A novel variant of SLC26A4 and first report of the c.716T > A variant in Iranian pedigrees with non-syndromic sensorineural hearing loss

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\textbf{ABSTRACT}

The autosomal recessive non-syndromic hearing loss (ARNSHL) can be associated with variants in solute carrier family 26, member 4 (SLC26A4) gene and is the second most common cause of ARNSHL worldwide. Therefore, this study aims to determine the contribution of the SLC26A4 genotype in the hearing loss (HL) of 40 ARNSHL pedigrees in Iran. A cohort of the 40 Iranian pedigrees with ARNSHL, having no mutation in the GJB2 gene, was selected. The linkage analysis with five short tandem repeat (STR) markers linked to SLC26A4 was performed for the 40 ARNSHL pedigrees. Then, two out of the 40 pedigrees with ARNSHL that linked to DFNB4 locus were further screened to determine the variants in all exons of SLC26A4 gene by direct DNA sequencing. The 21 exons of SLC26A4 were analyzed for the two pedigrees. A known variant (c.716T > A homozygote), it is the first reported incidence in Iran, a novel variant (c.493A > C homozygote) were detected in the two pedigrees and pathogenesis of c.493A > C confirmed in this study with review 100 hearing ethnically matched controls by PCR-RFLP analysis. The present study suggests that the SLC26A4 gene plays a crucial role in the HL occurring in Iranian pedigrees. Also, the results probably support the specificity and unique spectrum of SLC26A4 variants among Iranian HL patients. Molecular study of SLC26A4 gene may lead to elucidation of the profile of the population-specific variants which has importance in diagnostics of HL.

1. Introduction

Hearing loss (HL) is a common sensorineural disorder, with an incidence of one in every 500–1000 children [1,2]. At least half of the HL cases can be attributed to genetic factors and more than two-thirds of these individuals are classified as the autosomal recessive non-syndromic hearing loss (ARNSHL). HL may be caused either by unknown genetic factors or environmental factors [3], including pregnancy and childbirth trauma, vitamin B deficiency during pregnancy, immunization, bacterial infectious diseases such as meningitis, or a viral disease such as rubella [4]. Since HL is extremely heterogeneous, studying large-sized families from different ethnicities, such as the Middle Eastern populations, are helpful [5].

Based on several investigations, it is observed that the variants of SLC26A4 gene are the second most common cause of ARNSHL, after GJB2 mutations, in the world and that includes Iran [6]. These variants are also known to be the most common detectable cause of an enlarged vestibular aqueduct [2]. Pendrin is a protein that in humans is encoded by the SLC26A4 gene. It is a 110 kDa glycosylated protein, which functions as a membrane carrier protein that transports particles, such as chloride, iodide, bicarbonate, oxalate, sulfate, and secondary active sulfate across cell membranes [7]. Pendrin exists in different organs and tissues, such as the kidneys, liver, and the lining of airways, especially the inner ear and thyroid gland. In the inner ear, it is likely that this protein helps control and regulates the chloride and bicarbonate exchange [8]. Apart from maintaining the required ionic levels for development of the inner ear, it is believed that pendrin also influences the shape of the bony structures during this developmental phase [6].

The variants of SLC26A4 gene cause non-syndromic recessive HL and Pendred syndrome (deafness with goiter, OMIM #274600). To date, over 170 different alterations have been identified in SLC26A4...
The variant profile and allele frequency may vary among different populations. The hotspot mutation in HL genes is mainly single-gene biallelic mutation [9]. The SLC26A4 gene appears to have a more important role in the etiology of HL in the Middle East and Asia than in other parts of the world [10]. The determination of SLC26A4 mutations in different ethnic populations can provide new insights into the genetic causes of HL, thus enabling better prognostication and enhanced planning of management measures.

2. Materials and methods

2.1. Subjects

In this experimental study, the 40 Iranian pedigrees and each pedigree had at least 3 to 7 families. These families including 130 HL individuals with the ARNSHL were that these pedigrees were selected from > 400 Iranian HL pedigrees with autosomal recessive syndromic, autosomal dominant, X-linked recessive and mitochondrial. Also, there were not patients suspected to show Pendred syndrome-like symptoms in the 400 families with HL not involved in this study. At least two affected individuals with HL were existed in each pedigree. All cases were negative for GJB2 mutations in our previous study (submitted).

