



## Genetic Linkage Analysis of 15 DFNB Loci in a Group of Iranian Families with Autosomal Recessive Hearing Loss

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### Abstract

**Background:** Hearing loss (HL) is the most frequent sensory birth defect in humans. Autosomal recessive non-syndromic HL (ARNSHL) is the most common type of hereditary HL. It is extremely heterogeneous and over 70 loci (known as DFNB) have been identified. This study was launched to determine the relative contribution of more frequent loci in a cohort of ARNSHL families.

**Methods:** Thirty-seven Iranian families including 36 ARNSHL families and 1 family with Pendred syndrome each with  $\geq 4$  affected individuals, from seven provinces of Iran, were ascertained. DFNB1 contribution was initially studied by DNA sequencing of *GJB2* and linkage analysis using the relative STR markers. The excluded families were then subjected to homozygosity mapping for fifteen ARNSHL loci.

**Results:** Sixteen families were found to be linked to seven different known loci, including DFNB1 (6 families), DFNB4 (3 families + 1 family with Pendred syndrome), DFNB63 (2 families), DFNB2 (1 family), DFNB7/11 (1 family), DFNB9 (1 family) and DFNB21 (1 family). DNA sequencing of the corresponding genes is in progress to identify the pathogenic mutations.

**Conclusion:** The genetic causes were clarified in 43.2% of the studied families, giving an overview of the causes of ARNSHL in Iran. DFNB4 is ranked second after DFNB1 in the studied cohort. More genetic and epigenetic investigations will have to be done to reveal the causes in the remaining families.

**Keyword:** ARNSHL, Genetic linkage analysis, DFNB loci, Iran

### Introduction

Hearing loss (HL) is the most common sensory deficit in humans with an incidence of about one in 650 newborns. The prevalence continues to increase during childhood and reaches a rate of 2.7 per 1000 children before the age of five years and 3.5 per 1000 during adolescence. HL is a major public health concern in developing countries. Two thirds of the people who have HL worldwide live

in developing countries (1). A severe HL in early childhood prevents from proper speech acquisition and subsequent literacy and its later onset would negatively affect the quality of life (2). As sanitary indexes are improved, the figure would change in favor of the role of genetics (3, 4). HL can be classified by different criteria including severity (mild: 20 to 39 dB, moderate: 40 to 69 dB, severe: 70 to 89 dB, or profound:  $\geq 90$  dB), age of onset

(pre-lingual or post-lingual), origin (conductive, sensorineural or mixed) and presence or absence of associated features (syndromic or non-syndromic) (5).

It is estimated that at least 50% of pre-lingual HL has a genetic basis. The etiology for another 25% remains unclear, suggesting an additional role for genetics. Approximately 70% of genetic HL cases are non-syndromic (NSHL), where no other anomaly exists, whereas the remaining 30% are syndromic. Up to now, over 400 syndromic forms have been described; Usher syndrome and Pendred syndrome are the most common examples (6). Whilst in NSHL, autosomal-dominant (ADNSHL) comprises ~20% and only a minority of the causes include X-linked (~1%) and mitochondrial (<1%) forms, Autosomal-recessive forms (ARNSHL) encompasses ~ 80% of cases (3, 4). Notably, the frequency of ARNSHL becomes even higher in countries with high rate of consanguineous marriage (3). ARNSHL forms are usually pre-lingual and more severe in degree and are almost exclusively sensorineural (4, 7). The loci for ARNSHL, ADNSHL and X-linked HL are represented by DFNB, DFNA and DFN, respectively (4). Up to 1% of human genes are estimated to be involved in hearing process. Over 130 loci have been identified for NSHL so far. Thus, HL is one of the most heterogeneous human genetic trait (6, 8), of which more than 70 DFNB loci have been identified for ARNSHL (9).

Iran, with the average consanguineous marriage rate of 38.6% (10) and with a heterogeneous population due to different ethnicities, can provide a good opportunity for genetic research on ARNSHL. However, further research has been highly recommended to obtain an insight into the contributing loci, some of which might be new (11). Most studies in Iran on ARNSHL have only addressed certain loci with a special focus on DFNB1 (*GJB2*), the most common cause of ARNSHL. Studying other loci in the Iranian ARNSHL patients would provide insight into the role of other loci in pathogenesis of ARNSHL in this population. The results of such studies could be applied to a more efficient genetic screening of the disease and the concomitant DNA diagnostics and genetic counseling.

The present study was launched to determine the contribution of DFNB1 (*GJB2*, *GJB6*) and 14 other DFNB loci to ARNSHL in the study cohort.

## Materials and Methods

### *Subjects and clinical evaluation*

This study was approved by the Institutional Review Boards of Shahrekord University of Medical Sciences and the University of Antwerp in Belgium. In this descriptive study, a number of families with Iranian origin were collected from 7 provinces of Iran, including Charmahal va Bakhtiari, Fars, Guilan, Tehran, Khuzestan, Azerbaijan Sharghi and Kurdistan.

