Screening of 10 DFNB Loci Causing Autosomal Recessive Non-Syndromic Hearing Loss in Two Iranian Populations Negative for GJB2 Mutations

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Abstract

Background: Autosomal recessive non-syndromic hearing loss (ARNSHL), one of the global public health concerns, is marked by a high degree of genetic heterogeneity. The role of GJB2, as the most common cause of ARNSHL, is only <20% in the Iranian population. Here, we aimed to determine the relative contribution of several apparently most common loci in a cohort of ARNSHL Iranian families that were negative for the GJB2 mutations.

Methods: Totally, 80 Iranian ARNSHL families with 3 or more affected individuals from Isfahan and Hamadan provinces, Iran, were enrolled in 2017. After excluding mutations in the GJB2 gene via Sanger sequencing, 60 negative samples (30 families from each province) were analyzed using homozygosity mapping for 10 ARNSHL loci.

Results: Fourteen families were found to be linked to five different known loci, including DFNB4 (5 families), DFNB2 (3 families), DFNB7/11 (1 family), DFNB9 (2 families) and DFNB3 (3 families).

Conclusion: Despite the high heterogeneity of ARNSHL, the genetic causes were determined in 23.5% of the studied families using homozygosity mapping. This data gives an overview of the ARNSHL etiology in the center and west of Iran, used to establish a diagnostic gene panel including most common loci for hearing loss diagnostics.

Keywords: Autosomal recessive non-syndromic hearing loss (ARNSHL); DFNB loci; Homozygosity mapping; Iran

Introduction

Hearing loss (HL) is the most frequent form of sensory impairment in humans, with approximately 1-2 in 1000 infants being born with a serious HL (https://www.gov.uk/guidance/newborn-hearing-screening-programme-overview). Over 70% of pre-lingual HL cases can be classified as non-
syndromic HL (NSHL), where the hearing deficit is the only sign (1). HL can be inherited as autosomal recessive, autosomal dominant, mitochondrial, X- and Y-linked. Autosomal recessive mode of inheritance (ARNSHL) makes up 80% of the NSHL cases. ARNSHL is highly heterogeneous, for which over 100 mapped loci are known. The autosomal recessive loci are called DFNB followed by a number corresponding to the order that the locus was first explained; DFNB1 to DFNB105 have been reported so far (http://hereditaryhearingloss.org). However, more than seventy different DFNB loci have been mapped for ARNSHL by homozygosity mapping, an effective method to locate genes causing ARNSHL in large consanguineous families (2). The genetic etiology of ARNSHL in Iran has been studied by a number of independent studies with a special focus on the certain locus DFNB1 (GJB2), as the most common cause of HL all over the world (3). Variants in the GJB2 (NM_004004.5) could explain the etiology of ARNSHL in 4% to 35% of patients from different origin, suggesting that GJB2 gene mutations account for a part of ARNSHL in Iran. (4, 5). On the other hand, Iran is a large country with many ethnic groups and high rate of the consanguineous marriages (38.6% on average) (6). Thus, mutations in certain loci may be increased within some ethnic groups (7). Therefore, population-specific studies are necessary to identify other common loci and to determine the etiology of ARNSHL based on ethnicity. Until now, little data is available on the contribution of some frequent loci among the Iranian ARNSHL; and have mostly focused on mixed populations from different provinces (8). Mutations in at least 40 genes have been explained to cause ARNSHL in Iran. However, the contribution of their mutations do not appear to occur at the same frequencies across different ethnic groups (9).

We applied the homozygosity mapping strategy to identify the relative contribution of 10 DFNB loci to ARNSHL in the studied cohort including 60 ARNSHL families, which were negative for GJB2 mutations, from Isfahan and Hamedan provinces for the first time. This data could be applied to design a cheap and accurate panel for common genes causing ARNSHL in certain regions of Iran and would lead to affordable testing and improved management of HL (10).

Materials and Methods

Families and clinical evaluations
Totally, 60 unrelated consanguineous families with 3 or more hearing impaired patients and negative for GJB2 mutations were recruited from Isfahan and Hamedan provinces in the center and west of Iran in 2017. The Ethics Committee of the Isfahan University of Medical Sciences approved this project. All family members signed informed written consent prior to recruitment. They met the following criteria: 1) confirmation of HL by Pure tone audiology (PTA) from 250-8000 Hz 2) the autosomal recessive inheritance deduced through pedigree analysis 3) existence of three or more affected members within the pedigree. A complete clinical investigation was performed to exclude environmental exposures and to determine the presence of syndromic findings in each family.