All pedigrees had positive history of HL and were of Kurdish ethnicity. Also, for pathogenicity study 100 ethnically matched hearing controls were recruited. A written informed consent had been provided for all the patients in our previous study [11]. After filling out an informed consent form by subjects or their parents (children under age 18) about 5 ml of peripheral blood from all the ARNSHL pedigree members was collected in tubes containing EDTA (0.5 M).

Based on the interviews with the adult members of the pedigrees, informational questionnaires had been filled out and pedigrees drawn.

### Table 1

<table>
<thead>
<tr>
<th>Reverse primer sequence (R)^a</th>
<th>Forward primer sequence (F)^b</th>
<th>PCR product (bp)</th>
<th>Location (decode cM)</th>
<th>Location (Genethon cM)</th>
<th>Location (Marshfeld cM)</th>
<th>Length position (bp)</th>
<th>STR</th>
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<tr>
<td>AAATAATGACTGAGGCTAAAAACA</td>
<td>CCGTATGGAGGGCAAACCTA</td>
<td>240–290</td>
<td>116</td>
<td>120.70</td>
<td>119.81</td>
<td>106,889,928–106,890,211</td>
<td>D7S2420</td>
</tr>
<tr>
<td>GCTTACTGCTGATAANAAAGAAAA</td>
<td>AAGAAGCTGCAAGCGGACAAT</td>
<td>129–141</td>
<td>117.04</td>
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<td>D7S496</td>
</tr>
<tr>
<td>CGGCCCTAGTAAAAACCC</td>
<td>ACGAAGCTGCAAGCGGACAAT</td>
<td>140–152</td>
<td>117.72</td>
<td>120.70</td>
<td>119.81</td>
<td>107,233,501–107,331,642</td>
<td>D7S2459</td>
</tr>
<tr>
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<td>118.39</td>
<td>121.50</td>
<td>121.61</td>
<td>107,683,218–107,683,460</td>
<td>D7S2456</td>
</tr>
<tr>
<td>CCGTTGCCATGTTTTATCC</td>
<td>CTAGTCCCGAAAGACATCC</td>
<td>240–290</td>
<td>118.39</td>
<td>121.50</td>
<td>121.41</td>
<td>108,606,555–108,806,907</td>
<td>D7S2425</td>
</tr>
</tbody>
</table>

^a Reverse. ^b Forward.

Fig. 1. The haplotype analysis of the pedigree IR-15 is shown in up figure and being linked to the D7S2420 marker of the SLC26A4 gene is detected by the 15% polyacrylamide gel at this pedigree in down figure. The healthy individuals were heterozygous (1/2) and homozygous (2/2); three patients were homozygous (1/1).

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This study was approved by the Institutional Review Boards of the Shahrekord University of Medical Sciences, Shahrekord, Iran, in 2016 (Grant No. 2165). Two consanguineous pedigrees from Kurdistan (pedigree IR-15) and Kermanshah (pedigree IR-48) provinces of Iran, with three and two HL patients, respectively, linked to DFNB4 locus were further studied.

For all cases, pure-tone audiometric test (PTA) for air and bone conduction at frequencies 250 to 8000 Hz was performed to clarify HL severity as follows: mild: 21–45 dB; moderate: 46–60 dB; moderately severe: 61–75 dB; severe: 76–96 dB; and profound: ≥ 97 dB. Also, HL severity of all patients with conventional auditory brainstem response (ABR) testing was performed by certified audiologists (which are not shown in this study).

In general, a complete clinical evaluation including audiological, physical examinations, and ophthalmological was done to exclude environmental exposures and to detect the presence of Syndromic results in each pedigree. For the all patients of the linked pedigrees by temporal bone CT-scan was conducted using Somatom Sensation Emotion 16 — Slice Configuration (Siemens Medical Solutions, Erlangen, Germany) to clarify the vestibular aqueduct situation. According to this experiment was not observed enlarged vestibular aqueduct (EVA) in patients.