HL informational questionnaires were filled out for all families and the pedigrees were drawn based on the filled-out questionnaires and interview with the members of the families. Families with the possibility of exposure to known environmental risk factors (head trauma, the use of ototoxic drugs, etc.) were excluded from the study. In total, 37 families with at least 4 patients were included in this study. All, but one family with Pendred syndrome, were affected with ARNSHL. Participants signed an informed consent form before their inclusion into this study. Pure tone audiometric test for air and bone conduction at frequencies varying from 250 to 8000 Hz was performed.

### *DNA extraction*

DNA was extracted from EDTA-containing blood samples of participating individuals using the phenol/chloroform standard procedure (12). DNA qualities were checked on 1.2% agarose gel. A NanoDrop 1000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA) was used to determine DNA concentration and purity.

### *GJB2 mutation screening*

At least, one patient from every pedigree was subjected to DNA sequencing. The following primers were used as described elsewhere (13) F: 5'-CTCCCTGTTCTGTCCTAGCT- 3' R: 5'-CTCATCCCTCTCATGCTGTC-3'. PCR condition was as follow: 2 µl MgCl<sub>2</sub> (4 mM), 2.5 µl Taq PCR

buffer (10X), 1 µl of each of the primers (10 PM), 0.1 µl Taq DNA polymerase (5U/ul), 1 µl dNTP mix (10 mM) and 1 µl DNA (about 70 ng). The reaction was adjusted to the volume of 25 µl by ddH<sub>2</sub>O. Standard cycling conditions was performed in a thermocycler (ASTEPC PC-818; ASTEC, Fukuka, Japan) as follows: 95° C for 2 min; 35 cycles of 94° C for 30", 57° C for 45", 72° C for 45", and finally 72° C for 7 min. The PCR product of the *GJB2* gene was quality controlled on the 1.5% agarose gel. A single PCR product of 809 bp was obtained.

Subsequently, DNA sequencing of the PCR-amplified product was carried out bi-directionally on an ABI 3130 automated sequencer (Applied Biosystems) (Macrogen, South Korea) using the same primers.

#### ***SLINK analysis and selection of DFNB loci***

Power of the pedigrees for linkage analysis was simulated by calculating SLINK, using FastSLINK (version 2.51) option of Easylinkage plus version 5.05 software to predict the potential LOD score in a given family (14). Families with SLINK scores above 2.5 were considered informative enough for linkage analysis by screening several known loci. The threshold SLINK value of 3.3 was considered significant for genome-wide scan (GWS). Based on the literature review of the most frequent loci, both globally and regionally, 15 loci were selected for screening. Screening STR markers were selected based on their physical distance found at NCBI UniSTS and NCBI Map Viewer. STR markers of each locus and their primer sequences are listed in Table 1.

#### ***Pooling strategy***

A subset of the samples was subjected to DNA pooling prior to genotyping by the following protocol: The concentration of DNA in the individual samples to be pooled was first estimated by measuring UV light absorption at 260 nm. Samples were then diluted to 30 ng/ul and re-adjusted. The purity of samples was estimated by the absorption ratios of 260/280 and 260/230 nm. Equal amounts of each sample were pooled. Separate pools for

normal and affected samples were prepared for each nuclear family of the pedigree. Af (1, 2,...) and N (1, 2,...), representing different affected and normal pools, were used to label the pooled samples. In addition, critical samples with poor DNA qualities were analyzed individually.

#### ***Genotyping STR markers and Linkage Analysis***

Fluorescent PCR of STR markers was conducted according to the standard procedure. Fragment analysis was carried out by capillary electrophoresis with an ABI 3130 automated DNA sequencer (Applied Biosystems, California, USA). Alleles were assigned by Genescan software (Applied Biosystems, Foster City, CA, USA). At least, two screening markers were analyzed for every known locus. Upon encountering an uninformative marker or finding clues of linkage, further markers were genotyped. LOD score calculations were combined with haplotype analysis to confirm or exclude linkage. Two-point and multi-point parametric LOD scores were, respectively, calculated by Superlink version 1.6 (15) and Simwalk version 2.91, both options of Easylinkage plus version 5.05 software (14). While two-point LOD score is much faster than multi-point LOD score and provides an initial evaluation of the linkage status, multi-point LOD score is more comprehensive and is able to provide haplotype data. 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. Haplotypes were reconstructed via Simwalk and were visualized by HaploPainter software version 029.5 (16).

## **Results**

### ***Families and clinical data***

The majority of subjects of this study displayed bilateral, severe to profound sensorineural prelingual HL, whereas 3 families showed moderate to severe HL. Twenty-nine out of 37 families (78.4%) had at least one consanguinity loop within the pedigrees. Although the possibility of inbreeding was

not completely ruled out for the other families, particularly those living in isolated villages.

**Screening of GJB2**

DNA sequencing of the coding region of *GJB2* could reveal pathogenic variants in 6 out of 37 studied families. The homozygous *GJB2* mutations included 35delG (3 families), R127H (2 families) and 167delT (two families). These families were excluded from further analysis. As the coding region sequencing of *GJB2* cannot completely rule out the DFNB1 as the disease-causing locus, the remaining families were further analyzed for the linkage to DFNB1 using at least 3 informative markers. However, linkage analysis could not find any other family linked to DFNB1 locus.

