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Mutation Analysis of *GJB2* and *GJB6* Genes and the Genetic Linkage Analysis of Five Common DFNB Loci in the Iranian Families with Autosomal Recessive Non-Syndromic Hearing Loss

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Abstract

The incidence of pre-lingual hearing loss (HL) is about 1 in 1000 neonates. More than 60% of cases are inherited. Non-syndromic HL (NSHL) is extremely heterogeneous: more than 130 loci have been identified so far. The most common form of NSHL is the autosomal recessive form (ARNSHL). In this study, a cohort of 36 big ARNSHL pedigrees with 4 or more patients from 7 provinces of Iran was investigated. All of the families were examined for the presence of *GJB2* and *GJB6* (del D13S1830 and del D13S1854) mutations using direct sequencing and multiplex PCR methods, respectively. The negative pedigrees for the above-named genes were then tested for the linkage to 5 known loci including DFNB3 (*MYO7A*), DFNB4 (*SLC26A4*), DFNB7/11 (*TMC1*), DFNB21 *(TECTA)* and DFNB59 (*PJVK*) by genotyping the corresponding STR markers using PCR and PAGE. Six families had *GJB2* mutations. No *GJB6* mutation was found. Totally, 3 families showed linkage to DFNB4, 1 family to DFNB7/11 and 1 family to DFNB21. No family was linked to DFNB59. *GJB2* included 16.6% of the causes of ARNSHL in our study. In the remaining negative families, DFNB4 accounted for 10% of the causes. Other loci including DFNB7/11 and DFNB21 were each responsible for 3.3% of the etiology. Thus, DFNB1(*GJB2*) and DFNB4 are the main causes of ARNSHL in our study and *GJB6* mutations (del D13S1830, del D13S1854), DFNB3 and DFNB59 were absent. Totally, 30.5% of the ARNSHL etiology was found in this study.

Keywords: ARNSHL; Linkage analysis; Iran; *GJB2* (*CX26*); *GJB6* (*CX30*)

Introduction

Hearing loss (HL) is the most common sensory disorder in human. One per thousand neonates are with pre-lingual HL. Also, some degrees of HL negatively affect the normal interaction in 4% of individuals below

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the age of 45 and 10% of people at the age of 65 and older [1-3]. HL has a wide spectrum of clinical manifestations including: congenital or late-onset, conductive or neurosensory and syndromic or non-syndromic. Based on the severity, it is also classified as mild, moderate, severe and profound. Over 60% of HL is inherited. As the sanitary quality index improves, the contribution proportion of genetic to non-genetic factors increases [2]. Approximately, 80% of HL cases are non-syndromic (NSHL) with the autosomal recessive mode of inheritance (ARNSHL) making up the majority of cases. The phenotype is normally pre-lingual and more severe in these cases [1-3]. Up to 1% of human genes are estimated to be involved in hearing process and over 130 loci have been identified so far for NSHL. Thus, HL is the most heterogeneous human trait known [4,5]. For ARNSHL alone, over 70 loci have been identified [6].

Iran, with a high consanguinity marriage rate and with a heterogeneous population, offers a good opportunity to study rare autosomal recessive disorders including ARNSHL. Most studies on ARNSHL have only addressed certain loci with a special focus on *CX26 (GJB2)*, the most common cause of ARNSHL. Studies on the main loci involved in the Iranian ARNSHL patients seem to be essential in order to clarify their roles. The results of such studies could be applied to a more efficient genetic screening of the disease and the concomitant genetic counseling. The present research was launched to determine the contribution of several genetic factors to the studied ARNSHL series. Starting with the screening of the ARNSHL families with more prevalent genes including *GJB2*(MIM# 121011) and *GJB6* (*CX30*) (MIM# 604418) (del D13S1830 and del D13S1854), the study continued to the next step by the genetic linkage analysis for 5 known DFNB loci, including DFNB3 (*MYO7A*), DFNB4 (*SLC26A4*), DFNB7/11 (*TMC1*), DFNB21 (*TECTA*) and DFNB59 (*PJVK*) in the families negative for mutations in the two genes.