DNA extraction
Genomic DNA was extracted using Prime Prep Genomic DNA Extraction kit from blood (GeNet Bio, Korea) according to the manufacturer’s instruction. Qualitative and quantitative assessment of genomic DNA was checked using 1.2% agarose gel and Nanospec cube biophotometer (Nanolytik®, Dusseldorf, Germany).

SLINK analysis and selection of DFNB loci
Power of the pedigrees for linkage analysis was simulated by calculating SLINK, using FastSLink (ver.2.51) option of Easy linkage plus version 5.05 software to predict the potential LOD score in a given family (11). Based on the literature review of the most frequent loci, both globally and regionally, 10 loci were selected for screening. Screening sort tandem repeat (STR) markers were selected based on their physical distance found at NCBI UniSTS and NCBI Map Viewer.
Primers for STR markers of each locus were mostly obtained through the Probe database (https://www.ncbi.nlm.nih.gov/probe). STR markers of each locus and their primer sequences are listed in Table 1.

### Table 1: The list of 10 DFNB loci screened in this study. The corresponding genes and characteristic of screening markers are shown

| Locus (gene) | STR | Heterozygosity (%) | Size (bp) | Forward primer | Reverse primer |
|--------------|-----|-------------------|----------|----------------|----------------|
| DFNB7-11 (TMC1) | D9S1876 | 81 | 132-152 | GATGTACCCAGAAGATCTCG | AGTGTTACCCATTACCCAG |
| D9S1214 | 76 | 252-276 | GGTGCCACACATACACTACT | TCTAATCTCTCTTCCCTTG |
| D9S301 | 78 | 205-251 | CATGATGTTGTTCTTGG | GGTGGGCTCMAAGGAGTAC |
| D9S1799 | 91 | 139-178 | TTGACACATAATTTTGG | TGCAGTTTCAATCCACAT |
| DFNB3 (MYO15-A) | D17S953 | 81 | 119-131 | ACTATCCGCCCCCATACA | AAGGGCTGCTTGGAG |
| D17S1843 | 70 | 177-187 | TGCACAGGCCCATCCCTAC | TGCCTAAACTGCTTTCAGGT |
| D17S620 | 50 | 103-151 | CAGAAAGATGCTGAGGGGT | GGGCAGTTTACAGGAGTAG |
| D17S2196 | 81 | 139-256 | CACATTGTTGGCAGCCCAT | ATATTCTAATTTGGGAG |
| DFNB2 (MYO7-A) | D11S4179 | 72 | 140-200 | GGATGTAAGAGTAACTGGCTCCG | GAAAATGTTCTGCTTGAG |
| D11S4186 | 79 | 154-195 | AGTATTTGTGTACCTTATGTTATGTT | TCTGGCGTTTCTGCTTGAG |
| D11S4079 | 75 | 217-265 | CAGCAAGATCCTGTCTCAA | CTGCTTTAAGTGGGGAGT |
| D11S911 | 85 | 159-203 | TCTCTCATGCTTGACCATTT | CTATGGTGAGATGAGT |
| DFNB4 (SLC26-A1) | D7S2420 | 81 | 240-290 | CTTATGTTGTTCCTTATGTTATGTT | TCAATGGGAAATCTPACTT |
| D7S496 | 63 | 129-141 | AACAACAGTCAACCCACAAAT | GCCCCCTTAGCTAAAGAGCC |
| D7S2459 | 77 | 140-152 | ATTTCTCCCAATCTATCGCTC | CAGCGTTTCTGCTTGAG |
| D7S2456 | 78 | 238-252 | ATCTCATGGCTTGCACATTTT | CTGGTTAACATCGGACAT |
| DFNB9 (OTOF) | D2S365 | 85 | 164-200 | ATGATTTGTGTACCTTATGTTATGTT | TCAATGGGAAATCTPACTT |
| D2S2447 | 78 | 130-160 | TCCATCTTTGGTGTGC | GGTGGCCTTACAGT |
| D2S3674 | 65 | 203-221 | AGGCTGAATCCCACCTCC | TTAGAGCACACATGGTCA |
| D2S2223 | 63 | 182-200 | CAGCGCTTTAGCTACATTTT | ATCTGGTGAGATGAGT |
| DFNB21 (TECT-A) | D11S1774 | 60 | 206-226 | CAAAAGGCTTGGGCGTTT | GGGCTATTCCCATGGTA |
| D11S1295 | 85 | 173-195 | AGAACCAGGCTAGTAACTCTCG | TTAATGCAATTTGGACCAT |
| D11S4089 | 75 | 199-213 | TAAATCAATGGCTAGTTTATGG | ATCTGGCTTACATTTGGAA |
| D11S1407 | 70 | 172-212 | TACATCTAAGACAGTACATTAC | ATCTGGCTTACATTTGGAA |
| DFNB53 (CO11A2) | D13S1226 | 70 | 108-132 | GCACCTTGCTGCTGGTAA | AAGGGGCTGCTCTTCT |
| D13S1875 | 75 | 101-113 | TATTTGCTATCGTCATGCTG | TGCATCTCACAATATTG |
| DFN189 (PIF-K) | D2S2173 | 70 | 201-243 | GGAGACAGAGAGCTTTACATTTGAG | GCCACACTTTCCGATAC |
| D2S324 | 85 | 264-275 | TACACCGGCGGACGT | CAGCAATGCTTACAGG |
| DFN863 (LRTOMT) | D11S1314 | 78 | 209-227 | TCTGCTTACCTCCTTACT | GTGAAGGCGAAGATTGTAC |
| D11S1462 | 75 | 263-269 | GTCCTCAAGAGACAGACAC | GAGAGGCAACCATATTG |
| D11S1440 | 72 | 189-199 | TGGGCAAGTTCTCACAAT | CTTAGGTTGGAGGACAC |
| D11S1484 | 60 | 263-277 | CACACCTTATAAGT | GCATGTAAGGAGGAGT |
| DFN284 (RDX) | D11S1793 | 85 | 124-140 | AGTATGCTCATTCTCCTGTA | ATCTGGTACACATTTCT |
| D11S1391 | 75 | 158-178 | TCGATGCTACACACTACATACACTACA | CATCCACCTCTGCTTGCTG |
| D11S2017 | 70 | 109-133 | TTTTAGATGAAAGGTATGAGT | TTTGATAGGAAATGGAAGT |
| D11S1893 | 58 | 206-258 | TCTCTGGACACCTTGAT | TGAATGAGGCTCCTGAGGCTG |
**Genotyping STR markers and Linkage Analysis**