For assessing thyroid phenotype in all patients of the two pedigrees, thyroid-stimulated hormone (TSH), thyroxin (T4) and triiodothyronine (T3) levels were measured using a chemiluminescent immunoassay (Berthold Technology-CSA, Germany). Also ultrasonography was performed with a Sonoline G50 ultrasound system (Siemens Medical Solutions, Erlangen, Germany) to determine the thyroid size in all patients. Thyroid ultrasonography and hormone investigation results were interpreted according to sex and age of patients and these results were

Fig. 2. Electropherograms from the normal (a), the c.716T > A (p.V239D) homozygous (b), normal (c), and the c.493A > C (p.S165R) homozygous (d) genotypes are shown. Arrows show the location of the base change.
normal in all patients of the two pedigrees.

2.2. DNA extraction

DNA for all available members of the pedigrees, and normal controls was extracted using a DNA extraction kit (DNPTM, CinnaGen, Tehran, Iran). The DNA concentration and absorbance ratios were tested with the Nanodrop spectrophotometer (Thermo Scientific NanoDrop 1000 Spectrophotometer, Thermo Scientific, Wilmington, USA).

2.3. Genetic analysis of GJB2 and SLC26A4

Using sequence analysis, the 40 pedigrees were examined for mutations in the GJB2 gene. Pedigrees who did not possess mutation in this gene were then isolated (40 out of 50 the Iranian deaf pedigrees). Based on a previous study (submitted), these GJB2 negative pedigrees were screened for linkage to DFNB4 by genotyping the short tandem repeat (STR) markers (Table 1). The linkage analysis was followed by sequencing of SLC26A4 for two linked pedigrees.

2.4. Amplification of the STR markers and linkage analysis

Power of the 40 pedigrees for linkage analysis was carried out by evaluating SLINK, using the Fast Slink (version 2.51) option of Easy Linkage plus version 5.05 software [12]. STR markers and primers were selected based on NCBI Map Viewer and UniSTS data. LOD scores of two-point and multi-point were calculated using Super Link (version 1.6) and Gene hunter (version 2.91). For the LOD calculation, complete penetrance, autosomal recessive inheritance, disease allele frequency of 0.001, no phenocopies, and equal recombination frequencies for both males and females were assumed. Moreover, the reconstruction of haplotypes was done by Haplopainter software (version 029.5) (Fig. 1) [13].

Polymerase chain reaction (PCR) was applied for amplification of the five DFNB4 STR markers. The amplified conditions were as follows: reaction volume of 15 μl with a final concentration of ddH2O 7.5 μl, master mix 5.5 μl, forward primer (20 pmol/μl) 0.5 μl, reverse primer (20 pmol/μl) 0.5 μl, and template genomic DNA (100 ng/μl) 1 μl. The thermal cycle profile for PCR was performed using a thermal cycler (Program Temp Control System PC-700, ASTEC, Fukuoka, Japan) with the protocol as follows: an initial denaturation step of 1 min at 95 °C, followed by 30–35 cycles of 1 min of denaturation at 95 °C, 24 s of annealing for different markers at 51–59 °C, and a 35-s extension at 72 °C; with a final 8-min extension at 72 °C [14]. PCR amplicons were analyzed on a 15% polyacrylamide gel electrophoresis (PAGE) (Fig. 1).

2.5. Mutation analysis of SLC26A4 gene by sequencing analysis

In the two Iranian linked pedigrees with ARNSHL, the sequencing of 21 exons and exon-intron boundaries of SLC26A4 gene was done using specifically designed primers [15]. The amplification conditions were as follows: reaction volume was 15 μl with a final concentration of 7.5 μl, master mix 5.5 μl, forward primer (20 pmol/μl) 0.5 μl, reverse primer (20 pmol/μl) 0.5 μl, and template genomic DNA 1 μl (100 ng/μl). The thermal cycle profile for PCR (Program Temp Control System PC-700, ASTEC, Fukuoka, Japan) was as follows: initial denaturation step of 5-min at 95 °C, followed by 32–33 cycles of 30 s of denaturation at 95 °C, 24 s of annealing for different markers at 51–59 °C, and a 35-s extension at 72 °C; with a final 8-min extension at 72 °C [14]. PCR amplicons were analyzed on a 15% polyacrylamide gel electrophoresis (PAGE) (Fig. 1).

