and 72°C for 50 seconds (extension) and 32 cycles of 95°C for 50 seconds (denaturation), 53°C for 50 seconds (annealing), 72°C for 50 seconds (extension) and a final extension at 72°C for 8 minutes. PCR products were loaded on a 14% polyacrylamide gel electrophoresis (PAGE) and run at 28 mA for 2-8 hours. Silver staining was performed to visualize the bands on the gel following standard protocols.

Two-point and multi-point logarithm of odds (LOD) scores were calculated by SuperLink ver. 1.6), and SimWalk ver. 2.91 options of EasyLinkage ver. 5.05, respectively [18,19]. STR markers haplotypes were reconstructed by SimWalk and visualized using Haplopainter ver. 029.5 software after linkage analysis [20]. Autosomal recessive (AR) mode of inheritance, complete penetrance, disease-allele frequency of 0.001, existence of no phenocopy, equal allele frequencies for markers and identical meiotic recombination frequencies in both sexes were assumed for LOD score calculations.

Mutation screening of SLC26A4

All 21 exons (including 50-200 bp flanking regions) and promoter of *SLC26A4* were amplified using primers designed by Oligo ver. 6.7.1.0 (National Biosciences Inc., Plymouth, MN, USA) (Table 1).

Amplification was performed, with some modifications for each amplicons, in a 50 μL volume of reaction, containing 4 μL of $MgCl_2$ (50 Mm), 5 μL of Taq PCR buffer (10X), 0.6 μL of each primer (10 pM), 1 μL of dNTP mix (10 mM), 0.2 μL Taq DNA pol (5 U/μL), 4 μL DNA (50 ng), 34.6 of ddH20. PCR was done according to the following program: an initial denaturation at 95°C for 5 minutes, 36 cycles of 95°C for 1 minute (denaturation), 61°C for 1 minute (annealing), 72°C for 1 minute (extension) and a final extension at 72°C for 8 minutes. DNA sequencing of the PCR-amplified product was performed bi-directionally on an ABI 3730XL automated sequencer (Applied Biosystems, Macrogen, Seoul, Korea) using the same primers for amplification.

Pathogenicity investigation of novel variants

Mutation confirmation was done with co-segregation study of the novel variants and their absence in 100 ethnically matched normal control subjects. For investigation of c.881-882delAC variant pathogenicity, a 89 bp fragment of exon 7 harbouring c.881-882delAC location was amplified with primers 7K1F and 7K1R. For studying c.863-864insT pathogenicity, a 44 bp fragment of exon 7, containing the location of this variant, was amplified using 7K2F and 7K2R primers. 7K1 and 7K2 primers were designed by primer3 ver. 0.4.0 (http://frodo.wi.mit.edu/primer3/) and Oligo ver. 6.7.1.0 (National Biosciences Inc.), respectively (Table 1). PCR amplifications were performed using the conditions mentioned above. Amplification products were run on 14% PAGE at 28 mA for 3 hours. Allelic variant c.881-882delAC is 2 bp shorter than wild type alleles and c.863-864-insT is 1bp longer than normal type alleles.

RESULTS

Phenotype evaluation results

Audiological testing results

Audiological characterization of all patients is shown in Fig. 2. Mean PTA of thresholds for air conduction at frequencies 500,

Table 1. *SLC26A4* primers for amplification of promoter (P), 21 exons, a 89 bp (7k1) and a 44 bp fragments (7k2) of exon 7