PCR of STR markers was conducted according to the standard procedure. The touchdown program was used for markers amplification. Thermal cycling conditions for amplifying markers were in accordance with previous protocols (4). PCR conditions were as follows: 2 μl MgCl₂ (4 mM), 2.5 μl Taq PCR buffer (10X), 0.12 μl of each of the primers (10 PM), 0.15 μl Taq DNA polymerase (5U/ul), 0.9 μl dNTP mix (10 mM) and 1.2 μl DNA (about 70 ng). The reaction was adjusted to the volume of 25 μl by ddH₂O.

Standard cycling was done in a thermocycler (ASTEC PC-818; ASTEC, Fukouka, Japan). STR markers were selected based on their physical distance found at NCBI UniSTS. The criteria for selecting these markers are as follows: greater heterogeneity values, and lying near or at the known loci. At least 4 STR markers were selected for linkage analysis. Table 1 summarizes the general characteristics of the markers used in the study. PCR products were loaded on 12% polyacrylamide gel, followed by silver nitrate staining. Two-point and multipoint parametric LOD scores were calculated using Superlink (ver. 1.6) and GeneHunter (ver. 2.91), respectively. Haplopainter version 029.5 software package was used for reconstruction of haplotypes (12).

**Results**

**Families and clinical data**

After excluding mutations in the GJB2 gene, 60 Iranian families segregating (ARNSHL), from Isfahan and Hamedan provinces (30 families from each province) in the center and west of Iran, were screened for 10 ARNSHL loci. Totally, 634 individuals were studied 245 of whom were patients, with ages ranging from 6 months to 52 yr. For 45 families, PTA was consistent with profound HL (≥80 dB), 12 families showed severe HL (61-80 dB), whereas the 3 remaining families showed moderate HL (41-60 dB).

