Original Article

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Molecular diagnosis of *SLC26A4*-related hereditary hearing loss in a group of patients from two provinces of Iran

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SUMMARY The *SLC26A4* gene has been described as the second gene involved in most cases of autosomal recessive non-syndromic hearing loss (ARNSHL), after *GJB2*. Over 500 different *SLC26A4* mutations have been reported, with each ethnic population having its own distinctive mutations. Here, we aimed to determine the frequency and mutation profile of the *SLC26A4* gene from two different provinces (center and west) of Iran. This study included 50 nuclear families with two or more siblings segregating presumed ARNSHL. All affected tested negative for mutations in *GJB2* at the DFNB1 locus and were therefore screened for autozygosity by descent using short tandem repeat polymorphisms (STRPs) of DFNB4. Sanger sequencing was performed to screen the 20 exons of the *SLC26A4* gene for the families linked to this locus. *In silico* analyses were also performed using available software tools. Four out of 25 (16%) and 3 of 25 (12%) studied families of Isfahan and Hamedan provinces, respectively. were linked to DFNB4. Sanger sequencing led to the identification of six different mutations, one of which (c.919-2A>G) was recurrent and accounted for 31% of all mutant alleles. One out of 7 (14.3%) families with mutations were confirmed to be Pendred syndrome (PS). The *SLC26A4* mutations have a high carrying rate in ARNSHL Iranian patients. The identification of a disease causing mutation can be used to establish a genotypic diagnosis and provide important information to the patients and their families.

Keywords autosomal recessive non-syndromic hearing loss, *SLC26A4*, Iran, Pendred syndrome

1. Introduction

Hearing loss (HL) is the most common sensory disorder affecting 2-3 out of 1,000 births (*http://hearing.screening.NHs.UK/nationalprog*); over 70-80% of the etiology are genetic factors. It is estimated that 70% of HL includes non-syndromic forms (NSHL), where the hearing deficit is the only sign. Approximately, 80% of cases in this group follow autosomal recessive inheritance (ARNSHL) (*1*). The autosomal recessive loci are called DFNB followed by a number corresponding to the order that the locus was first described; DFNB1 to DFNB108 have been reported so far (see Hereditary Hearing Loss Homepage at *https://hereditaryhearingloss.org/*).

Mutations in the *SLC26A4* gene have been described for DFNB4 non-syndromic hearing loss (NSHL, MIM # 600791) and Pendred syndrome (PS, MIM # 274600). Pendred syndrome is associated with sensorineural deafness, congenital and severe to profound temporal bone abnormalities, goiter and iodide organification defects. In the absence of thyroid dysfunction, patients are considered to be forms NSHL DFNB4 (*2*).

SLC26A4 (OMIM: 605646) was identified by Everett *et al.* after using positional cloning on chromosome 7q22-31. This gene encodes pendrin, an anion transporter, which is expressed in the kidneys, inner ear, and thyroid (*3*). Pendrin is composed of 780 amino acids and has a molecular weight of 86 k DA. In the inner ear, pendrin was found in the endolymphatic sac and hair cells, where it is involved in pH homeostasis, acting in bicarbonate/chloride exchange. Mutations in the *SLC26A4* gene can affect pendrin activity, causing an imbalance of ions and fluid levels in the inner ear (*4*). Different investigations have suggested that *SLC26A4* mutations are among the most frequent causes of genetic HL in the world populations, including Iranians (*5*). The genetic

etiology of ARNSHL in Iran, has been shown by a number of independent studies with a special focus on DFNB1 (*GJB2*) (*6-12*), the most common cause of HL in the world (*13-15*). In the previous study, we showed that variants in the *GJB2* (NM_004004.5) can explain the etiology of ARNSHL in 22.5% and 20% of patients from Isfahan and Hamedan provinces of Iran, suggesting that *GJB2* gene mutations only represent a part of ARNSHL in the center and west of Iran (*16*). Thus, more studies are necessary to identify other common loci and determine the etiology of ARNSHL based on ethnicity (*17,18*).

In the present study we applied a homozygosity mapping strategy and Sanger sequencing to identify the spectrum and mutation type of the *SLC26A4* gene contribution to ARNSHL. The study cohort included 50 ARNSHL families, negative for *GJB2* mutations, from Isfahan and Hamedan provinces in the center and west of Iran for the first time. This study is part of a larger study, which aims to complete the genetic map of HL in Iran by investigating the pedigrees of families with hearing impaired members in each province. The result of this study should have implications in improved genetic counseling and prevention of HL using pre-implantation genetic diagnosis (PGD) and prenatal diagnosis.