**SLINK calculation**

Fifteen families were of SLINK values  $\geq 3.3$ , Seven families had SLINK values of  $\leq 2$ . the rest of the families present value between 2-3.3. Later on, however, the lower families together with a subset of others were examined by DNA pooling strategy.

**DNA pooling, genotyping, and linkage analysis**

Screening loci for homozygosity mapping in this study were composed of: DFNB1 (*GJB2*), DFNB2 (*MYO7A*), DFNB3 (*MYO15A*), DFNB4 (*SLC26A4*), DFNB6 (*TMIE*), DFNB7/11 (*TMCI*), DFNB8/10 (*TMPRSS3*), DFNB9 (*OTOF*), DFNB12 (*CDH23*), DFNB21 (*TECTA*), DFNB22 (*OTOA*), DFNB28 (*TRIOBP*), DFNB35 (*ESRRB*), DFNB59 (*PJVK*) and DFNB63 (*LRTOMT*).

Ten out of the 31 families, all negative for DFNB1 locus, were linked to six different loci, 4 of which had been found by performing the locus screening on the pools of the related families. The results were confirmed by individually genotyping the family members for the same markers, as well as additional markers. Table 2 shows the linked families and the maximum values for SLINK, two-point and multi-point LOD scores. DFNB4 (4 families) was the most frequent locus, after DFNB1, in the studied ARNSHL series. The haplotypes of the linked families are shown in Figure 1 (a-J).

**Table 1:** The list of 15 DFNB loci screened in this study. The corresponding genes and details of screening markers are shown. Categories and functions of their encoded proteins are mentioned (52)

Locus (gene), Physical location(bp), Category& function	Marker	Physical position (bp)	PCR product range	Forward primer (5'→3')	Reverse primer (5'→3')
<b>DFNB1(<i>GJB2</i>, <i>GJB6</i>)</b>					
<i>GJB2</i> : 20761602..20767114	D13S1236	22696180-22696305	108-132	GCACTTGGCCTGGGTAA	AAGGGGCTGGCTCTCA
<i>GJB6</i> : 20796101..20806534)	D13S1275	-	180-214	ATCACTTGAATAAGAAGC CATTG	CCAGCATGACCTTTACCAG
Ion homeostasis proteins: Connexins	D13S175	20848506-20848618	101-113	TATTGGATACTTGAATCT GCTG	TGCATCACCTCACATAGGTTA
<b>DFNB2 (<i>MYO7A</i>)</b> 76839310..76926286)	D11S4179	76396260-76396495	200-256	GGATGTAAGAGTAACTGG CTCCG	GAAAATGTTCTGCCTGAGGG
Hair bundle morphogenesis proteins: Motor proteins	D11S4186	76968518-76968685	154-175	ATTCTCCCAATCTATCG CTC	GGGCAGTAATGATGATGTG
	D11S4079	77119447-77119701	217-265	CAGCAAGATCCTGTCT CAA	CTCCTTAAAGTGGGGGAGTT
	D11S911	77448583-77448769	159-203	CTTCTCATGCTTGACC ATTT	CTTCTGAACAATTGCCACAT
<b>DFNB3 (<i>MYO15A</i>)</b> 18012020..18083116)	D17S921	14260705-14260882	169-185	CTTGGACTCCTACAA ATCCTGGCA	GGCCACCATAATCATGTC AGACAAT
Hair bundle morphogenesis proteins: Motor proteins	D17S953	16102497-16102619	119-131	ACTATCCGCCAATACA	AAGGGCTTGCTTTGAC
	D17S2196	17264482-17264618	139-163	CCAACATCTAGAATT AATCAGAATC	ATATTTCAATATTGTAACC AGTCCC

Table 1: Continued...