**SLINK calculation, genotyping and linkage analysis**

Totally, 9 families were of SLINK values ≥3.2, 24 families had SLINK values of 2.5-3.2. The rest of the families presented values 1.8-2.5. Screening loci for homozygosity mapping in this study were composed of: DFNB2 (MY07.A), DFNB3 (MY015.A), DFNB4 (SLC26.A4), DFNB7/11 (TMC1), DFNB9 (OTOF), DFNB21 (TECTA), DFNB24 (RDX), DFNB59 (PJVK), DFNB63 (LRTOMT) and DFNB53 (COL11.A2). The family members were individually genotyped for these markers. After genotyping of STR markers and linkage analysis, 14 out of the 60 families, negative for GJB2 mutations, showed linkage to five different loci (Table 2). DFNB4 was the most frequent locus in the studied ARNSHL series in both provinces (36.9% of the etiology). Three out of 30 (10%) and 2 of 30 (6.6%) families were linked to DFNB4 in Isfahan and Hamedan provinces, respectively. DFNB2, DFNB3, and DFNB9 were ranked next after DFNB4. Table 3 shows the linked families and the maximum values for SLINK, two-point and multipoint LOD scores. One family (3.3%) from every single province was linked to DFNB9. One family from Hamedan Province was linked to DFNB7/11 (7.7% of the etiology). The haplotypes of 3 selected linked families are shown in Fig. 1(a-c).

We did not find linkage to the other loci (DFNB21, DFNB24, DFNB59, DFNB63 and FNB53) among the studied families.

**Discussion**

We determined the contribution of 10 DFNB loci to HL in 60 Iranian families affected with ARNSHL and negative for the GJB2 mutations. We have obtained 20% and 26% involvement of GJB2 mutations in ARNSHL in Hamedan and Isfahan provinces, respectively (unpublished data).
Fig. 1: Pedigree and haplotypes of the 3 Iranian families with ARNSHL, negative for GJB2 mutations, linked to 2 known loci. a) ISF-5 is linked to DFNB4: b) IR-3 is linked to DFNB3 c) IR-14 is linked to DFNB4. Hearing impaired patients show homozygosity for markers. The order of markers is based on the Marshfield map.
Table 2: Genetic etiology of ARNSHL in 60 families negative for GJB2 mutations from Isfahan and Hamedan provinces.

| Number | Locus  | Gene     | Number of diagnoses | % of diagnoses | % of cohort |
|--------|--------|----------|---------------------|----------------|------------|
|        |        |          | Isfahan  | Hamedan | Isfahan | Hamedan | Isfahan | Hamedan |
| 1      | DFNB2  | MYO7A    | 1        | 2       | 7.2     | 15.4     | 3.3       | 6.6       |
| 2      | DFNB3  | MYO15A   | 1        | 2       | 7.2     | 15.4     | 3.3       | 6.6       |
| 3      | DFNB4  | SLC26A4  | 3        | 2       | 21.5    | 15.4     | 10        | 6.6       |
| 4      | DFNB7-11| TMC1    | 0        | 1       | 0       | 7.7      | 0         | 3.3       |
| 5      | DFNB9  | OTOF     | 1        | 1       | 7.2     | 7.2      | 3.3       | 3.3       |
| Total  |        |          | 6        | 8       | 19.9    | 26.4     |           |           |

Table 3: Maximum SLINK and LOD score (two-point and multi-point) values for the linked families.