Materials and Methods

Sampling and DNA Extraction

The study was approved by the Institutional Review Boards of Tehran University of Medical Sciences and Shahrekord University of Medical Sciences. In this descriptive study, 36 families with 4 or more cases were collected form 7 provinces of Iran, including Charmahal va Bakhtiari, Fars, Guilan, Tehran, Khuzestan, Azarbaijan Sharghi and Kurdestan. A pre-designed questionnaire separately filled out for each family together with clinical evaluations helped exclude non-syndromic forms of HI. Informed consent was taken from all the family members, 5-10 ml of whole blood was collected in 0.5 M EDTA containing tubes. Genomic DNA was extracted by a standard phenol-chloroform method. DNA concentration and purity was measured by spectroscopy (UNICO 2100, USA) [6,7].

Screening of GJB2 by Direct PCR-Sequencing

One patient was sequenced from each part of the pedigrees. Following primers were designed using Primer 3 web-based software: F: 5'-CTCCCTGTTCTG TCCTAGCT-3' R: 5'-CTCATCCCTCTCATGCTGTC-3'. A single PCR product of 809 bp was obtained using the following conditions for each reaction: 2 µl MgCl2 (50 mM), 2.5 µl Taq PCR buffer (10X), 1µl of each of the primers (10 PM), 0.1 µl Taq DNA polymerase (5U/ul), 1 µl dNTP mix (10 mM) and 1µl DNA (about 50 ng). The reaction was adjusted to the volume of 25ul by ddH2O. Standard cycling conditions was performed in a thermocycler (ASTEC PC818-Japan) as follows: 95°C for 3 min; 35 cycles of 94°C for 30", 57°C for 45", 72°C for 45", and a final extension at 72°C for 7 min. PCR product was run in a 6-8% polyacrylamide gel electrophoresis (PAGE) (Merk, Germany) at 40 mA for 2 h. Bands were visualized by silver staining [9]. 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.

Screening of GJB6 Deletions

A multiplex PCR assay was performed to detect del (GJB6-D13S1830) and del (GJB6-D13S1854) using following primers:

F1: 5'-CACCATGCGTAGCCTTAACCATTTT-3'

R1: 5'-TTTAGGGCATGATTGGGGTGATTT-3'

(for amplification of the del (GJB6-D13S1830) breakpoint junction)

del (GJB6-D13S1854):

F2: 5'-CAGCGGCTACCCTAGTTGTGGT-3'

R2: 5'-TCATAGTGAAGAACTCGATGCTGTTT-3'

(for amplification of the del (GJB6-D13S1854) breakpoint junction);

GJB6 (exon 1):

F3: 5'-CATGAAGAGGGCGTACAAGTTAGAA-3'

R3: 5'-CGTCTTTGGGGGTGTTGCTT-3'

(for amplification of *GJB6* exon 1).

The logic behind the multiplex PCR is that by any of the deletions the breakpoint junctions will amplify: del(GJB6-D13S1830) and del(GJB6-D13S1854) create

amplicons of 460 bp and 564 bp, respectively.

While, the segment containing GJB6 exon 1 (F3R3) will be lost in homozygous state. In the wild type, we should only have F3R3 amplicon (333bp) [8]. Each reaction contained: 1 MgCl2 (50 mM), 2.5 µl Taq PCR buffer (10X), 0.5 µl of each of the primers (10 PM), 0.1 µl Taq DNA polymerase (5U/ul), 0.5 µl dNTP mix (10 mM) and 1µl DNA (about 50 ng). The total volume was adjusted to 25 µl by ddH2O. A touch down thermal program was performed as follows: One cycle of denaturation at 95°C for 3 minutes; five touchdown cycles of denaturation at 94°C for 40", annealing at 64°C for 40" in the first cycle with 1°C reduction per cycle, and extension at 72°C for 45"; 27 cycles of denaturation at 94°C for 45", annealing at 60°C for 40", and extension at 72°C for 45"; and a final extension step of 72°C for seven minutes. PCR products were resolved by 6-8% PAGE. A positive control, heterozygous for del(GJB6-D13S1830), was used.