2. Materials and Methods

2.1. Families and phenotype investigation

Fifty consanguineous multiplex families with at least 2 hearing impaired individuals in each family and negative for *GJB2* mutations were recruited for this study. The Ethics Committee of Isfahan University of Medical Sciences approved this project. All family members signed an informed written consent prior to recruitment. They met the following criteria: confirmation of HL by pure tone audiometry (PTA) from 250 to 8000 Hz, autosomal recessive inheritance through pedigree analysis (*3*), existence of three or more affected members within the pedigree. A complete clinical evaluation, including audiology, ophthalmological, and physical examinations were performed to exclude environmental exposures and to determine the presence of syndromic findings in each family.

In two families, both parents were hearing impaired, suggesting the presence of the same identical mutation by descent mutation.

2.2. Genotyping STR markers and linkage analysis

Genomic DNA of patients was extracted from peripheral blood lymphocytes using a standard salting out procedure (*19*). Qualitative and quantitative assessment of genomic DNA was checked using 1.2% agarose gels and a Nanospec cube biophotometer (Nanolytik®, Dusseldorf, Germany).

Linkage analysis was performed using at least four informative Short Tandem Repeat (STR) markers located at or tightly linked to each locus. Primer sequences were obtained from the Probe database. Table 1 summarizes the general characteristics of the markers used in the study. Touchdown PCR was performed in a thermal cycler machine (ASTEC PC-818, Fukuka, Japan) to amplify STR markers. PCR products from family members were genotyped using polyacrylamide gel electrophoresis. We used 12% polyacrylamide (29:1) to detect STR bands. As some STRs were uninformative in some of the families, we had to test more STRs to find at least 4 informative markers for each locus among families to confirm or reject linkage. Two-point and multi-point parametric LOD scores under a recessive model were, respectively, calculated by Superlink version 1.6 and Simwalk version 2.91 (assuming a risk allele frequency of 0.001 and complete penetrance) (easyLINKAGE program package) (*20,21*). Simwalk and Haplopainter software version 029.5 were used to reconstruct and visualize haplotypes, respectively (*22*).

2.3. Mutation detection of SLC26A4

All 20 coding exons (numbered from 2 through 21) of the *SLC26A4* gene were amplified by polymerase chain reaction (PCR) using previously reported primers (*23*). The PCR products were sequenced using a ABI3130 sequencer (Applied Biosystem, Foster City, CA, USA). The resulting sequences were edited and compared against the published NCBI Homo sapiens *SLC26A4* DNA sequence reference assembly (accession NC_000007region: 107301080.107358254).

2.4. Computational analyses

We used Bioinformatics predictive tools including

Table 1. STR markers used and their characteristics

Locus (gene)	STR	Heterozygosity (%)	Size (bp)	Forward Primer	Reverse Primer
DFNB4 (*SLC26A4*)	D7S2420	81	240-290	CCTGTATGGAGGGCAAACTA	AAATAATGACTGAGGCTCAACA
	D7S2496	63	129-141	AACAACAGTCAACCCACAAT	GCTATAACCTCATAACCAAAA
	D7S2459	77	140-152	AAGAAGTGCATTGAGACTCC	CCGCCTTAGTAAAACCC
	D7S2456	78	238-252	CTGGAAATTGACCTGAAACCTT	ACAGGGTCTCTCAATATTA

STR, Short Tandem Repeat.

MutationTaster, PolyPhen and SIFT (*24,25*) to assess possible effects of mutations on the protein structure. Multiple sequence amino acid alignment of the pendrine protein and visualization of conserved amino acids was performed using the ConSurf Server. The American College of Medical Genetics and Genomics (ACMG) guidelines for variant interpretation were used to categorize identified variants (*26*).

2.5. Clinical investigation

Post-test genetic counselling and clinical examination were carried out for all affected members of the linked families, to determine whether the phenotype was related to DFNB4 (NSHL) or PDS (SHL).