| Number | Family ID | SLINK value | Linked locus | Two-point LOD score | Multi-point LOD score | Severity HL |
|--------|-----------|-------------|--------------|---------------------|-----------------------|-------------|
| 1      | ISF-5     | 2.82        | DFNB4        | 1.7                 | 2.2                   | Severe-profound |
| 2      | ISF-15    | 2.9         | DFNB4        | 2.6                 | 2.8                   | Moderate-profound |
| 3      | ISF-6     | 3.8         | DFNB4        | 3.1                 | 3.2                   | Moderate-severe |
| 4      | IR-14     | 3.28        | DFNB4        | 2.4                 | 2.6                   | Profound    |
| 5      | IR-9      | 1.8         | DFNB4        | 2.2                 | 2.3                   | Severe-profound |
| 6      | IR-13     | 2.53        | DFNB2        | 1.9                 | 2.3                   | Severe-profound |
| 7      | IR-30     | 2.8         | DFNB2        | 2.3                 | 2.6                   | Moderate-profound |
| 8      | ISF-17    | 1.9         | DFNB2        | 2                   | 2.2                   | Profound    |
| 9      | IR-19     | 3.4         | DFNB9        | 2.8                 | 3.9                   | Severe-profound |
| 10     | ISF-14    | 2.8         | DFNB9        | 1.9                 | 2.3                   | Profound    |
| 11     | IR-3      | 2.3         | DFNB3        | 2.1                 | 2.4                   | Profound    |
| 12     | IR-27     | 3.1         | DFNB3        | 2.9                 | 3.1                   | Profound    |
| 13     | ISF-23    | 2.4         | DFNB3        | 2.1                 | 2.3                   | Severe-profound |
| 14     | IR-7      | 2.1         | DFNB7-11     | 2                   | 2.2                   | Severe-profound |

For the first time, we investigated the role of 10 other loci in the etiology of HL in GJB2 negative families in the west and center of Iran. DFNB4 was found to be the mostly linked locus among the studied families, with 3 out of 30 (10%) and 2 of 30 (6.6%) showing linkage to it in Isfahan and Hamedan provinces, respectively. Mutations in its corresponding gene (i.e. SLC26A4) are the second most common cause of ARNSHL, after GJB2, all over the world (13). About 5% of ARNSHL cases in South Asia have been related to SLC26A4 mutations (14). Variants in this gene can cause both ARNSHL at the DFNB4 locus and Pendred Syndrome (PS), known as one of the most common forms of syndromic HL. PS is correlated to hypothyroidism (goiter) later in life. Present data show no sign of PS in the studied families. Several reports have revealed SLC26A4 mutations in ARNSHL in Iranian populations. In a study, 12 families out of 80 (15%) Iranian families with 2 or more ARNSHL patients were linked to DFNB4 locus (15). In a recent study 12 out of 121 (9.9%) families were linked to DFNB4 (16). Thus, our result shows that DFNB4 contributes mainly to ARNSHL in the west and center of Iran and is ranked second after DFNB1, which is in agreement with previous studies reported from Iran (Table 4). The next most frequently linked locus in our cohort was DFNB2, which accounts for about 6.6% of HL etiology in Hamedan and 3.3% in Isfahan. The related gene MYO7A, encoding myosin VIIA, is an action-based molecular motor converting energy from ATP hydrolysis into mechanical force. It also interacts with actin to participate in the formation of the mechatransduction complex, which is critical for detecting sound (17).
### Table 4: The overview of DFNB loci frequencies obtained from in the studies on the Iranian ARNSHL patients

| Loci    | Number of cases | Number of affected | Frequency (%) | References               |
|---------|-----------------|--------------------|---------------|--------------------------|
| DFNB2   | 144             | 4                  | 2.77          | Babanejad et al.         |
|         | 302             | 15                 | 4.96          | Heggen et al.            |
|         | 60              | 3                  | 5             | This study               |
| DFNB3   | 144             | 8                  | 5.5           | Babanejad et al.         |
|         | 302             | 29                 | 9.6           | Heggen et al.            |
|         | 60              | 3                  | 5             | This study               |
| DFNB4   | 80              | 12                 | 15            | Kahrizi et al.           |
|         | 302             | 37                 | 12.25         | Heggen et al.            |
|         | 60              | 5                  | 8.3           | This study               |
| DFNB7-11| 144             | 4                  | 2.7           | Babanejad et al.         |
|         | 54              | 1                  | 2.2           | Dahaghani et al.         |
|         | 60              | 1                  | 1.66          | This study               |
| DFNB9   | 144             | 1                  | 0.69          | Babanejad et al.         |
|         | 38              | 1                  | 2.38          | Mahdieh et al.           |
|         | 60              | 2                  | 3.3           | This study               |

DFNB2-linked families have been reported from Iran (18). 2.8% of DFNB2 involvement in ARNSHL etiology were reported in the studied Iranian cohort (19). Similarly, Sloan-Heggen et al. (20) using a custom targeted genomic enrichment method in a cohort of 302 GJB2-negative Iranian families, found the DFNB2 contribution to be 5%, which is in accordance with our results (Table 4).