Primer name	Sequence (5′ → 3′)	Primer name	Sequence (5′ → 3′)	PCR product length (bp)
F1	CTGGCCATTGTTCCTCTT	R1	TTGAGCAAGTCTCTCCCC	618
F2	ACTCGCTTCAAGTTTGGG	R2	GCGAGTTTCCCAGGTAAG	664
F3	GCACTTCAGGGTTATTATTTTC	R3	AAGAGAACTCTAAGGAAGGGG	461
F4	GAAAAACAGAATGGTTGTATGG	R4	GAAAAAGCAGGCAAAACAC	496
F5	GATGGGGTTTTACTATGTTGC	R5	CTCTCATCCTCAATTGAATCAC	689
F6	ATTTTTGTGCTATAGGCAGG	R6	ATGAGGTCTCACGTCTCAAA	459
F7	ATCACCCAGTTTTTCCTTTC	R7	GGGATGGATTTAACAATGC	595
F8	ATAGACGCTGGTTGAGATTTT	R8	AGAAAAAAGAGCATATACGGG	558
F9	CAGCCAGTAAGATAACACCAA	R9	AAAGCAAAGTGATGCAGTGT	538
F10	TTATCGAGAGCAATGAGACC	R10	TCAGTTGTTATTGACCACAGC	606
F11	TAGATGCCATTTTTGTTCAGTT	R11	ACACAGCTGCATAAACATCC	468
F12	CATCTCTGCTGCGATTGT	R12	CCAAAGGTGTATGAATGAGC	556
F13	AATCCAGAAGATGGAGGC	R13	AAATCTTAGCTCTGCCACG	593
F14	CCAGCTGTTCATTTCAGAGT	R14	AAAAGTTTTCATGACACTCCC	378
F15	ACTGTGACTTGACTCCTTGC	R15	TTTAATTCTCATTGCCCTACAC	321
F16	TTGTCTTTTACTGTCTTGGAGC	R16	TTGCACTTATTTTGTTCCTTTC	626
F17	CACAATCATCCAGAAAACAAA	R17	CAGATTAAGCAACTTGCCC	689
F18	CTGGATGTTGCCATCTCTT	R18	CTGTCTTTGGCCTTTTCTG	496
F19	TAGGGTGTGCCCTGTAGTC	R19	TACACAAATCCCAGATCACAA	676
F20	CAGAGGGGGTGACTTGTTA	R20	TAGGTATCAAATCAGGAGCAGT	628
F21	GGGCAACAGTGAGTGAGAT	R21	GTGATGTAGATCAGCAGCGT	494
FP	TGGGGAGGAGTTCTGAGT	RP	ATCCTCACTCATCCCGTT	715
F7K1	TGCTCACCATTGTCGTCTGT	R7K1	AATTACTTCTATAGGAATAGGGACTGG	89
F7K2	GTCGTCTGTATGGCAGTT	R7K2	TGTCTAAACCGATCATTT	44

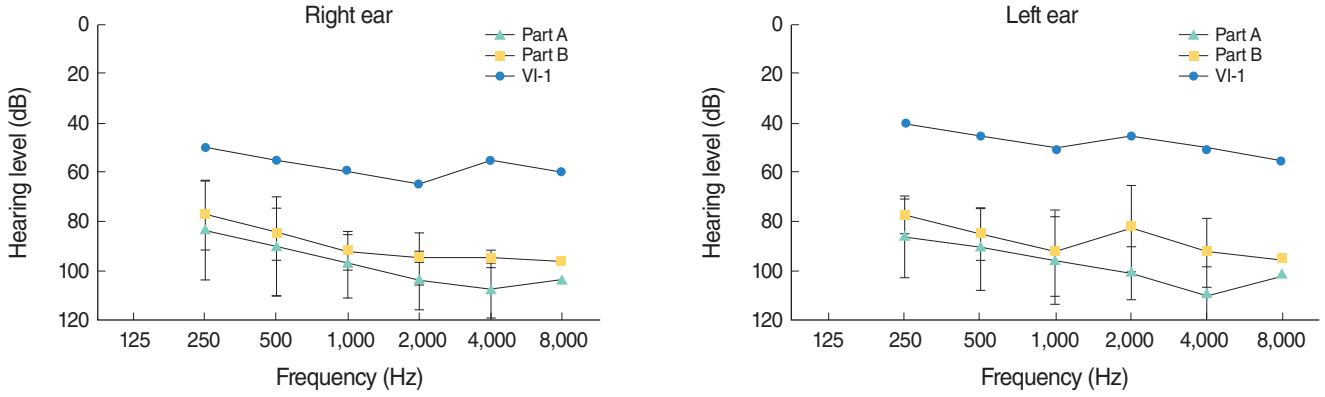

Fig. 2. Mean audiometric hearing thresholds for right and left ears of all patients of part A (triangles), patients V-3 and V-5 of part B (squares), and audiogram of both ears of patient VI-1 (circles) of the family Iranian 3.

1,000, 2,000 Hz was 97.22±7.09 for part A, 90.83±5.20 for part B, 60±5 for patient VI-1 (right ear) and 95.83±5 for part A, 86.67±5.20 for part B and 46.67±2.89 for patient VI-1 (left ear). The severity of HL for all patients has been shown in Table 2.

Thyroid phenotype testing results

All patients had multinodular goiter and normal level of T_3, T_4, and TSH hormones (euthyroid goiter), except VI-1, who had a normal size thyroid and normal levels of thyroid hormones (Table 2, Fig. 3)

CT scan of temporal bone

Vestibular aqueduct enlargement was observed in all the patients assayed for temporal bone in the present study. MD was also detected in three of the patients inspected (Table 2, Fig. 4).