<b>DFNB4 (SLC26A4)</b> 107301080..107358254) Ion homeostasis proteins: Ion channels	D7S2420	106889928- 106890211	240-290	CCTGTATGGAGGGCAA CTA	AAATAATGACTGAGGCTCA AAACA
	D7S496	107154713- 107154849	129-141	AACAACAGTCAACCCAC AAT	GCTATAACCTCATAANAA ACCAAAA
	D7S2459	107331501- 107331642	140-152	AAGAAGTGCATTGAGA CTCC	CCGCCTTAGTAAAACCC
	D7S2456	107683218- 107683460	238-252	CTGGAAATTGACCTGAAA CCTT	ACAGGGGTCTCTCACA CATATTA
	D7S2425	108347079- 108347322	234-246	CTAGTCCTGAGAA GACATTACCC	CCTGTTTCAGATGTT TTATCCA
<b>DFNB6 (TMIE)</b> 46742823..46752413) Poorly understood function: integral membrane protein	D3S3658	40903258- 40903371	104-126	AAAAGTTAGCAAA CACAATCCTATC	CTGGACTAAATCTAAGT TGGTTATG
	D3S2420	48067370- 48067462	93-108	ACAAGTGCGAAAC TCTGCCT	CAGGAGCCTCTAAGT CAGCA
	D9S1837	75185129- 75185367	205-251	CATGATGGTGGTC TCTGG	GGTGGGGCTCAAAG AGTAG
<b>DFNB7/11(TMCI)</b> 75136717..75451267) poorly understood function: integral membrane protein	D9S1806	74201357- 74201620	216-266	TTTTAGGTGTTCTCAGTA CATGC	GGGAGCAACATTTT GACATT
	D9S1124	75224065- 75224327	252-276	GGTGCCACCATACACT ACT	TCTAATCCTTCCTT CCCTCG
	D9S1876	75232791- 75232938	132-152	GATGTACCAGAGAAGT CTCG	AGTGGTTACCATTTA CCCAAG
	D9S301	73802720- 73802954	209-237	AGTTTTTCATAACACAAAA GAGAACA	ACCTAAATGTTTCATCA AAAGAGG
	D9S1822	74930323- 74930483	157-163	AAGTTTGGCTTCTGCTGT AAGGGTC	AATCCCCCAGGCT GAGTG
<b>DFNB8/10 (TMPRSS3)</b> 43791999..43816200) Poorly understood function: Serine protease	D9S1799	73366891- 73367055	139-178	TTGCCAACTATTTTAG CCC	TGCAGTTTCAATCC ACATC
	D21S1890	44848178- 44848330	143-173	GGTCTGACCACAGAT TTCC	AAAAACACTCTGAACG ATTAAGG
	D21S1260	42796042- 42796251	200-214	TCCAAGGGGTTTCATCC	CCCAAGGCACT GTTCC
<b>DFNB9 (OTOF)</b> 26680071..26781566) Poorly understood function: Exocytosis at auditory ribon synapse	D2S365	28606342- 28606533	164-204	ATGATTTGTGTACCTTA TGTATGTT	TCAATGGAGGAAT CCTACTT
	D2S2247	27303911- 27304064	130-160	TCCATCTTTTGCGTGC	CCGTGCTCTATG CCAG
	D2S174	26839873- 26840075	203-221	AGGCTGAATCCCACCTCC	TTAGAGCACACATGG TCACTCC
<b>DFNB12 (CDH23)</b> 73156704..73575704) Hair bundle morphogenesis proteins: Adhesion proteins	D2S2223	26559144- 26559325	182-200	CACTGCGCCTAGCCTC	GGCGATTTATGAATA ATCCTGC
	D10S1432	74659396- 74659569	157-185	CAGTGGACACTAAACAC AATCC	TAGATTATCTAAATGGT GGATTTC
<b>DFNB21 (TECTA)</b> 120973375..121061515) Extracellular matrix proteins: Structural component tectorial membrane	D10S1146	74659314- 74659555	164-246	ATTGCACTCCAGCCTGGGT	CACAATCCAATCACA TGGATG
	D11S4107	121049124- 121049321	172-212	TCATTCTACAAGACTAG CATTACC	GCTTGATCATGGGTGA TTATCTT
	D11S925	120828264- 120828438	172-199	AGAACCAAGGTCGTAA GTCCCTG	TTAGACCATTATGG GGCAA
	D11S912	128624097- 128624205	101-123	TCGTGAGANTACTGC TTTGG	TTTTGTCTAGCCATG ATTGC
	D11S4151	126292160- 126292309	145-155	GTCTTCCACCTTGGAT ATGGGTA	AATGGGCACCTCCACCC TATTAGT
<b>DFNB22 (OTOA)</b> 21689835..21772050)	D11S4089	120989673- 120989875	199-213	ATTCCTAGTTCCTCAT AAACACTG	TAATCAAAGGCTGTAGT GAATTGG
	D11S4110	126971672- 126971780	93-107	TGAGCCTCCAGTA CCTACC	GTTTGTGGCAGAG CCCTAAG
	D16S3046	20886507- 20886610	84-108	CCCAGAATAAACTG CGTG	TTCATGGACCCCC TATTG