Our data show that one out of 30 families (3.3%) from Isfahan province and two out of 30 families (6.6%) from Hamedan were linked to DFNB3. The DFNB3 locus was first identified in the village of Indoesia, Bengkala, with a frequency of 9.4% among the inbred population (21). Since then, many mutations have been reported from different countries such as India, Iran, Turkey, and Brazil (22, 23). In Pakistan, DFNB3 is the third locus for ARNSHL accounting for 5% of ARNSHL (24). In a previous study on 40 Iranian ARNSHL families from Qom and Markazi provinces, from center of Iran, 2 families were linked to DFNB3 (5.8%) (25). Therefore, the locus could be one common cause of ARNSHL both in the west and center of Iran.

In the present study, one family (3.3%) from every single province was linked to DFNB9 (OTOF). Mutations in the OTOF gene (named as homology to Ferlin (Fer-1)) encoding otoferlin at DFNB9 results in ARNSHL which is sometimes associated with auditory neuropathy. Otoferlin is a member of the ferlin protein family; its role is in vesicle recycling and efficient and linear encoding of low-intensity stimulate the synapse between inner hair cells and auditory nerve fibers (26). OTOF mutations have been reported from many countries such as Pakistan, Spain, Italy, and Japan with different mutation spectrum (26, 27). The frequency of the OTOF gene mutations has been reported to be 2.7% in Iran (28). It was screened 37 Iranian ARNSHL families from 7 different provinces for 15 loci using linkage analysis and found 1 family (2.7%) to be linked to the DFNB9 locus. Similarly, in our present study, one family (3.3%) from each of the two provinces was linked to DFNB9 (OTOF). Our study involves 3 families with moderate to severe HL highlighted by a "U" shaped audiogram. DFNB21 and DFNB93 are related to this audio profile (18, 29). Interestingly, none of the 3 families was linked to DFNB21. Thus, the next step for these families involves investigating the DFNB93 contribution (30).

The contribution of DFNB7/11 in Hamedan Province was found to be 3.3% (1/30), in line with the prevalence figures of 4% in Pakistan,
and 3.4% in Turkey. The related gene TMC1 (Transmembrane channel-like gene I) is required for postnatal hair cell development. TMC1 might be an ion channel or transporter which mediates K⁺ homeostasis in the inner ear (31). The TMC1 gene was initially mapped to chromosome 9q13–q21 in two consanguineous Indian families with prelingual, severe-to-profound defining the DFNB7/11 locus (NM_138691.2). In the study on 144 GJB2-negative subjects, using linkage analysis and direct sequencing, 4 out of 144 families (2.7%) were linked to DFNB7-11 (19). Moreover, Dehaghani et al. (32), using homozygosity mapping on 45 ARNSHL families, detected 1 family (2.2%) to be linked to DFNB7/11. Therefore, the locus could be one common cause of ARNSHL in the west of Iran.

The next step of the study involves DNA sequencing of the corresponding genes of the DFNB loci in the linked families to identify the pathogenic mutations. Until now, 4 homozygous mutations have been identified in the related families, including 3 missense and 1 splice site mutations (7, 33). The identified mutations and their characteristics are listed in Table 5. The large size of some of the related genes hinders detection of their related mutations (34). However, the remaining families have been considered for sequencing of the related genes.

In the present study, despite the high heterogeneity of ARNSHL, we could detect the genetic etiology in 6 out of 30 (20%) and 8 out of 30 (26.6%) ARNSHL families, negative for GJB2 mutations, from Isfahan and Hamedan provinces, respectively. Thus, nearly 23.5% of ARNSHL families negative for GJB2 mutations are linked to 5 loci including DFNB4, DFNB2, DFNB3, DFNB9 and DFNB7/11. The remaining genes and loci could be either rare or yet to be identified (2, 28). The study is in progress by subjecting some of the remaining families to the next-generation sequencing, which is a rapid and cost-effect method.