Fig. 3. The haplotype analysis of the pedigree IR-48 with the novel variant c.493A > C was shown and the results of the co-segregation is determined for this variant.

Fig. 4. Pure tone audiometry is shown of the two probands with the two SLC26A4 variants (c.493A > C and c.716T > A).
with a final extension for 5-min at 72 °C. The sequencing was carried out at in SEQLAB (Sequence Laboratories, Gottingen, Germany) (Fig. 2).

2.6. Confirmation of pathogenicity for c.493A > C

The pathogenicity study was based on the co-segregation (the presence of the homozygous variant in patients and its absence or heterozygosity in the non-affected family members) (Fig. 3), absence or heterozygosity of variants in at least 100 hearing ethnically matched controls using PCR-RFLP analysis. The restriction enzyme AcI1 was selected for PCR-RFLP analysis, using NEBCutter (http://tools.neb.com/NEBcutter2/). A 689 bp length fragment of exon 5 containing c.493A > C site was amplified in 100 ethnically matched normal controls and all available members of the IR-48 pedigree.

PCR amplicons were analyzed on a 10% polyacrylamide gel electrophoresis (PAGE). The wild and mutant alleles have one and two AcI1 cutting site, respectively. After digestion, the normal allele had 605 bp and 84 bp, but the mutant allele had 365 bp, 240 bp, and 84 bp fragments. In c.493A > C variant serine (polar without charge), this difference in the load can, to some extent, enhance the pathogenicity of this variant.

3. Results

3.1. The audiological data of SLC26A4 mutation patients

All patients of pedigrees IR-15 and IR-48 had bilateral profound HL (≥ 97 dB) (Fig. 4).

3.2. Radiological information of temporal bone

The enlarged vestibular aqueduct (EVA) was not observed in the patients from the two pedigrees.

3.3. Thyroid status

All of the patients from the two pedigrees had normal TSH, T4, T3 hormone levels, and thyroid size.

3.4. Family data

In the current research, the 40 pedigrees with at least two the ARNSHL patients with negative GJB2 mutation were selected for investigation. All related conditions with ARNSHL were evaluated by physical examination and audiology tests to discriminate ARNSHL from age-related HL. Mainly, the two pedigrees showed linkage to the DFNB4 locus.

3.5. SLINK and linkage analysis results

SLINK value theoretically predicts the LOD score for a pedigree. SLINK, two-point and multi-point LOD scores were 1.33, 1.32, and 1.4 for the pedigree IR-15 and 0.72, 0.73 and 0.72 for the pedigree IR-48, predicting possible linkage to the DFNB4 locus.

3.6. SLC26A4 sequence analysis result

The molecular analysis of the SCL26A4 variants in patients of the linked pedigrees revealed a known pathogenic variant (c.716T > A homozygote) in exon 6 for the pedigree IR-15, and a novel pathogenic variant (c.493A > C homozygote) in exon 5 for the pedigree IR-48. All patients were homozygous and individuals: I.1, I.2, II.1, II.4, and II.5 were heterozygous for variant c.716T > A in the pedigree IR-15. Also, the all patients were homozygous and the individuals: I.1, I.2, and II.3
were heterozygous for variant c.493A > C (p.S165R) in the pedigree IR-48. The c.716T > A (p.V239D) variant had earlier been reported in a Palestinian family as a novel mutation. Interestingly, in the Iranian ARNSHL population, this was detected for the first time during the present investigation. Also, in this study, we have detected five novel variants and the two known variants detected as polymorphisms in these two pedigrees (Table 2). In silico analysis predicted their importance in combination with other pathogenic alleles.

3.7. Mutation confirmation analysis

The c.493A > C variant was not detected in the 100 ethnically matched control individuals, without HL and segregated with the disease in the pedigree. In the c.493A > C variant serine (polar without charge) in this position is replaced by arginine (polar with positive charge), which may cause the defects in folding of the protein. These results suggest that the c.493A > C variant is most likely to be pathogenic. Furthermore, this c.493A > C variant was pathogenic in this study.

4. Discussion

Our data suggests that the SLC26A4 pathogenic variants constitute 4% of the etiology of the ARNSHL in Iran that may be involved in the severity of HL, and the phenotype may be modified by epistatic interactions. In the present research, all the pedigrees were bilateral and all of the patients had profound sensorineural HL.