Genotyping STR Markers, SLINK and Linkage Analysis

Short tandem repeat (STR) markers were amplified by PCR. The following general protocol was carried out with some modifications for some STR markers. Each reaction contained: 3 MgCl2 (50 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 1µl DNA (about 50 ng). The total volume was adjusted to 25 µl by ddH2O.

A touch down thermal cycling was programmed as follows: One cycle of denaturation at 95°C for 3 minutes; six touchdown cycles of denaturation at 94°C for 45", annealing at 59°C for 45" in the first cycle with 1°C reduction per cycle, and extension for 45" at 72°C; 30-33 cycles of denaturation at 94°C for 45", annealing at 54°C for 45", and extension for at 72°C for 45"; and a final extension step of 72°C for seven minutes. PCR products were run on 8-12% PAGE at 50 mA for 3-6 h.

Silver staining was followed and bands were visualized [9]. Alleles were assigned by visual inspection. At least, two screening markers were analyzed for every known locus. The selection of STR markers was based on their physical distance found at NCBI UniSTS and NCBI Map Viewer. Upon encountering an uninformative marker, further markers were examined. The primer sequences are available upon request.

SLINK analysis was performed by FastSlink version 2.51. Two-point and multi-point parametric LOD scores were calculated by Superlink version 1.6 and Simwalk version 2.91 options of Easylinkage plus version 5.05 software [11]. After linkage analyses, haplotypes were reconstructed via Simwalk and were visualized by Haplopainter software version 029.5 [9].

Results

Screening of GJB2 and GJB6 Genes

Out of the 36 studied pedigrees, 6 pedigrees were found to have *GJB2* mutations. In the remaining 30 pedigrees, *GJB6* deletions were investigated. No deletions of *GJB6* were found and all the individuals showed the normal 333 bp band (Fig. 1).

Genotyping STR Markers, SLINK and Linkage Analysis

We initially analyzed a part of a pedigree, that included one consanguinity loop comprising parents, two non-affected and two affected individuals, by at least two informative microsatellite markers. In case, all the patients were homozygous (i.e. showing one single band), hence, different from the pattern seen for the non-affected individuals, including carriers and non-carriers, all parts of the pedigree would be analyzed for confirmation of the linkage and more informative markers were genotyped. Priority of screening was with intragenic markers. A sample of linked genotype is shown in Figure 2.

Thirty families that were negative for *GJB2* and *GJB6* gene mutations were analyzed by the genetic linkage analysis for 5 DFNB loci including DFNB3, DFNB4, DFNB7/11, DFNB21 and DFNB59. Three families showed linkage to DFNB4. We found also one family linked to DFNB7/11 and one family linked to DFNB21. No family was linked to DFNB3 or DFNB59. We used D17S921, D17S953, D17S2196 and D17S2206 as the screening markers of DFNB3 and D2S2981 , D2S2173, D2S 324, D2S 2314 as the screening markers of DFNB59 in this study.

Table 1. Calculated SLINK and LOD score (two-point and multi-point) values for the linked families. SLINK value theoretically estimates the LOD score for a given family

Family	Linked locus	SLINK value	Two-point LOD score	Multi-point LOD score
IR-GHA	DFNB7/11	1.8	1.6	2.0
IR-JOL	DFNB4	2.4	2.1	2.4
IR-SH9	DFNB4	6.2	3.5	5.1
IR-ABY	DFNB4	7.4	3.4	4.6
IR-JAF	DFNB21	2.9	2.6	3.1

The haplotypes of the linked families are shown in Figure 3 (a-e). SLINK and LOD score values (two-point and multi-point) are illustrated for the linked families (Table 1). As evident, the LOD score values match to some extent with SLINK values. SLINK would predict a theoretical estimation for LOD score in a given family. For LOD score calculations, equal recombination for both sexes, autosomal recessive pattern of inheritance, with complete penetrance and no phenocopy, and disease allele frequency of 0.001 were assumed.