### Table 5: The mutations detected in the linked families in this study

| Number | Family ID | Gene   | Type of mutation | Nucleotide change | Amino acid change |
|--------|-----------|--------|------------------|-------------------|------------------|
| 1      | IR-14     | SLC26A4| missense         | c.416 G>T         | p.Gly139Val      |
| 2      | ISF-5     | SLC26A4| Splice site      | c.919-2 A>G       | -                |
| 3      | IR-27     | MYO15A | missense         | c.6442 T>A        | p.Trp2148Arg     |
| 4      | IR-13     | MYO7A  | missense         | c.6487 G>A        | p.Gly2163Ser     |

### Conclusion

Using homozygosity mapping, we detected 23.5% of the genetic etiology of GJB2 mutation negative ARNSHL in Isfahan and Hamedan provinces. This finding is interesting in view of the extreme genetic heterogeneity of ARNSHL (with over 50 genes discovered so far). Investigation of a limited number of genes could lead to an acceptable level of diagnostic yield.

### Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

References

1. Norouzi V, Azizi H, Fattahi Z, Esteghamat F et al (2011). Did the GJB2 35delG mutation originate in Iran? *Am J Med Genet Part A*, 155:2453-2458.
2. Masoudi M, Ahangari N, Zonouzi AAP et al (2016). Genetic Linkage Analysis of DFNB3, DFNB9 and DFNB21 Loci in GJB2 Negative Families with Autosomal Recessive Non-syndromic Hearing Loss. *Iran J Public Health*, 45:680-7.
3. Sanaz Arzhangi B, Kevin Booth B, Ahmad Daneshi M et al (2016). Heterogeneity of Hereditary Hearing Loss in Iran: a Comprehensive Review. *Arch Iran Med*, 19:720-728.
4. Chaleshtori MH, Zohour MM, Rad LH et al (2006). Autosomal recessive and sporadic non-syndromic hearing loss and the incidence of Cx26 mutations in a Province of Iran. *Iran J Public Health*, 35:88-91.
5. Azadegan-Dehkordi F, Bahrami T, Shirzad M et al (2019). Mutations in GJB2 as Major Causes of Autosomal Recessive Non-Syndromic Hearing Loss: First Report of c. 299-300delAT Mutation in Kurdish Population of Iran. *J Audiol Otol*, 23(1):20-26.
6. Saadat M (2005). Epidemiology and mortality of hospitalized burn patients in Kohgiluyeh va Boyerahmad province (Iran): 2002–2004. *Burns*, 31:306-309.
7. koohiyan M, Ahmedi A, koohian F et al (2019). An update of spectrum and frequency of GJB2 mutations causing hearing loss in the south of Iran: a literature review. *Int J Pediatr Otorhinolaryngol*, 119:136-140.
8. Koohiyan M, Chaleshtori MH, Salehi M et al (2018). GJB2 mutations causing autosomal recessive non-syndromic hearing loss (ARNSHL) in two Iranian populations: Report of two novel variants. *Int J Pediatr Otorhinolaryngol*, 107:121-126.
9. Ghasemnejad T, Khaniani MS, Zarei F et al (2017). An update of common autosomal recessive non-syndromic hearing loss genes in Iranian population. *Int J Pediatr Otorhinolaryngol*, 97: 113-126.
10. Yan D, Xiang G, Chai X, Qing J et al (2017). Screening of deafness-causing DNA variants that are common in patients of European ancestry using a microarray-based approach. *PloS one*, 12:e0169219.
11. Lindner TH, Hoffmann K (2005). easyLINKAGE: a PERL script for easy and automated two-/multi-point linkage analyses. *Bioinformatics*, 21:405-407.
12. Fishelson M, Geiger D (2004). Optimizing exact genetic linkage computations. *J Comput Biol*, 11:263-275.
13. Koohiyan M (2019). A systematic review of SLC26A4 mutations causing hearing loss in the Iranian population. *Int J Pediatr Otorhinolaryngol*, 125:1-5.
14. Wu C-C, Lu Y-C, Chen P-J et al (2010). PhenoTypic analyses and mutation screening of the SLC26A4 and FOXI1 genes in 101 Taiwanese families with bilateral nonsyndromic enlarged vestibular aqueduct (DFNB4) or Pendred syndrome. *Audiol Neurotol*, 15:57-66.
15. Kahrizi K, Mohseni M, Nishimura C et al (2009). Identification of SLC26A4 gene mutations in Iranian families with hereditary hearing impairment. *Eur J Pediatr*, 168:651-3.