The linkage analysis and haplotype analysis for the DFNB4 locus revealed linkage in the two pedigrees. The DNA sequencing of SLC26A4 in all affected individuals of these linked pedigrees led to the identification of the five different homozygous variants: (c.493A > C and c.716T > A) were the pathogenic variants and c.416-114A > G, g.28351T > A, and c.1001 + 131G > Ti were the polymorphism variants. The novel variants, (g.862T > A, heterozygote), (c.493A > C, homozygote), (g.13368T > A, heterozygote), (g.28351T > A, heterozygote), (g.28375G > A, heterozygote), and (g.34248G > C, heterozygote) as well as the known variants (g.23034G > T, homozygote), and (g.13416A > G, homozygote) are the polymorphism variants and typically found in ARNSHL.

In this study, we detected a novel variant (c.493A > C) that linked to DFNB4 in an Iranian pedigree. It is in the CDS region of exon 5; the key feature of this variant is its topological domain which is in an extracellular local between the third and fourth transmembrane domains of the pendrin homozygous. In the c.493A > C variant serine (polar without charge) in this position is replaced by arginine (polar with positive charge). Therefore, it is possible for the substitution to detect an effect on the structure, charge and function of the protein. Moreover, running Consurf server (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) determined a rather high conservation score for serine at position 165 (Fig. 5). This is the reason of rather high conservation degree of serine 165 among orthologs. (Polyphen prediction = 1 [-14 < conserves < +6] and PhastCons prediction = 0.04 [0 < conserves < 1] (http://mutationtaster.org). The c.716T > A variant is as a transversion mutation in exon 6 that valine (nonpolar and without charge) and in this position is replaced by aspartic acid (polar with negative charge) and highly conserved (Polyphen prediction = 3.021 and PhastCons prediction = 0.984) (http://mutationtaster.org), is known to cause changes in the fifth and sixth transmembrane domains of the third extracellular loop in pendrin (Fig. 6). Valine in this position is conserved in Mus musculus, Rattus norvegicus and Homo sapiens [16]. According, these variations are in exons and highly conserved, therefore, it is possible for the substitutions to alter the structure and function of the protein and this may enhance the pathogenicity. The cause of HL in the remaining pedigrees does not detect to be related to mutations in SLC26A4 and other unidentified loci may be involved, though environmental factors could not be fully in creation HL. The present research detected the c.716T > A variant in the Iranian ARNSHL population for the first time. It had previously been reported as a novel mutation in a Palestinian family [17].

The c.716T > A pathogenic variant was found in 16 Pakistani families and haplotype analyses suggested that it is a founder variant. The families with the c.716T > A variant had an inherited autosomal recessive early-onset, sensorineural HL [18]. The c.716T > A variant in the pendrin protein is associated with a significant decrease in the transportation activity. Moreover, it is assumed that the residual function of pendrin with c.716T > A could be improved by an activator of pendrin [19].

Studies reported folding-defective pendrin protein (with c.716T > A) is retained in the endoplasmic reticulum [17]. Additionally, this functional malfunction may be caused by unidentified factors, proteins and modifier genes [20]. The frequency of this variant varies worldwide, from 5% in the Middle East and South Asia to 0% in Africa [21]. The assumed founder effect of the c.716T > A variant is in the Middle East (30% of the mutant alleles of SLC26A4 are in Pakistan) [18]. However, future investigations are required to confirm this hypothesis. In the present study, the frequency of this mutation in the
Iranian pedigrees with ARNSHL was 2%. Different studies have detected at least 200 variants in the SLC26A4 gene and this gene is known as the second most common cause of ARNSHL in the world [21].

5. Conclusion

Despite many studies conducted on the role of SLC26A4 in HI, the spectrum and frequency of its different mutations remain to be accurately exposed for the different ethnic groups. This is probably due to the allelic heterogeneity, the large size of SLC26A4, racial diversity, and the large number of known and unknown mutations occurring across this gene. However, these studies help in clinical decision-making, genetic counseling, and a suitable treatment strategy for ARNSHL patients.

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Conflicts of interest

The authors have declared that no competing interests exist. All authors have approved this manuscript.

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References