Molecular studies results

Slink calculation, DFNB4 STR marker genotyping and linkage analysis

Slink, two-point and multi-point LOD scores were respectively as follows: 4.90, 5.17, and 6.57 (Fig. 5). LOD score >3 confirmed linkage to DFNB4.

Mutation screening of SLC26A4 results

Finding of two different homozygous haplotypes in the patients of part A and part B of the pedigree and the simultaneous presence of both haplotypes in patient V1-1 predicted possible compound heterozygosity (Fig. 1), which was later confirmed by DNA sequencing: two novel variants including c.863-864insT (p.Leu-288PhefsX3) and c.881-882delAC (P.His294GlnfsX35) were identified in exon 7 of *SLC26A4* (Fig. 6). Homozygous c.863-864insT variant was found in Patients V-3 and V-5, while subjects V-4, V-7, IV-20, IV-19, IV-18, IV-17, IV-13, and IV-10 were heterozygous for this variant. Individuals II-4, III-1, III-2, III-3, IV-1, IV-2, and IV-3 were heterozygous for c.881-882delAC. Patients IV-4, IV-5, IV-8, IV-9, V-1, and V-2 were homozygous for this variant. Compound heterozygosity for both of these variants was revealed in patient VI-1.

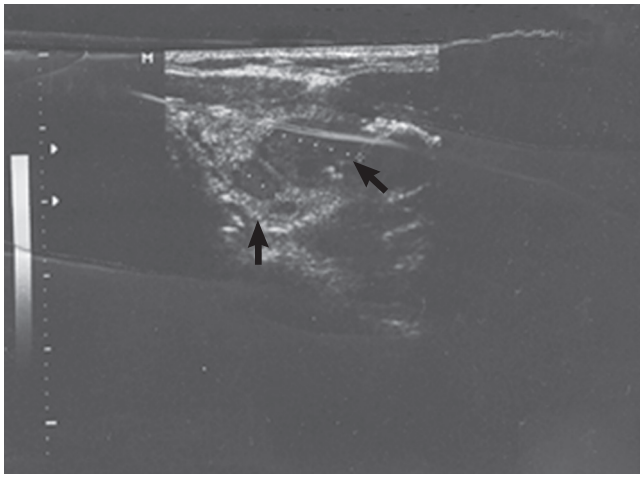

Fig. 3. Thyroid ultrasonography result of patient V-5 (from family Iranian 3) with multinodular goiter. The arrows show nodules. The view is axial, and ultrasound probe is positioned in the neck region.

Pathogenicity confirmation of novel variants

The variants c.881-882delAC and c.863-864insT were found in a homozygous state in patients of the pedigree but were not detected, or were in heterozygous state, in normal subjects of the family. None of these variants were detected in 100 ethnically matched normal control subjects. With respect to these results and the nature of the two variants (frameshift), we suggest the pathogenicity role of both variants.

Table 2. Clinical and genetic evaluation results of all patients of the family Iranian 3

ID no.	Age (year)	Sex	Audiogram of both ears	CT scan	Thyroid (T_3, T_4) and TSH hormones	Thyroid ultrasonography	Variant	Homozygous or Heterozygous
V-2	19	M	Profound	NA	Normal	Multinodular goiter	c.881-882del AC	Homozygous
IV-4	46	M	Profound	EVA, MD	Normal	Multinodular goiter	c.881-882del AC	Homozygous
IV-5	40	M	Severe	EVA	Normal	Multinodular goiter	c.881-882del AC	Homozygous
IV-8	31	M	Severe	EVA	Normal	Multinodular goiter	c.881-882del AC	Homozygous
IV-9	30	F	Profound	EVA	Normal	Multinodular goiter	c.881-882del AC	Homozygous
V-3	32	M	Profound	EVA, MD	Normal	Multinodular goiter	c.863-864insT	Homozygous
V-5	22	F	Severe	NA	Normal	Multinodular goiter	c.863-864insT	Homozygous
VI-1	8	F	Moderate	EVA	Normal	No goiter	c.863-864insT/ c.881-882del AC	Compound heterozygous

CT, computed tomography; TSH, thyroid-stimulated hormone; EVA, enlarged vestibular aqueduct; MD, Mondini dysplasia; NA, not available.

Fig. 4. Computed tomography scan of the temporal bone for patient IV-8 (from family Iranian 3) with bilateral enlarged vestibular aqueduct.