Function, structure, and size of the thyroid were evaluated, to determine the presence of goiter, associated with PDS. The levels of thyroid stimulating hormone (*27*), T3 and T4 were measured in all patients by means of Elecsys (Chemiluminescent Immunoassay) to evaluate the function of the thyroid. The size and structure of the thyroid were assessed using a Tc99m thyroid scan. The Perchlorate Discharge Test (PDT) was done in order to confirm the clinical features of PDS. One gram of perchlorate ($KClO_4$) was administered two hours after the administration of 131-iodine (50 mCi). Then, the discharge of iodide was measured. A discharge less than 10% of the incorporated iodide is expected in normal individuals (*28*). All three siblings (family IR-20) also underwent a high-resolution computed tomography (CT) scan of the temporal bone, using a Somatom Sensation 16 (Siemens, Erlangen, Germany), to determine if there were alterations in the cochlea and vestibular aqueduct. When the diameter at the midpoint between the common crus and the external aperture was equal to or greater than 1.5 mm, it was described as Enlarged Vestibular Aqueduct (EVA) (*29*).

3. Results

3.1. Family data and linkage analysis

After excluding mutations in *GJB2*, a total of 50 Iranian families segregating ARNSHL were recruited from Isfahan and Hamedan provinces (25 families from each province) in the center and west of Iran. Among the 50 families (456 individuals), there were 205 patients, with ages ranging from 6 months to 52 years. In 12 studied families, PTA was consistent with severe HL (61-80 dB) and the remaining had profound HL (≥ 80 dB).

The linkage results were confirmed by individually genotyping the family members for the same markers, as well as additional markers. After genotyping of STR markers, 7 out of the 50 families, negative for *GJB2* mutations, showed linkage to DFNB4. 4 out of 25 (16%) and 3 of 25 (12%) ARNSHL families of Isfahan and Hamedan provinces, respectively, were linked to this locus. Two-point and multi-point LOD score values for the seven linked families are shown in Table 2. Our study included two deaf-to-deaf marriages, none of whom were linked to DFNB4.

3.2. SLC26A4 mutation screening

A total of 6 different *SLC26A4* mutations including c.416G>T, c.1334T>G, c.1156A>G, c.1238A>G, c.1295C>T and c.919-2 A>G were detected in 13 out of the 100 (13%) total studied alleles (Table 3). In one

Table 2. LOD score (two-point and multi-point) values for linked families to DFNB4

Number	Family ID	Two-point LOD score	Multi-point LOD score	Severity HL
1	IR-9	3.1	3.2	Severe-profound
2	IR-14	3.8	4.1	Moderate-profound
3	IR-20	2.8	3.2	Severe
4	ISF-5	3	3.3	Severe-profound
5	ISF-6	3.5	3.9	Profound
6	ISF-15	29	3.3	Profound
7	ISF-21	3.2	3.6	Severe-profound

Table 3. Identified *SLC26A4* mutations, their frequencies and *in silico* analyses

Mutations	No (%)		Mutation type	Location	Functional effect		
	Isfahan	Hamedan			PolyPhen Prediction	CADD score	SIFT
c.416 G>T	0	2 (4)	Missense	Exon 05	probably damaging	24.8	Damaging
c.1156 A>G	1	0	Missense	Exon 10	probably damaging	22	Damaging
c.1238 A>G	2 (4)	0	Missense	Exon 10	probably damaging	26.2	Damaging
c.1259 C>T	2 (4)	0	Missense	Exon 10	probably damaging	23.8	Damaging
c.1334 C>T	0	2 (4)	Missense	Exon 11	probably damaging	24.1	Tolerated
c.919-2 A>G	2 (4)	2 (4)	Splice-site	Exon 07	NA	27	NA
Normal	43	44					
Total	50	50					

