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Specific Distribution of *GJB2* Mutations in Kurdistan Province of Iran; Report of a Relatively Isolated Population

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Abstract

Hearing Loss (*HL*) represents high genetic heterogeneity with an incidence of almost 1 out of 500 newborns in most populations. Approximately half of the cases have a genetic basis that most of them are autosomal recessive non-syndromic (*ARNSHL*) with *DFNB1*-related defect in many worldwide populations. Given the heterogeneity of the trait together with the unique infrastructure of Iranian population, made it necessary to study other loci than GJB2 in various ethnic groups in order to elucidate the genetic etiology of *HL* in Iran. The present study was designed to determine the contribution of four DFNB loci in Kurdistan Province, west Iran. In this connection, we performed *GJB2* analysis and homozygosity mapping using STR markers covering the *DFNB21*, *DFNB4* and *DFNB7/11* loci in 20 pedigrees with *ARNSHL*. *GJB2* mutations were the cause of *HL* in 20% of the patients. However, surprisingly, we only observed *35delG* and *IVS1+1G>A* mutation among the study population. We also did not find any family linked to *DFNB21, DFNB4* and *DFNB7/11*. Our results indicate the contribution of other loci in etiology of HL in Kurdistan that needs to be carefully investigated.

Keywords: *ARNSHL*; Iranian Kurdish people; *GJB2*; DFNBs.

Introduction

Hearing Loss (*HL*) is the most prevalent sensorineural disorder, affecting one out of every 500 newborns [1]. The majority (~70%) of cases are non-syndromic (non-syndromic hearing loss, *NSHL*) with the remaining 30% being associated with other clinical findings (syndromic hearing loss, *SHL*). The autosomal recessive form of *NSHL* (*A1RNSHL*) accounts for over 80% of *NSHL* and is expected to be even more prevalent in families from consanguinity belt, including Iran [2, 3].

Identification of over 100 loci and 60 genes (www.hereditaryhearingloss.org), emphasizes the heterogenic nature of *ARNSHL*. In spite of this heterogeneity, DFNB1 (*GJB2*) accounts for up to 50%

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of *ARNSHL* in many populations [4-7], with more than 300 variants described so far (www.hgmd.org). *GJB2* mutations also remain the major cause of *ARNSHL* in Iranian population but only approaching an average of ~16-18% of cases [8, 9]. On the other hand, analysis of *GJB2* mutations in different Iranian ethnic groups shows a remarkable difference. Therefore, it worth considering the molecular etiology of *ARNSHL* differs considerably among different Iranian ethnic groups.

SLC26A4 which encodes for the anion exchanger pendrin, is expressed in cells bordering the endolymph and spiral ganglion [10]. Mutations in the *SLC26A4* at DFNB4 locus cause both *ARNSHL* and Pendred syndrome, which is characterized by thyroid enlargement and *HL*. The *HL* phenotype is associated with abnormal inner ear development, identifiable by temporal bone imaging [10, 11]. To date, over 200 mutations have been reported in *SLC26A4* [MORL homepage. http://www.medicine.uiowa.edu/pendredand bor], making it the second cause of congenital *HL* after *GJB2*.

TMC1 gene encodes for transmembrane channel-like 1 protein which is a member of the subfamily A of transmembrane channel-like (*TMC*) gene family. *TMC1* mutations can cause both Autosomal recessive (DFNB7/11) and autosomal dominant (DFNA36) mode of hearing impairment [12, 13].

The *TECTA* gene encodes a-tectorin, which is one of the main noncollagenous parts of the tectorial membrane in the inner ear. Mutations in *TECTA* have been detected in both autosomal recessive and autosomal dominant *HL* (DFNB21and DFNA8/12) [14, 15].

Iranian population is heterogenous with high rate of consanguineous marriage; however, few investigations have ever addressed the prevalence of DFNB loci other than DFNB1 in individual ethnic groups. This study investigates the contribution of *GJB2* and the prevalence of 3 frequent DFNB loci including DNB4, DFNB7/11 and DFNB21 in 20 Iranian *ARNSHL* families from Kurdistan province, west of Iran. These results give a preliminary overview of the molecular epidemiological characteristics of hereditary *HL* in this region and will provide data for better genetic diagnostics and counseling.