Discussion

In this study, we analyzed 36 ARNSHL families from 7 provinces of Iran including Charmahal va Bakhtiari, Fars, Guilan, Tehran, Khuzestan, Azarbayjan Sharghi and Kurdestan. After screening *GJB2* mutations and two *GJB6* deletions including D13S1830 and D13S1854, the genetic linkage and haplotype analysis was carried out by STR markers for the 5 studied DFNB loci. Since *GJB2* mutations account for 18.29% of ARNSHL and 12.7% of sporadic cases, and for the extreme heterogeneity of the disease and population diversity of Iran, studying other loci seems to be essential and is expected to provide us with an insight into the roles of other loci in pathogenesis of ARNSHL in Iran [15].

In the present study, 6 families were homozygous to *GJB2* gene which were set aside for the rest of the study. This fits well with the 16.6% [16] or 18.29% [15] rate of *GJB2* involvement in ARNSHL. This also emphasizes the heterogeneity of the Iranian population and puts forth the fact that other loci as well as novel ones may be involved. DFNB1 is the first locus to be identified and is the most common locus in ARNSHL. Its rate of involvement (0-50%) is partly population-dependent and has been estimated to account for up to 50% of ARNSHL cases in the North American, Mediterranean and most of the European populations [10-12].

GJB6 lies adjacent to *GJB2,* that causes HL, in DFNB1 locus. Its two common deletions include del(GJB6-D13S1830) and del(GJB6-D13S1854). The former deletion is 309 Kb and causes HL in such countries as Spain, France, UK, Brazil, USA, Belgium and Australia either in homozygous status or as compound heterozygous with other *GJB2* mutations [13-15]. In one study, the prevalence of the deletion was found to be between 5.9 to 9.7% of all the DFNB1 alleles in the populations of Spain, France, UK and Brazil [18]. Therefore, it was nominated as the second mutant allele after 35delG, the single most frequent *GJB2* mutation in some studied populations. Notably, 10-50% of HL individuals having *GJB2* mutation are heterozygous and del(GJB6-D13S1830) is the second mutant allele in 30 to 70% of these cases [14] However, it must be noted that the deletion has no role in pathogenesis of HL in a variety of European and Asian populations including Austria, Croatia, China and India [16-20].

del(GJB6-D13S1854) is a 232 Kb deletion which has been found more recently in *GJB6* in the same region of the other deletion. This deletes a shorter region upstream of *GJB2* as compared to del(GJB6-D13S1830) [19]. The distribution of the two deletions is different in various populations. In fact, del(GJB6-D13S1830) is much more prevalent and has been reported from various countries. In contrast, del(GJB6-D13S1854) has been reported from a limited number of families [8]. Both mutations have been reported from Spain and UK in 10.6% and 9.8% of mutant DFNB1 alleles, respectively, and are listed among the five more frequent DFNB1 mutations. Interestingly, unlike the

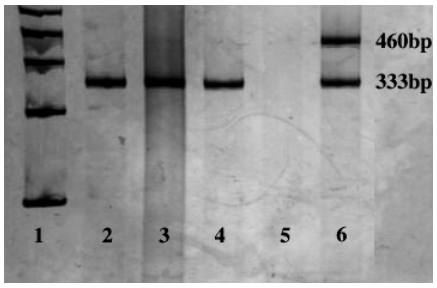

Figure 1. Analysis of two common *GJB6* deltions in ARNSHL families. Column order is as follows: 1: marker, 2-4: patients, 5: negative control (all PCR reaction materials except DNA), 6: positive control (a heterozygous del(*GJB6*-D13S1830)/wt). The 333 bp band indicates the normal status of *GJB6*.

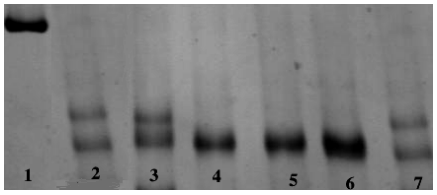

Figure 2. Gnotyping STR markers using Polyacrylamide gel electrophoresis (PAGE) 12% for a DFNB4 marker in a DFNB4 linked family is shown. Column order is as follows: 1: marker, 2: father, 3: mother 4-6: affected siblings 7: a non-affected sibling. A single (homozygous) band is observed for affected individuals.

high prevalence of del(GJB6-D13S1830) in France, there has been no report of del(GJB6-D13S1854). In Belgium, del(GJB6-D13S1830) is not a prevalent allele and thus far, del(GJB6-D13S1854) has not been reported [18]. Previous studies in Iran have not detected del(GJB6-D13S1830) in populations from the Western, North-Eastern and Central Iran [21-23]. This is in agreement with our results and suggests that it may have no role in pathogenesis of ARNSHL in our country. Neither did we find any positive case for del(GJB6-D13S1854). This is the first evidence to address the deletion in Iran and suggests that it may not contribute to ARNSHL in Iran.