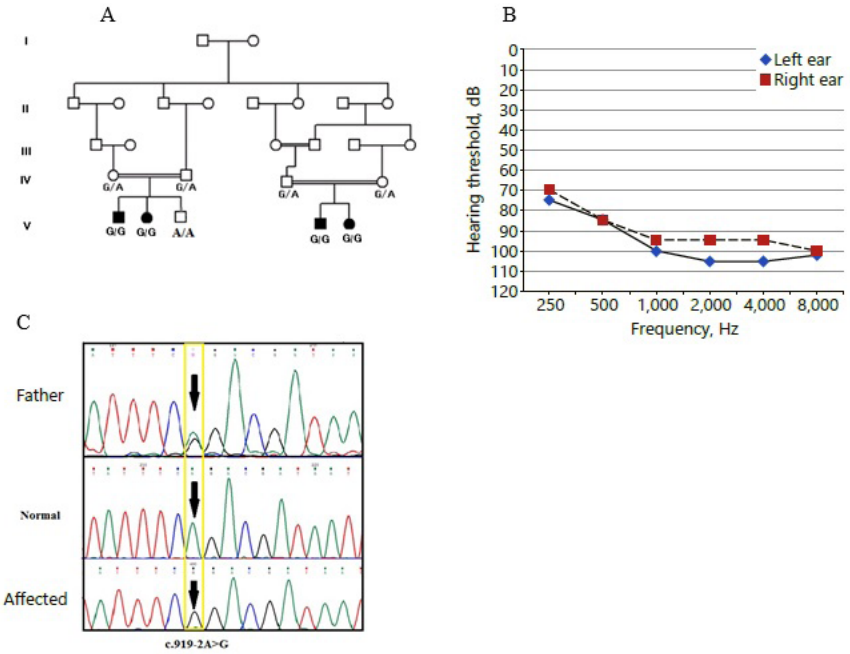

Figure 1. (A) Pedigree and segregation of the c.919-2A>G mutation in Iranian family ISF-5; **(B)** Right and left ear audiograms in the (V: 1) patients from ISF-5 family; **(C)** Sequencing results of the c.919-2A>G mutation.

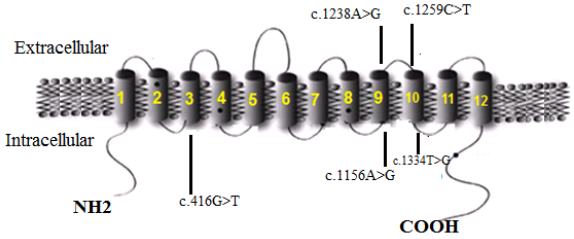

Figure 2. Location of *SLC26A4* mutations at the protein level (The mutation 919-2A>G as an intronic mutation does not change amino acid at the protein level).

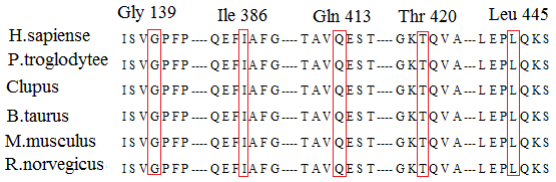

Figure 3. Multiple amino acid alignment of protein homologs was conserved. The identified mutations c.416G>T, c.1334T>G, c.1156A>G, c.1238A>G and c.1295C>T occur at highly conserved positions (Gly 139, Ile 386, Gln 413, Thr 420 and Leu 445) in the pendrin protein.

of the DFNB4 linked families, only one heterozygous mutation was identified, and the remaining five families were homozygotes. Notably, the result of the present study might be a digenic state: interaction with one of the two genes interacting with *SLC26A4*. The missing cause is most likely a non-coding mutation or a large deletion in the coding region. It is certainly possible that a homozygous mutation in another gene could be the underlying cause of HL in this family.

One mutation was a splice site and the others were missense (Table 3). One mutation (c.919-2A>G), was seen in two linked families (IR-9 and ISF-5) from both Isfahan and Hamedan province (Figure 1). All the mutations showed co-segregation with HL in the related family members. Figure 2 shows the distribution of the identified mutations in the schematic structure of pendrin. *In silico* program prediction is shown in Table 3. The ConSurf Server (*http://conseq.tau.ac.il/*) revealed that the causative variants were located at a well conserved site (Figure 3).

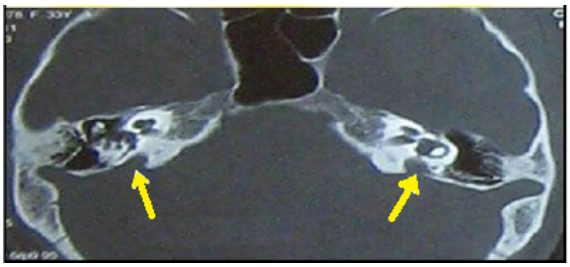

Figure 4. Temporal bone CT scan results of an affected member with EVA.