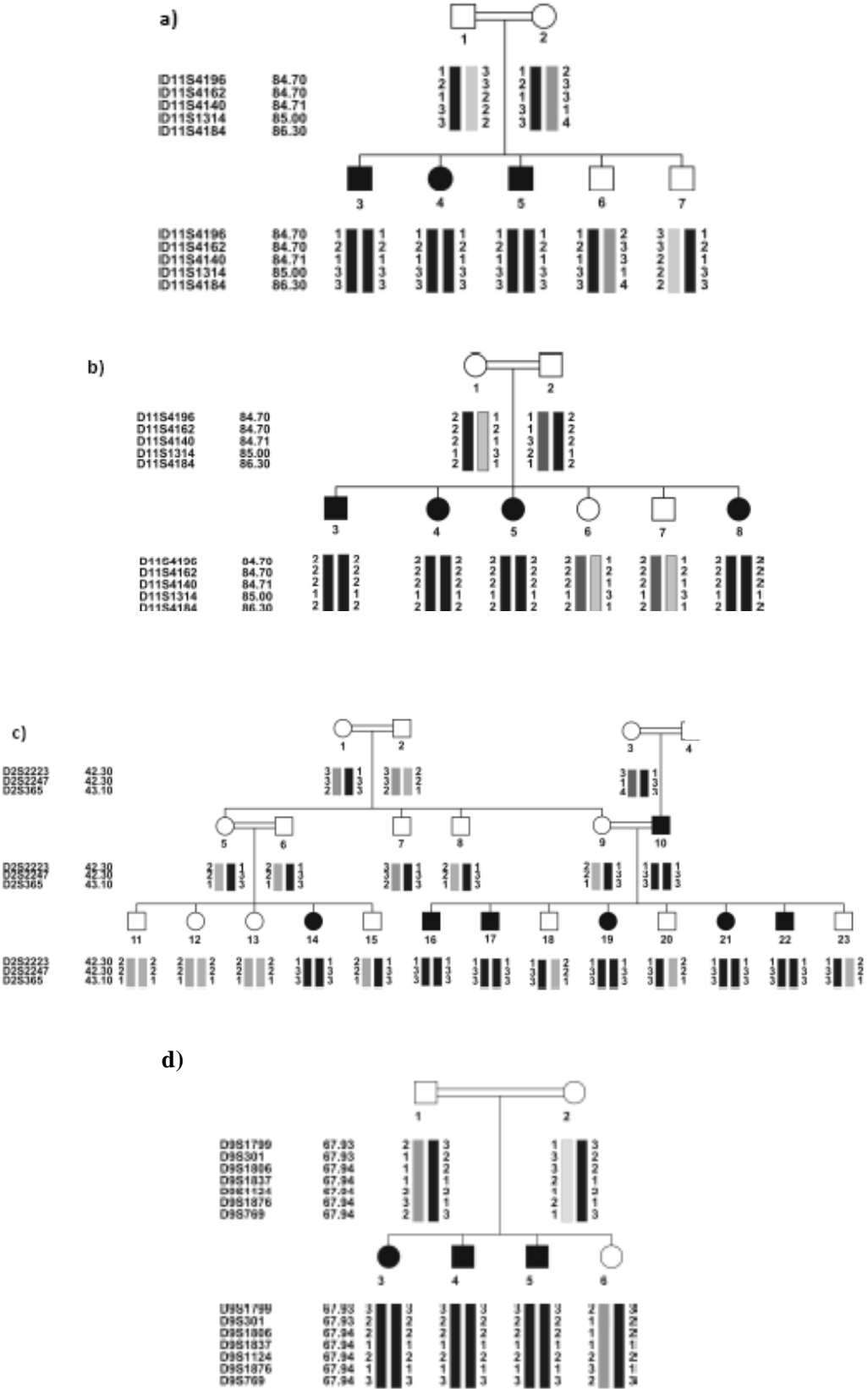
**Table 1:** Continued...

Extracellular matrix proteins: Anchoring proteins between cellular gels and non-sensory cells	D16S403	23037651-23037790	134-152	GTTTTCTCCCTGGGA CATTT	TATTCATTTGTGT GGGCATG
<b>DFNB28 (TRIOBP)</b> 38092995..38172563) Hair bundle morphogenesis proteins: Proteins of the cytoskeleton	D22S1156	38381771-38381926	130-162	TGAGGTAGTCACA CGAGGCA	AATTCACTGGGCTCC GAGG
	D22S1045	37536298-37536453	140-158	GCTAGATTTTCCCC GATGAT	ATGTAAAGTGCTCTCAAG AGTGC
<b>DFNB35 (ESRRB)</b> 76837726..76967208) Transcription factors	D14S1015	92736133-92736396	224-264	GAATGCCATTATTTT GTCCT	TTAGAAAAACCCGAG CAGA
	D14S77	73570540-73570772	203-251	GCGTGAGTCACTGTGCC	CAGACAGAAATTAACC AGAGTTGAA
	D14S1045	0	240-246	AGGGCTGGTGACAATG CATAAAAGGCTTA	GTAAGGNCTTGGGTGG CAAACACAGAGAACAG
	D14S48	88428727-88429001	260-265	TTGGTTTG	AGTAG
	D2S2981	176173334-176173578	234-262	AAAATATGCAGGTA ATGACTTGG	CAAGCAAAAAGTGCAG GGTAGG
<b>DFNB59 (PJKV)</b> 179316163..179326113) Poorly understood function: Signaling of hair cells and neurons	D2S301	217887163-217887396	224-240	CATAGGACTGAAG GGGTGTA	GGAAAATCTCGAATG TACCAAT
	D2S2173	178445536-178445768	201-243	GGAGACAGAGAGTT TACATTTGAG	GCCACACTTTCCT GAATC
	D2S324	179656244-179656508	264-275	TTACCCACCGGGACAGT	CAGCAAATGCTTCT AGGTCA
	D2S307	204654566-204654784	205-221	CATGACCTGAAATA AACATAGACA	AGCTTTTCCTGTAGG CTGTC
	D2S2314	176862406-176862505	96-118	GGTGTCAGTGA GACCCTGT	ATTTCTAGCGGCCCT AAAAC
	D11S1314	72323192-72323414	209-227	TTGCTACGCACTC CTCTACT	GTGAAGGCAGGAAA TGTGAC
<b>DFNB63 (LRTOMT)</b> 71791382..71821828) leucine-rich methyltransferase	D11S4132	119948397-119948596	176-214	GTGCAAGTTTTGG CTTCGTC	ACTCCAGCCTGGG TGAAA
	D11S4162	70975752-70976016	263-269	GTTCTCCAGAGAG ACAGCAC	GAGAGCAACACTA TTGCC
	D11S4140	71945684-71945874	189-199	TGCAACAAGGT TCCACACT	CTTATGGGTGAGGG CACAG
	D11S4196	0	200-240	GAACGTTNTTCAT GTAGGCGT	TAATGGTCGCTG TCCC
	D11S4184	72670843-72671103	263-277	CCCAGCCTTA CATATTCC	GCTGATGAGCAGA GGTAG

Physical positions were determined from the National Center for Biotechnology Information (NCBI) Build 37.1 sequence-based physical map (International Human Genome Sequence Consortium 2009).