Materials and Methods

Subjects

Totally 20 large pedigrees compatible with autosomal recessive mode of inheritance, with at least 2 affected members were recruited. The families were referred to the Center for Genetic Counseling, Center of the Welfare and Rehabilitation Organization, Sanandaj, Kurdistan province. All patients had bilateral prelingual hearing loss. The detailed history could not attribute *HL* to known environmental factors (such as CMV, trauma, aminoglycoside drugs). In addition, the possibility of *SHL* was excluded by detailed clinical examination. *HL* was confirmed with pure tone audiometry. After the Informed consent was obtained, peripheral blood samples were collected in 0.5 M EDTA containing tubes and genomic *DNA* was extracted using the salting out method [16]. Concentration and purity of *DNA* was measured (Nanodrop®TM1000-Thermo Scientific) and approximately 50 ng of the genomic DNA was used to genotype each sample. The study was approved by the Ethics committee of Tehran University o Medical Sciences (*TUMS*).

PCR-Sequencing and Screening of GJB2 Exons

From each pedigree, one affected member was sequenced for exon1 and 2 of *GJB2* gene. Following primers were used for amplification of exon1 forward: 5'CGACCACAGCCATCCCTGAACC-3' and reverse: 5'-GGACGTGTGTTGGTCCAGCC-3' which yielded a 684 bp band. Each *PCR* reaction condition was: : 0.8µl of each of the primers (10 PM), 7ul Mastermix (amplicon® Mastermix Contains MgCl2, Taq *PCR* buffer, *Taq DNA* polymerase and *dNTPs*) and 1µl *DNA* (50ng/ul) adjusted to 25 µ by ddH2O. *PCR* reactions were performed under the following condition: initial denaturation at 95°C for 2 minutes, followed by 33 cycles of denaturation at 95 °C for 30 s, 72 °C for 60 s, and final extension at 72 °C for 5 min.

Exon 2 was amplified based on the protocol described before. *PCR* products were run in 1/5% agarose gel electrophoresis. Bands were visualized by *EtBr* staining. Afterward,the *PCR*-amplified products were sequenced on an *ABI* 3130 automated sequencer (Macrogen®, South Korea). Sequencing data were analyzed by chromas software. After identification of each variant, ensemble.org, dbSNP (http//: www.ncbi.hlm.nih.gov/snp), 1000 genome databases (http//:browser.1000genome.org) and HGMD (http:// www.hgmd.cf.ac.uk/ac/index.php) were searched for previously known variants. Sequence variant numbering was based on the transcript ENST00000382848 for *GJB2*.

Genotyping and linkage analysis

At least, three informative short tandem repeat (*STR*) markers were analyzed for each locus. The *STR* markers were chosen based on their physical distance found at *NCBI* UniSTS and Map Viewer. Further markers were examined upon encountering an uninformative marker.