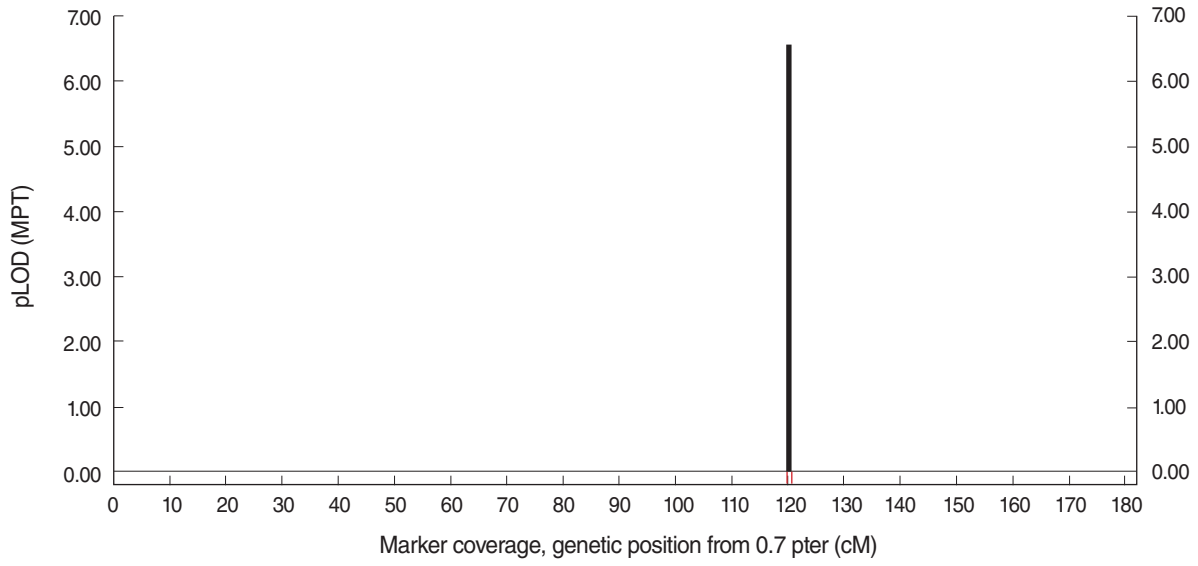

Fig. 5. Multipoint logarithm of odds (LOD) score calculation of the family Iranian 3 with SimWalk ver. 2.91 showed a score of 6.57 confirming linkage to DFNB4 locus.