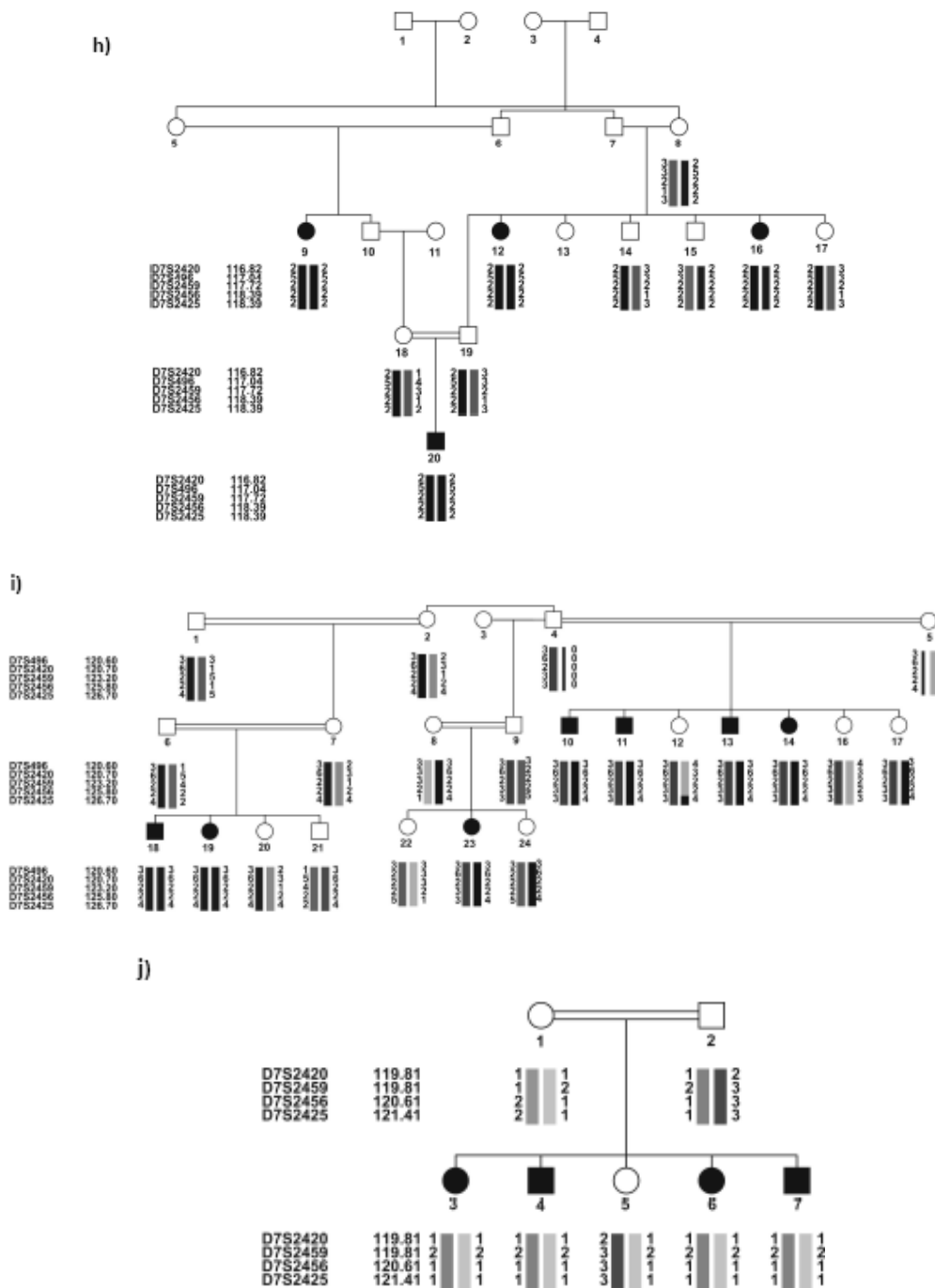
**Table 2:** Maximum SLINK and LOD score (two-point and multi-point) values for the linked families. Asterisk shows the family with Pendred syndrome

Family ID	Linked locus	SLINK value	Two-point LOD score	Multi-point LOD score
IR-JOL	DFNB4	2.4	2.1	2.4
IR-SH17*	DFNB4	5.2	3.3	4.1
IR-SH9	DFNB4	6.2	3.5	5.1
IR-ABY	DFNB4	7.4	3.4	4.6
IR-GHA	DFNB7/11	1.8	1.6	2.0
IR-JAF	DFNB21	2.9	2.6	3.1
IR-SH5	DFNB9	3.9	3.2	3.3
IR-SH6	DFNB2	4.4	3.6	4.2
IR-HEM	DFNB63	2.8	1.9	2.4
IR-SH11	DFNB63	2.1	2.1	2.1