Out of the 30 remaining families negative for *GJB2* and *GJB6* mutations, 3 families (10%) were linked to DFNB4. The locus contains *SLC26A4* gene. In a study aimed at identifying the causes of HL in the South Asia, 212 Pakistani and 106 Indian families with 3 or more HL patients were investigated. About 5% of ARNSHL cases in South Asia as well as other populations were suggested to be caused by *SLC26A4* mutations [24,25]. DFNB4 accounts for both syndromic and non-syndromic forms of HL. In a study, out of 80 Iranian families with 2 or more HL patients, 12 families (15%) were linked to DFNB4 locus with clues for 5 families to be syndromic [26]. 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 2nd After DFNB1 [27].

In our study, one family (3.3%) was linked to DFNB7/11. Linkage to the locus has been obtained in 10 families out of 230 consanguineous families from India and Pakistan and a novel gene called TMC1 was found [31]. TMC1 mutations seem to be a rather common cause of ARNSHL in India and Pakistan. Mutations of the gene have also been found in Turky [31]. In a research in the North East and East of Turky, using homozygosity genome scan analysis, 4 out of 65

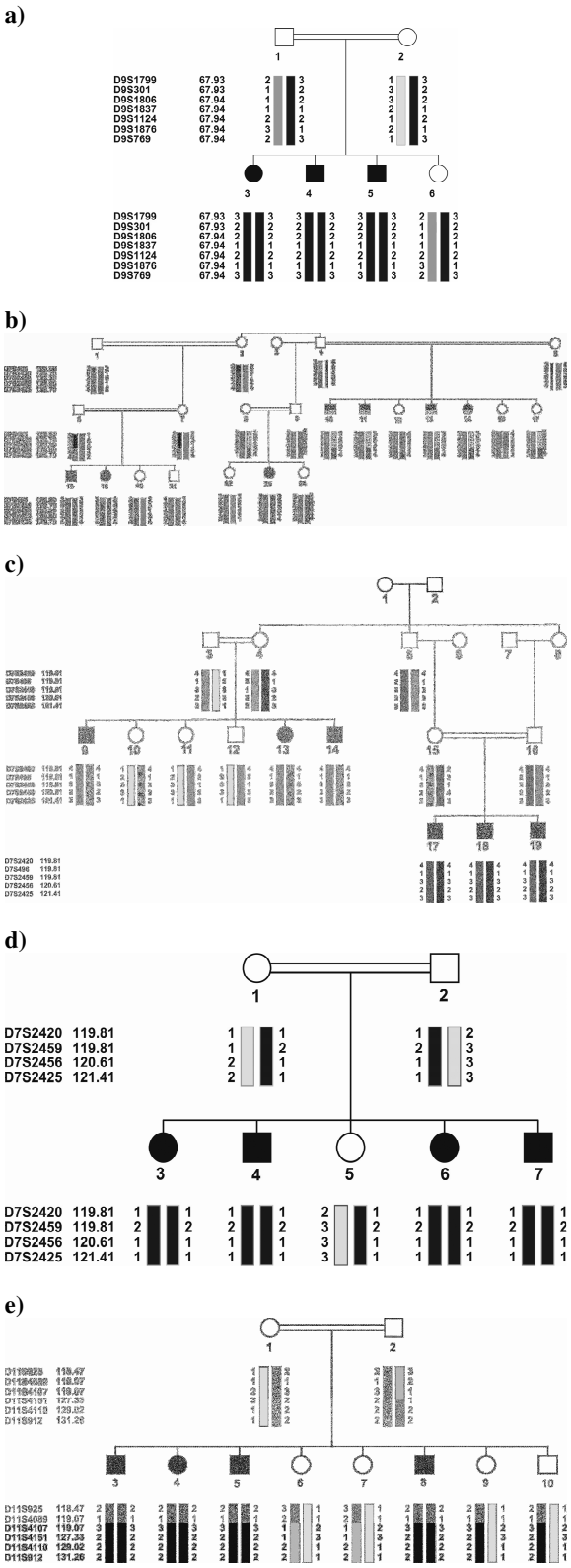

Figure 3. Haplotypes of families linked to DFNB7/11 (a), DFNB4 (b-d), DFNB21 (e). Patients are shown in black. a) Family IR-GHA linked to DFNB7/11. Markers D9S1837, D9S1124 and D9S1876 are intragenic.The order of markers is based on the Marshfiled map. b) In IR-ABY a cross-over in one of the upper generations of individual 4 between markers D7S2459 (which is an intragenic marker) and D7S2456, has created two haplotypes segregating with HL in two parts of the pedigree. c) Family IR-SH9 linked to DFNB4. Marker D7S2459 is intragenic.The order of markers is based on the Marshfiled map. d) Family IR-JOL linked to DFNB4. Marker D7S2459 is intragenic. The order of markers is based on the Marshfiled map. e) Family IR-JAF linked to DFNB21. Marker D11S4089 and D11S4107 are intragenic. The order of markers is based on the Marshfiled map.

(6.15%) families, negative for *GJB2*, were shown to be linked to DFNB7/11 [28]. In one study, out of 32 ARNSHL families with 2 or more patients and from different parts of Iran, 3 families (9.4%) showed linkage to DFNB7/11. It was concluded that the locus could be one of the most common causes of ARNSHL in the Iranian population [33].