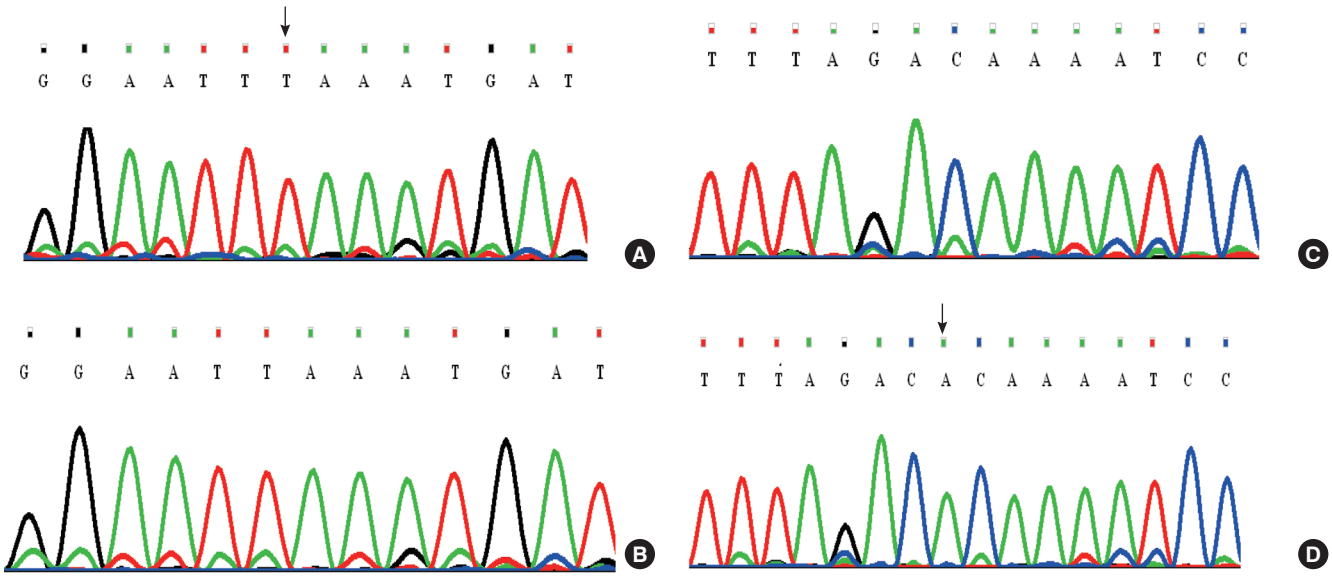

Fig. 6. Electropherogram results of two novel *SLC26A4* variants in family Iranian 3 and normal alleles. (A) A patient with homozygous c.863-864insT allele. (B) A normal subject without c.863-864insT allele. (C) A patient with homozygous c.881-882delAC allele. (D) A normal subject without c.881-882delAC allele.

DISCUSSION

In the present study, we detected two novel variants (c.863-864insT and c.881-882delAC) in a large consanguineous Iranian family with 9 patients with HL linked to DFNB4 locus. c.863-864insT with a frameshift at codon 288 causes a premature stop at position 290 and c.881-882delAC results in a frameshift at codon 294 and a premature stop at position 328.

There is no definite structure for pendrin but a few models have been predicted for this protein. Based on pendrin structural modeling, predicted by Everett et al. [3] and the model described in Pendred/Bor homepage (http://www.healthcare.edu/labs/pendredandbor/domains.htm), c.863-864insT and c.881-882delAC lead to amino acid changes at intracellular region between the sixth and seventh transmembrane domains of the protein (Fig. 7). Thus, these variants can delete more than half of pendrin and cause a defective protein without a long C terminal segment which probably is not processed to reach the plasma membrane and would not be functional.

However, further molecular studies on mRNA expression might

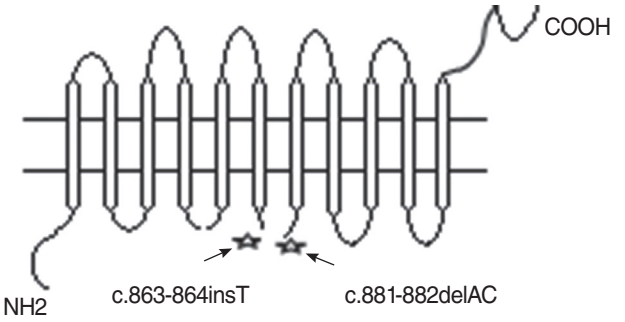

Fig. 7. c.863-864insT and c.881-882delAC variants which cause amino acid change at intracellular region (most probably, between the sixth and seventh transmembrane domains) of the pendrin protein in the Everett model.

be necessary to help gain a more comprehensive understanding of the exact effect of these two novel variants on structure and function of the resulting protein.

In this study, patient VI-1, aged 8 years old, had normal thyroid size and function and a moderate HL. She was compound heterozygous for c.863-864insT and c.881-882delAC. The combined presence of the two variants may lead to a different phenotype from that caused by any single variant alone in the homozygous state. Alternatively, PS-related phenotypes in the patient might be progressive and could be more severe with age. As goiter does not usually manifest until adulthood and even after that, the patient VI-1 may show goiter at an advanced age. Furthermore, according to Scott et al. [21] study, PS and DFNB4 *SLC26A4* mutations are different functionally. Mutations, leading to PS are usually associated with no transporting ability, but those accounting for DFNB4 HL may show some residual iodide and chloride transporting function.

However, no obvious relationship has been reported between *SLC26A4* mutations and phenotype by some investigators [22, 23]. These contradictory results could suggest that some other modifier genes, epigenetic and environmental factors such as nutritional iodide uptake can influence the clinical manifestations due to *SLC26A4* mutations. Other patients in the pedigree had mostly severe to profound HL, and were positive for EVA and euthyroid goiter. These results show c.863-864insT and c.881-882delAC would most probably cause PS with rather similar phenotypic patterns.

It has been suggested that *SLC26A4* would play a more considerable role in HI of Asian populations, such as Iranians, than other populations [11-15,24]. Up to now a considerable number of all mutations, which have been detected in Iranian HL patients have been novel [13,25,26]. In the present study, we detected two different novel variants segregating with HL in a large deaf family. Taken together, these different studies probably confirm the high frequency and specificity of *SLC26A4* mutations and the critical role of these defects in causing HI in Iranian heterogenous population. Therefore it is beneficial to consider screening of *SLC26A4* mutations in molecular diagnostics of HL. Furthermore, Detection of mutations in this gene may have implications for better understanding of the exact structure and function of pendrin as well as designing of comfortable and more cost effective strategies in genetic diagnosis of HI.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

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