**Fig. 1:** Pedigree of the 10 Iranian families with ARNSHL, negative for *GJB2* mutations, linked to 6 known loci. Black symbols indicate affected individuals. For conciseness, some of the pedigrees are partly shown. haplotypes are shown below each symbol, For individual families, the corresponding genetic map of the markers is shown in parenthesis. (a,b) linked to DFNB63: a) IR-HEM and b) IR-SH11 (LDB map), c) IR-SH5 linked to DFNB9 (LDB map), d) IR-GHA linked to DFNB7/11(Marshfiled map), e) IR-SH6 linked to DFNB2 (LDB map), f) IR-JAF linked to DFNB21 (Marshfiled map), (g-j) linked to DFNB4: g) IR-SH9 (Marshfiled map), h) IR-SH17 (decode mapand i) IR-ABY(LDB map) j) IR-JOL (Marshfiled map). In IR-ABY (i) a cross over must have happened in one of the upper generations of individual 1 between markers D7S2459 (which is an intragenic marker) and D7S2456, thus creating two haplotypes segregating with HL in two parts of the pedigree.

## Discussion

The locus DFNB1, harboring *GJB2* and *GJB6*, was the first to be excluded in our study cohort composed of 37 families. It plays a major role in the pathology of ARNSHL worldwide. *GJB2* mutations have been estimated to account for up to 50% of ARNSHL cases in the North American, Mediterranean, and most of the European populations (17-19). Thus, *GJB2* was tested by both gene mutation screening and linkage analysis to strictly rule out the cause in the studied families. Furthermore, *GJB6* deletions were investigated (13) with no finding of the mutations. DFNB1 linkage analysis confirmed the results and showed no more positive family with DFNB1 involvement. Totally, 16.2% of the families were homozygous for *GJB2* mutations which were set aside from further analysis. This fits well with the 16.6% (20) or 18.3% (11) rate of *GJB2* involvement in ARNSHL in previous studies on the Iranian population and would emphasize the diversity of the Iranian population and the fact that the contribution of other loci should be questioned. Interestingly, two mutations 35delG (3 families) and R127H (2 families) that had been previously reported as the first and second prevalent mutations in Iran (11), were found to cause HL in 5 of the 6 families with a mutation in *GJB2* in this study. Two families (IR-JAF and IR-Sh11), both heterozygous for the *GJB2* mutation V37I, were included in homozygosity mapping for two reasons. At first, the pathogenicity of this variant has been doubted by some investigators (21). Secondly, there is a possibility to be carrier for this mutation but the real disease-causing gene would be different. However, both families showed linkage to DFNB21 and DFNB63, respectively. Three out of 36 (8.3%) ARNSHL families of the study cohort were linked to DFNB4. One additional family, diagnosed as Pendred syndrome due to co-segregation of hypothyroidism (goiter) in later ages was also linked to the region containing *SLC26A4*, also known as *PDS*, which encodes pendrin, a chloride and iodide transporter (22). Its molecular pathology has been linked to both ARNSHL and Pendred syndrome. Although the

prevalence of Pendred syndrome is not known exactly, it seems to be the most common form of syndromic HL (23). Actually, distinction between Pendred syndrome and NSHL can be challenging since not all affected individuals may co-segregate thyroid disease (24).

About 5% of ARNSHL cases in South Asia have been tied to *SLC26A4* mutations (25, 26). In a study, 12 families out of 80 (15%) Iranian families with 2 or more HL patients were linked to DFNB4 locus with clues for 5 families to be syndromic (27). In a recent study, out of 34 families negative for *GJB2*, 3 families (8.8%) were linked to DFNB4. Thus, DFNB4 contributes significantly to HL in Iran and is ranked second after DFNB1.

In our study, two families showed linkage to DFNB63. The locus which contains *LRTOMT*, has been reported in families of Turkish, Tunisian, and Pakistani origin (28, 29). In a study, a mutation in *LRTOMT* was identified in one out of 192 screened Iranian families (30). However, the true frequency of the locus might be more in the series since only catechol-O-methyltransferase (*LRTOMT2*) had been addressed and the possibility that mutations in isoforms of *LRTOMT1* could lead to HL, can not be discounted. The finding of two DFNB63-linked families in our cohort may substantiate the above hypothesis.

One out of 36 families (2.7%) was linked to DFNB2. The related gene *MYO7A*, encoding myosin VIIA, is a cytoskeletal motor proteins facilitating the movement of cell components along actin filaments (31). DFNB2-linked families have been reported from Iran (32, 33) and one family out of 40 (2.5%) was found to be linked to DFNB2 (32).

In our study, one family (2.7%) was linked to DFNB7/11. The novel gene, called *TMCI*, is required for postnatal hair cell development. *TMCI* mutations seem to be a rather common cause of ARNSHL in India and Pakistan (34). In a research in the North East and East of Turkey, four out of 65 (6.2%) families, negative for *GJB2*, were shown to be linked to DFNB7/11 (35). The locus could be one a common causes of ARNSHL in the Iranian population.