16. Yazdanpanahi N, Tabatabaiefar MA, Farrokhii E et al (2013). Compound heterozygosity for two novel SLC26A4 mutations in a large Iranian pedigree with Pendred syndrome. *Clin Exp Otorhinolaryngol*, 6:201-208.
17. Hilgert N, Kahrizi K, Dietijens N et al (2009). A large deletion in GPR98 causes type IIC Usher syndrome in male and female members of an Iranian family. *J Med Genet*, 46:272-276.
18. Tabatabaiefar M, Alasti F, Shariati L, Farrokhii E (2011). DFNB93, a novel locus for autosomal recessive moderate-to-severe hearing impairment. *Clin Genet*, 79:594-598.
19. Babanejad M, Fattahi Z, Bazazzadegan N et al (2012). A comprehensive study to determine heterogeneity of autosomal recessive...
nonsyndromic hearing loss in Iran. *Am J Med Genet Part A*, 158:2485-2492.
20. Sloan-Heggen CM, Babanejad M, Beheshtian M et al (2015). Characterising the spectrum of autosomal recessive hereditary hearing loss in Iran. *J Med Genet*, 52:823-829.
21. Friedman TB, Liang Y, Weber JL et al (1995). A gene for congenital, recessive deafness DFNB3 maps to the pericentromeric region of chromosome 17. *Nat genet*, 9:86-91.
22. Cengiz FB, Duman D, Sarmae A et al (2010). Recurrent and private MYO15A mutations are associated with deafness in the Turkish population. *Genet Test Mol Biomarkers*, 14:543-550.
23. Zarepour N, Koohiyan M, Taghipour-Sheshdeh A et al (2019). Identification and Clinical Implications of a Novel MYO15A Variant in a Consanguineous Iranian Family by Targeted Exome Sequencing. *Audiol Neurootol*, 24(1):25-31.
24. Bashir R, Fatima A, Naz S (2012). Prioritized sequencing of the second exon of MYO15A reveals a new mutation segregating in a Pakistani family with moderate to severe hearing loss. *Eur J Med Genet*, 55:99-102.
25. Sadeghi A, Sanati MH, Alasti F et al (2009). Contribution of GJB2 mutations and Four common DFNB loci in autosomal recessive non-syndromic hearing impairment in Markazi and Qom provinces of Iran. *Iran J of Biotechnol*, 7:108-111.
26. Choi BY, Ahmed ZM, Riazuddin S et al (2009). Identities and frequencies of mutations of the otoferlin gene (OTOF) causing DFNB9 deafness in Pakistan. *Clin genet*, 75:237-243.
27. Chiu Y-H, Wu C-C, Lu Y-C et al (2010). Mutations in the OTOF gene in Taiwanese patients with auditory neuropathy. *Audiol Neurootol*, 15:364-374.
28. Tabatabaiefar M, Alasti F, Zohour MM et al (2011). Genetic linkage analysis of 15 DFNB loci in a group of Iranian families with autosomal recessive hearing loss. *Iran J Public Health*, 40:34-48.
29. Khosrofam, Pourreza MR, Asgharzadeh S et al (2017). Genetic Linkage Analysis of the DFNB21 Locus in Autosomal Recessive Hearing Loss in Large Families from Khuzestan Province. *Arak Medical University Journal*, 20:31-38.
30. Schrauwen I, Helfmann S, Inagaki A et al (2012). A mutation in CYP2B2, expressed in cochlear hair cells, causes autosomal-recessive hearing impairment. *Am J Med Genet*, 91:636-645.
31. Kawashima Y, Géléoc GS, Kurima K et al (2011). Mechanotransduction in mouse inner ear hair cells requires transmembrane channel–like genes. *J Clin Invest*, 121:4796-809.
32. Davoudi-Dehaghani E, Zeinali S, Mahdieh N et al (2013). A transversion mutation in non-coding exon 3 of the TMC1 gene in two ethnically related Iranian deaf families from different geographical regions; evidence for founder effect. *Int J Pediatr Otorhinolaryngol*, 77:821-826.
33. Reisi S, Sanati MH, Tabatabaiefar MA et al (2014). The study of SLC26A4 gene causing autosomal recessive hearing loss by linkage analysis in a Cohort of Iranian Populations. *Int J Mol Cell Med*, 3:176-82.
34. Practice P, Committee G (2014). American College of Medical Genetics and Genomics guideline for the clinical evaluation and etiologic diagnosis of hearing loss. *Genet Med*, 16:347-355.

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