3.3. Clinical testing results

CT scan showed EVA in all linked families (Figure 4). The levels of thyroid hormones (TSH, T3, and T4) were normal in all linked families, but thyroid scan detected enlarged multinodular goiter in one family (IR-20). There were multiple cold and functioning isoactive nodes on both lobes, which is strong evidence

Table 4. Characterization of Iranian families with *SLC26A4* mutations

Nucleotide Change	Amino Acid Change	Location	Mutation Type	Authors (Year)
c.65-66insT	p.Ser23ValfsAla64	Exon 02	Frameshift	Yazdanpanahi *et al.* (2015)
c.269C>T	p.Ser90Leu	Exon 03	Missense	Sloan-Heggen *et al.* (2015)
c.235C>T	p.Arg79*	Exon 03	Nonsense	Kahrizi *et al.* (2009)
c.347G>T	p.Gly116Val	Exon 04	Missense	Sloan-Heggen *et al.* (2015)
c.416G>T	p.Gly139Val	Exon 05	Missense	Reisi *et al.* (2014)
c.481T>A	p.Phe161Ile	Exon 05	Missense	Sloan-Heggen *et al.* (2015)
c.683C>A	p.Ala228Asp	Exon 06	Missense	Sloan-Heggen *et al.* (2015)
c.716T>A	p.Val239Asp	Exon 06	Missense	Sloan-Heggen *et al.* (2015)
c.863-864insT	p.Leu288PhefsGly3	Exon 07	Frameshift	Yazdanpanahi *et al.* (2015)
c.881-882delAC	p.His294GlnfsGlu35	Exon 07	Frameshift	Yazdanpanahi *et al.* (2015)
c.919-2A>G	–	Intron 07	Splice site	Yazdanpanahi *et al.* (2015)
c.965insA	p.Asn322KfsAla8	Exon 08	Frameshift	Sloan-Heggen *et al.* (2015)
c.959-960insA	p.Gly320LeufsLys20	Exon 08	Frameshift	Sloan-Heggen *et al.* (2015)
c.1001G>T	p.Gly334Val	Exon 08	Missense	Sloan-Heggen *et al.* (2015)
c.1102G>A	p.Gly368Arg	Exon 09	Missense	Sloan-Heggen *et al.* (2015)
c.1156 A>G	p.Ile386Val	Exon 10	Missense	Present study
c.1174A>T	p.Asn392Tyr	Exon 10	Missense	Sloan-Heggen *et al.* (2015)
c.1197delT	p.Cys400Valfsx32	Exon 10	Missense	Kahrizi *et al.* (2009)
c.1226G>A	p.Arg409His	Exon 10	Missense	Kahrizi *et al.* (2009)
c.1229C>T	p.Thr410Met	Exon 10	Missense	Yazdanpanahi *et al.* (2015)
c.1238A>G	p.Gln413Arg	Exon 10	Missense	Yazdanpanahi *et al.* (2015)
c.1259C>T	p.Thr420Ile	Exon 10	Missense	Sloan-Heggen *et al.* (2015)
c.1334T>G	p.Leu445Trp	Exon 11	Missense	Kahrizi *et al.* (2009)
c.1412delT	p.Leu471Argfsx17	Exon 12	Missense	Yazdanpanahi *et al.* (2015)
c.1343C>T	p.Ser448Leu	Exon 12	Missense	Sloan-Heggen *et al.* (2015)
c.1489G>A	p.Gly497Cys	Exon 13	Missense	Yazdanpanahi *et al.* (2015)
c.1517T>G	p.Leu506Arg	Exon 13	Missense	Sloan-Heggen *et al.* (2015)
c.1546C>T	p.Pro516Ser	Exon 13	Missense	Sloan-Heggen *et al.* (2015)
c.1574C>T	p.Pro525Leu	Exon 13	Missense	Sloan-Heggen *et al.* (2015)
c.1614+1G>C	–	Intron 14	Splice site	Sloan-Heggen *et al.* (2015)
c.1717G>T	p.Asp573Tyr	Exon 16	Missense	Sloan-Heggen *et al.* (2015)
c.1790T>C	p.Leu597Ser	Exon 16	Missense	Kahrizi *et al.* (2009)
c.2027T>A	p.Leu676Gln	Exon 19	Missense	Sloan-Heggen *et al.* (2015)
c.2106delG	p.Lys702Asnfsx19	Exon 19	Missense	Yazdanpanahi *et al.* (2015)
c.2162C>T	p.Thr721Met	Exon 19	Missense	Kahrizi *et al.* (2009)
c.2145G>T	p.Lys715Asn	Exon 20	Missense	Kahrizi *et al.* (2009)

supporting that c.1334 C>T is associated with PDS.