One family (2.7%) was linked to DFNB9 (*OTOF*). In a study from Lebanon, 3 out of 30 families (10%) were linked to the locus (36). Other studies have reported DFNB9 from India (37) and UAE (38). *OTOF* mutations cause ARNSHL, which may be accompanied by auditory neuropathy in about half of cases, with important implications in DNA diagnostics (39).

We found one family linked to DFNB21 in this study. The corresponding gene, *TECTA*, can cause both dominant and recessive HL. *TECTA* encodes  $\alpha$ -tectorin, a major non-collagenous constituent of the tectorial membrane that bridges the stereocilia bundles of the sensory hair cells (40). Interestingly, the DFNB21-linked family in our study, showed the distinctive audio profile (moderate to severe HL, more pronounced in the mid-frequencies) that has been suggested for DFNB21-linked families and is important in DNA diagnostics (41). In a study, linkage to DFNB21 was found in 3 (6.6%) out of 45 Iranian consanguineous families which were negative for *GJB2* mutations (42). In another study on 75 Iranian families segregating ARNSHL, 1 family (1.33%) was linked to DFNB21 (41) and finally, in a genetic linkage study of forty ARNSHL families living in Markazi and Qom provinces of Iran with at least 3 affected individuals per family, no instance of linkage to DFNB21 was found (32). No linkage was found to DFNB3 in any of the study families. The corresponding gene, *MYO15*, codes for myosin XV that is necessary for actin organization in hair cells (43). It has been suggested that at least 5% of the studied Pakistani population are caused by the gene (44). Unlike our study, with no DFNB3-linked family, in a recent study on 40 Iranian ARNSHL families from Qom and Markazi provinces of Iran with 3 or more patients, 2 families were linked to DFNB3 (5.8%) (32). Thus, the prevalence of the DFNB3 HL may vary among Iranian populations. More ARNSHL families have to be studied before reaching any definite conclusion in this regard. No family was linked to DFNB59. The locus has been mapped to 2q31.2, and the corresponding gene, *PJVK* encoding pejvakin, has been identified in 4 Iranian families (45). Pejvakin plays a role in action potential propagation or intracellular traf-

ficking. Like *OTOF*, its defects can sometimes cause auditory neuropathy (45). Thus, in case of auditory neuropathy, both DFNB9 and DFNB59 are strong candidates from the DNA diagnostic standpoint. Screening of 67 Turkish ARNSHL families led to finding of a linked family. It was concluded that it was not playing a significant role in the pathogenesis of HL in the Turkish patients (46). In a study on 30 Iranian ARNSHL families, 2 families (6.7%) were found to be linked. The investigators proposed checking the locus in the Iranian ARNSHL families (47). Based on our study, it is possible that DFNB59 plays no major role in the pathogenesis of ARNSHL.

As a sub-goal of the study, we successfully performed the DNA pooling strategy for a subset of the families. In the DNA pooling, the DNA samples are segregated into pools based on the contrast phenotype. Thus, in an inbred family linked to a given locus, it is expected that affected individuals would be homozygous for a single marker allele closely linked to the given locus and is thus "hunted" (48). While the attraction of pooling lies in reducing time and expense for genotyping individuals, some information, that could have been obtained by individual genotyping, might be lost (49). This can specially occur if more than one cause of hearing loss is segregating in a family. Other pitfalls include differential amplification of alleles and the STR stutter bands, the PCR artifacts associated with STR markers that can sometimes complicate allele calling of individual pools (50). As to tackle with some of its limitations, for the pedigrees showing possible clues of digenic inheritance, based on the structure of the pedigrees or audiometric profiles, a number of screening markers were independently checked on the individuals. In this experiment, we applied a set of adjustments to successfully conduct the DNA pooling and could find 4 linked families which were confirmed by further genotyping in the corresponding family individuals. Our result shows that the strategy could easily be applied to the studies of genetic linkage covering the extraordinary big pedigrees or those with SLINK values lower than 2.5, which are often discarded from additional analyses.

In the present study, we could only clarify the genetic etiology of 16 out of 37 (43.2%) HL families, including 36 ARNSHL families and one with Pendred syndrome, and 15 out of 36 (41.6%) ARNSHL families. This could be explained by the extreme heterogeneity of the disease arising from the complexity of the auditory system, and the lower contribution of DFNB1 (*GJB2*) in Iran as compared to some other populations. The results of the present study confirm those of previous studies in Iran. The results would also give an overview of the most frequent loci: DFNB1, DFNB4, DFNB2, DFNB21 and DFNB7/11. It might be worthwhile including also DFNB63 in the locus-screening list of future studies on Iranian ARNSHL patients. The design and practice of similar studies on the different populations of Iran will provide a wealth of population-specific knowledge for genetic diagnosis and genetic counseling of the families. The next phase of the study involves the DNA sequencing of the known DFNB genes we have already mapped in the linked families as to identify their underlying pathogenic mutations in the families. Several families with  $SLINK \geq 3.3$  are currently being subjected to GWS by the Illumina SNP array utilizing 6K SNP Linkage Panel IVb (Illumina Inc., San Diego, CA). Thus far, one novel locus (DFNB93) for hearing loss has been identified (51).

### 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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