Table 1. List of the STR markers used in this study

Locus (gene), Physical location(bp), Category& function	Marker	Physical position (bp)	PCR product range	Forward primer (5'→3')	Reverse primer (5'→3')
	D7S2420	106889928-106890211	240-290	CCTGTATGGAGGGCAAACTA	AAATAATGACTGAGGCTCAAAACA
	D7S496	107154713-107154849	129-141	AACAACAGTCAACCCACAAT	GCTATAACCTCATAANAAACCAAAA
DFNB4(*SLC26A4)*	D7S2459	107331501-107331642	140-152	AAGAAGTGCATTGAGACTCC	CCGCCTTAGTAAAACCC
107301080..107358254)	D7S2456	107683218-107683460	238-252	CTGGAAATTGACCTGAAACCTT	ACAGGGGTCTCTCACACATATTA
	D9S1837	75185129-75185367	205-251	CATGATGGTGGTCTCTGG	GGTGGGGCTCAAAGAGTAG
	D9S1806	74201357-74201620	216-266	TTTTAGGTGTTCTCAGTACATGC	GGGAGCAACATTTTGACATT
	D9S1124	75224065-75224327	252-276	GGTGCCCACCATACACTACT	TCTAATCCTTCCTTCCCTCG
DFNB7/11(*TMC1)*	D9S1876	75232791-75232938	132-152	GATGTACCCAGAGAAGTCTCG	AGTGGTTACCATTTACCCAAG
75136717..75451267)	D9S301	73802720-73802954	209-237	AGTTTTCATAACACAAAAGAGAACA	ACCTAAATGTTCATCAAAAGAGG
	D9S1822	74930323-74930483	157-163	AAGTTTGGCTTCTGCTGTAAGGGTC	AATTCCCCCAGGCTGAGTG
	D9S1799	73366891-73367055	139-178	TTGCCAACTATTTTAGCCC	TGCAGTTTCAATCCACATC
	D11S4107	121049124-121049321	172-212	TCATTCTACAAGACTAGCATTACC	GCTTGATCATGGTGTATTATCTT
	D11S925	120828264-120828438	172-199	AGAACCAAGGTCGTAAGTCCTG	TTAGACCATTATGGGGGCAA
DFNB21*(TECTA*)	D11S912	128624097-128624205	101-123	TCGTGAGANTACTGCTTTGG	TTTTGTCTAGCCATGATTGC
120973375..121061515	D11S4151	126292160-126292309	145-155	GTCTTCCCACCTTGGATATGGGTA	AATGGGCACCTCCACCCTATTAGT
	D11S4089	120989673-120989875	199-213	ATTCCTAGTTCCCTCATAAACACTG	TAATCAAAGGCTGTAGTGAATTGG
	D11S4110	126971672-126971780	93-107	TGAGCCTCCCAGTACCTACC	GTTTGTGGCAGAGCCCTAAG

The used primer sequences are shown in Table 1.

STR markers were amplified by touch down *PCR* in an *ABI* thermal cycling 2720 (Applied Biosystems®, *USA*). *PCR* protocol and thermal cycling program are available upon request. Generally, PCR products were run on 8-12% *PAGE* at 15 mA for 4-6 h. Bands were visualized by silver staining. Alleles were assigned by visual assessment.

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 [17]. After linkage analyses, haplotypes were reconstructed via Simwalk and were visualized by Haplopainter software version 029.5 [18].

Results

Screening of GJB2 Exons

Totally 20 large pedigrees including 46 affected and 60 healthy members were recruited , among which four pedigrees showed *GJB2* mutations. Interestingly, three of four were compound hetrozygous for *35delG* and splice site mutation of *IVS1+1G>A*, while one pedigree was homozygous for *35delG*. Pedigrees of the families positive for *GJB2* mutations and electropherograms of the mutations are depicted in Figure 1.

Genotyping STR Markers and Homozygosity Mapping

Families negative or heterozygote for DFNB1 mutations were screened for DFNB4, DFNB21 and DFNB7/11 loci by linkage analysis which no family showed to be linked to the mentioned loci.

Discussion

HL is the most common sensory defect with high clinical and genetic heterogeneity [19]. Up to now, over 100 loci have been mapped and about 60 genes identified for *ARNSHL* (http://www.hereditary hearingloss.org). *GJB2* mutations in the DFNB1 locus