No linked family was found for DFNB3 in our study. DFNB3 contains *MYO15*. In a study on consanguineous families of Pakistan, 10% were linked to DFNB3 (11 out of 112). Thus, it was suggested that at least 5% of the studied Pakistani ARNSHL population were caused by the gene [34]. In a study on 40 Iranian families from Qom and Markazi provinces of Iran with 3 or more ARNSHL, 2 families were linked to DFNB3 (5.8%) [27]. It is likely that a more frequent involvement of DFNB3 exists in some populations of Iran comparing to the others. More ARNSHL families have to be studied before reaching any definite conclusion on the issue.

We found one family linked to DFNB21 in this study. The corresponding gene, *TECTA,* can cause both dominant and recessive HL. The first report of ARNSHL family caused by the locus was on a large Lebanese family [29]. In a study, linkage to DFNB21 was found in 3 (6.6%) out of 45 Iranian consanguineous families which were negative for *GJB2* mutations [30]. In another study on 75 Iranian families segregating ARNSHL, 1 family (1.33%) was linked to DFNB21 [31] and finally, in a genetic linkage study of fourty 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 [35].

We were unable to find any linked family to DFNB59. The locus, mapped to at 2q31.2, and the corresponding gene*, PJVK*, has recently been identified in 4 Iranian families [32]. The protein was named pejvakin, derived from a Persian word meaning echo. 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 [33]. 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 [34]. In a recent study carried out by PCR-RFLP for two exons of *PJVK, on* 100 ARNSHL individuals from Shahrekord city, Chaharmahal va Bakhtiari provinve of Iran, no mutation was found. Since the study was not at all comprehensive, the possibility of DFNB59 involvement could not be ruled out [35]. Based on our study, It is possible that DFNB59 plays no major role in the pathogenesis of ARNSHL.

In the present study, after excluding the *GJB2* and *GJB6* causes, linkage analysis was performed for 5 DFNB loci including DFNB3, DFNB4, DFNB7/11, DFNB21 and DFNB59. Totally, the causes of 30.5 % of the studied ARNSHL families were clarified which is of importance given the lower prevalence of *GJB2* in the Iranian ARNSHL families as well as the extreme heterogeneity of the disease, with the engagement of over 70 loci. Other genetic and epigenetic factors should be investigated to clarify the etiology of the remaining families. The next phase of the study involves screening for other known loci and the genome scan analysis of the remaining families with high statistical power (SLINK≥ 3.3). 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.

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