4. Discussion

Mutations of *SLC26A4* are a well-known cause of ARNSHL globally. Many studies have been performed to demonstrate the role of *SLC26A4* mutations in ARNSHL among various ethnic cohorts (*30-32*). In a recent multicenter study performed by Sloan-Heggen *et al.*, 37 out of 302 *GJB2*-negative multiplex Iranian families (12.3%) were attributable to variants in the *SLC26A4* gene (*33*), while the frequency of the *SLC26A4* mutations has been reported in Pakistan (*34*) and Turkey to be 5.4%, and 1.8% (*35*) respectively. Mutations of *SLC26A4* are the second most common cause of HL, and have been identified in the sequence encoding all of the transmembrane (TM1-12) segments of this protein (*36*).

The most comprehensive previous study was done on 80 *GJB2*-negative Iranian subjects using linkage analysis and Sanger sequencing showing ~10% ARNSHL families are related to the *SLC26A4* gene and ranked second after DFNB1 (*37*). Our study shows that 4 out of 25 (16%) and 3 of 25 (12%) ARNSHL families of Isfahan and Hamedan provinces, respectively, were linked to DFNB4. The spectrum of *SLC26A4* mutations in our cohort of 50 Iranian families involves mostly missense mutations (70%), which was observed in the other Iranian cohort studies (Table 4) as well as other populations (*38,39*).

c.1156A>G is reported for the first time in Iran. This variant is the adenine to guanine transversion in exon 10 at codon 386 resulting in substitution of isoleucine by valine (P. Ile 386Val). The isoleucine 386 residue is highly conserved among species. Moreover, substitution of residues with a residue, which has different physicochemical properties might result in damaging effects. Isolucine is a nonpolar amino acid with a big hydrophobic structure, while Valine is a small non-charged amino acid. This substitution would affect its contacts with neighboring residues, thereby influencing the folding of the pendrin protein with the mutated residue (*36*).

Three out of 6 (50%) mutations (c.416 G>T, c.919-2A>G and c.1238A>G) were reported in our previous study for the first time in Iran (*23,40*). They may

be unique to the Iranian population, which should be further investigated. Here, exon 10 had the most number of mutations (38.5%). c.1156 A>G, c.1238 A>C and c.1259 C>T mutations are located in exon 10 within the 10th loop putative transmembrane segment of the pendrin protein. Previous studies showed that near 16% of *SLC26A4* mutations are observed within exon 10 (*39*), suggesting that the TM10 region is functionally relevant for pendrin activity (*36*).

In the current study, one out of the 7 (14.3%) families with *SLC26A4* mutations had PS syndrome. In the previous studies in Iran, half of the families with *SLC26A4* mutations were diagnosed with PS (*23*). These results suggest that PS is probably a prevalent syndromic form of HL, which has to be considered in molecular diagnostics of HL in Iran. EVA was a constant feature among all the tested patients in this study.

Unlike *GJB2*, the *SLC26A4* gene has a larger role in Asian than Caucasian populations (*34,41,42*). Studies on NSHL have revealed biallelic *SLC26A4* mutations in 2% to 3.5% of Caucasian patients (*39,43,44*), but in 5.5% to 12.6% of East Asian patients (*42,45,46*). P.Val239Asp was the most common mutation in Turkey (33.3%) and Pakistan (35.6%) while c.919-2A>G and c.1334C>T mutations are more prevalent in East Asia (*34,47*); c.919-2>G has also been found in two of the families in our study. In our patient group, the most frequent mutation is c.919-2A>G, detected in 31% of the mutated alleles. This mutation, which has previously been reported in other ethnic groups, was shown to be a founder mutation in Chinese (*48*) and was reported with a frequency of 31% in a Korean cohort (*49*). However, apart from the present study, no other studies have reported such a high frequency of the c.919-2A>G mutation in Iran (*37*). Notably, c.1238A>G, detected in our previous study, was more common in our population (*23*). The spectrum of mutations in *SLC26A4* in our patients from Isfahan and Hamedan provinces might not be broad, with mutations affecting 3 of 20 exons and exon/intron boundaries. This finding will help design simplified routine DNA testing, targeting the most frequent mutation(s) rather than sequencing all 20 coding exons in patients. Interestingly, the analysis could be started with exon 10, where we found the majority of pathogenic mutations.

The limitation of this study was the sampling of all members of a family. Because both healthy and deaf people had to be sampled, and some members lived in different cities.

5. Conclusion

In the current study, we performed homozygosity mapping analyses and detected 16% and 12% of the studied families to be associated with the *SLC26A4* gene in Isfahan and Hamedan provinces. Our finding will extend the mutation spectrum of the *SLC26A4* gene, and could be applied to prepare a targeted approach to cover the mutations of the *SLC26A4* gene for molecular diagnostics in central and west Iran.

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