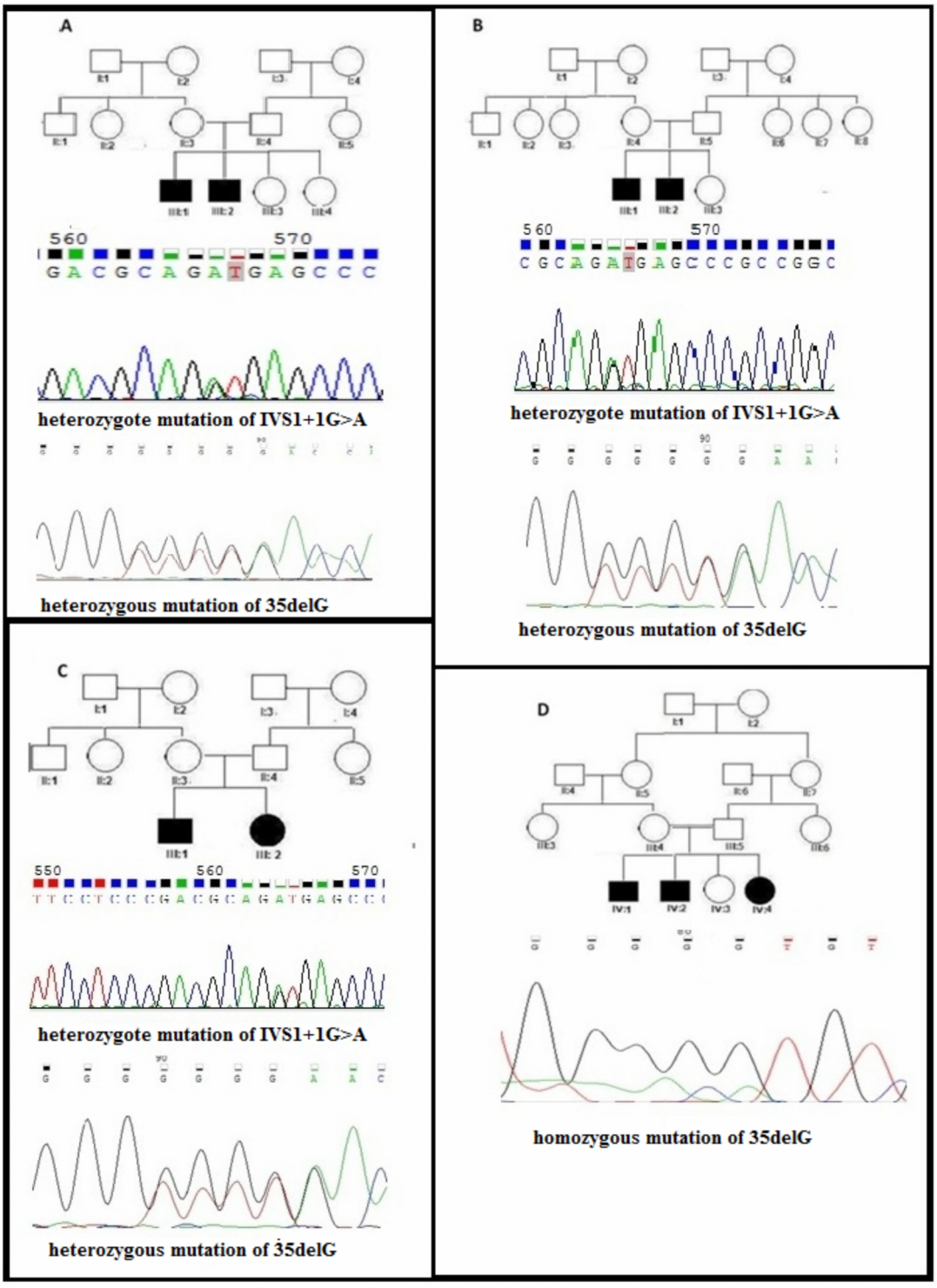

Figure 1. Pedigrees carried mutations at DFNB1 locus

are the main cause of *ARNSHL* in many ethnic groups, including Iran [20-22]. The prevalence of *HL* in Iran is 2–3 times greater than other populations [19] while *GJB2* mutations are responsible for 16.7% of ARNSHL cases and this prevalence may vary among different ethnic groups.

Kurdistan population is located in the west part of Iran. Kurdish population of Iran is distributed among 4

different provinces including: Kurdistan, Kermanshah, Ilam and Azerbayjan while the population of Kurdistan province remains isolated by culture and religious approach. Findings of the current study showed that contribution of *GJB2* mutations in Kurdistan province of Iran is 20% that almost seem to be consistent with results of Mahdieh et al [23] also conducted on a Kurdish population. However, distribution of the variants is not exactly the same. In the current study, only 2 *GJB2* variants, including *35delG* and *IVS1+1G>A,* were observed while the other study found six variants including *35delG, R127H, del120E, R32H, IVS1+1G>A* and *R184P* [24]. It worth mentioning that study population of Mahdieh et al, mostly originate from Kermanshah province, which is less isolated in comparison to Kurdistan. This might be a possible explanation for the different mutational spectrum observed. This discrepancy may also arise due to differences in sample size which was larger in Mahdieh's et al.

In the Iranian Azeri patients, a frequency of 28% of mutant *GJB2* have been reported [25]. In HL population of Oman [26], Pakistan [27],Turkey [28], and Slovakia [29], contribution of *GJB2* gene mutations is 0%, 6.1%, 25%, and %45.6, respectively. In addition, some studies indicated 31.7% contribution of *GJB2* mutation in Turkey [30], in neighboring of Iranian Azeri Turkish residents.

Many allelic variations have been reported in *GJB2* (http://www.hgmd.cf.ac.uk/ac/index.php). Among different deafness-causing alleles, *35delG* mutation is the most common one. This frame shift mutation results in a truncated protein [19]. The frequency of this allele differs among different Iranian ethnic groups [21], for example, it is 71.6 % in Iranian Azeri Turkish population [31]. The present findings indicate a frequency of 62.5% and 37.5% for 35delG and *IVS1+1G>A* among Kurdish people from Kurdistan province of Iran, respectively. *R127H, delE120, W24X, V27I+ E114G, R184P, -3170G4A* and *235delC* are the other most common mutations of *GJB2* in Iranian population [19] which were not observed in this study.

Here, we found no family linked to DFNB4, DFNB21 and DFNB7/11.

SLC26A4 gene, located on the DFNB4, 7q31[32], is considered to be the second leading cause of autosomal recessive HL in many populations [33]. Studies had reported frequencies of 10% and 4.8% for SLC26A4 mutations in Iran [34, 35]. Interestingly, unlike the previous reports, no mutation was found in *SLC26A4* in our study population which consequently suggests that DFNB4 may not be a major locus in etiology of HL in Kurdistan Province of Iran.

TMC1 at the DFNB7/11 locus on chromosome 9q31-21 (MIM 606706, GeneID 117531), encodes transmembrane channel-like 1 protein [38]. Mutations of this gene has been reported as a frequent cause of *ARNSHL* in several populations, including Pakistan, India and also Iran [39]. In a heterogeneous Iranian study population, 9.4% of *HL* families were linked to DFNB7/11 [40]. However another study failed to replicate this frequency and only a 2% contribution was observed [34]. Here, our results show that *TMC1* mutations are not an important cause for *ARNSHL* in Kurdistan population.

TECTA gene which has been mapped on chromosome 11q22–q24 (MIM 602574, GeneID 7007) [41, 42], encodes a protein of 2155 amino acids. *TECTA* mutations have been found in French, Belgian, Swedish and Austrian families [41]; in Iran, they are responsible for 4.1% of ARNSHL cases [43-45]. However, unlike previous studies, no mutation in *TECTA* was observed in our study population which suggests its minor contribution in etiology of HL Kurdish people from Iran.

Conclusion

In this study, we could find the genetic basis of *HL* in only four out of 20 pedigrees of the studied population, while the etiology in about 80% remains to be identified. *GJB2* mutations were the most common cause of *HL*, accounting for about 20% of the families. Regarding high frequency of *35delG* and *IVS1+1G>A*, it worth to detect carrier frequency of these two variants among normal population.

Other loci should also be investigated in order to clarify the molecular etiology of hearing loss in Kurdistan province. Our results highlight the heterogeneity of *HL* and provide clues for a different panel of DFNB loci involved in the etiology of hearing loss in the province.

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