Diverse pattern of gap junction beta-2 and gap junction beta-4 genes mutations and lack of contribution of DFNB21, DFNB24, DFNB29, and DFNB42 loci in autosomal recessive nonsyndromic hearing loss patients in Hormozgan, Iran

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Background: We aimed to determine the contribution of four DFNB loci and mutation analysis of gap junction beta-2 (*GJB2*) and *GJB4* genes in autosomal recessive nonsyndromic hearing loss (ARNSHL) in South of Iran. **Materials and Methods:** A total of 36 large ARNSHL pedigrees with at least two affected subjects were enrolled in the current study. The *GJB2* and *GJB4* genes mutations were screened using direct sequencing method. The *GJB2* and *GJB4* negative families were analyzed for the linkage to DFNB21, DFNB24, DFNB29, and DFNB42 loci by genotyping the corresponding STR markers using polymerase chain reaction-PAGE method. **Results:** We found a homozygous nonsense mutation W77X and a homozygous missense mutation C169W in 5.55% of studied families in *GJB2* and *GJB4* genes, respectively. Five heterozygous mutations including V63G, A78T, and R127H in *GJB2* gene, and R103C and R227W in *GJB4* gene were detected. We identified two novel variations V63G in *GJB2* and R227W in *GJB4*. *In silico* analysis predicted that both novel variations are deleterious mutations. We did not unveil any linkage between DFNB21, DFNB24, DFNB29, and DFNB42 loci and ARNSHL among studied families. **Conclusion:** This is the first report of *GJB2* and *GJB4* mutations from Hormozgan population. According to the previous publications regarding *GJB2* and *GJB4* mutations, the distribution of the mutations is different from other parts of Iran that should be considered in primary health-care programs. Further investigations are needed to evaluate the contribution of other loci in ARNSHL subjects in South of Iran.

Key words: Connexin 30.3, deafness, gap junction beta-2, linkage analysis

INTRODUCTION

Congenital hearing loss (HL) is considered as a most prevalent sensorineural disorder associated with both genetic and environmental factors that affect 1–2 in 1000 neonates worldwide.[1] In Iran, the incidence of HL has been estimated one case in 166 live births.[2]

This high frequency of HL, at least in part, steams from high consanguineous marriage rate (38.6%) in Iranian population.[3] In the last decades, myriad investigations have been carried out to disclose etiopathogenesis of HL that revolutionized our knowledge concerning molecular basis of this disorder. However, owing to high genetic heterogeneity and multiple phenocopies of HL, more studies in this area are needed.

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Genetic causes account for approximately 50% of HL and can be inherited as either syndromic (~30%) or nonsyndromic (~70%) types with a spectrum of inheritance patterns. Autosomal recessive nonsyndromic HL (ARNSHL) is the common hereditary type of HL that has been causally linked to mutations in over eighty different loci nominated as DFNB loci.[4] Among various loci, DFNB1 (gap junction beta-2 [*GJB2*] gene, MIM 121011) has been identified as a master switch. *GJB2* gene encodes connexin 26 protein that its mutations are responsible for nearly half of ARNSHL cases in many populations. Since the initial reports of the identification of *GJB2* gene mutations in ARNSHL, more than 100 variants have been identified throughout the gene.[5] With regard to ethnicity, the contribution of *GJB2* gene mutations in ARNSHL ranges from 22% (at Northwest) to <5% (at South) in Iranian cases; however, considering the total number of ARNSHL patients, 4.17% of them carry a single heterozygous mutation of *GJB2* gene.[2,6] Therefore, the *GJB2* gene mutations only explain a fraction of ARNSHL and the reported rates illustrated the possible role of other loci in Iranian cases. Studies in Western countries illustrate *GJB6* gene to be involved in ARNSHL as a common second mutated allele; however, regarding the investigations about *GJB6* gene in Iranian cases, it does not seem that this gene highly contributes to the pathogenesis of ARNSHL as a second allele.[7,8] As the encoding gene for connexin 30.3, *GJB4* has been investigated in the recent studies including a study in Iran by Kooshavar *et al.* in 2013 that indicates its possible role as a second mutated gene in digenic inheritance of ARNSHL.[6] This study reveals several missense (c.451C>A/Wt; c.507C>G/Wt; c.542C>T/Wt), frameshift (c.155_158delTCTG/Wt), and silent (c.219C>T/Wt) heterozygote variations in GJB4 gene of which the missense c.542C>T variation seems to have a pathogenic role since it was detected only among patients but not in control individuals.[6] Thus, *GJB4* stands as a potential candidate gene in patients with compound heterozygote inheritance pattern that provokes us to investigate it among our population.

There are evidences to support an association between DFNB21 (*TECTA*, MIM 602,574), DFNB24 (Radixin [*RDX*], MIM 179,410), DFNB29 (claudin 14 [*CLDN14*], MIM) and DFNB42 (*ILDR1*, MIM 609,739) loci, and ARNSHL.[9] The autosomal recessive locus DFNB21 is characterized by prelingual, severe HL phenotype that harbors *TECTA* gene. *TECTA* encodes a major noncollagenous component of the tectorial and otolithic membranes called alpha tectorin, and its mutations lead to disruption of sound transmission to the stereocilia.[10] *RDX* is mapped on chromosome 11q23, designated DFNB24. It is believed that *RDX* functions as cross-linkers between plasma membranes and actin filaments.[11] *CLDN14* gene, located on DFNB 29 (chromosome 21q22.13), encodes a tight junction protein which has been implicated in the inner ear ion

homeostasis. Mutations in *CLND14* disrupt ionic permeability of the paracellular barrier of the reticular lamina and lead to the death of hair cells, ultimately.[12] The *ILDR1* gene which encodes immunoglobulin-like domain containing receptor 1, maps to chromosome 3q13.33 while the precise function of *ILDR1* is unknown, a possible role in epithelial barrier functions is postulated.[9]

To the author's knowledge, there is no data available for the association of DFNB21, DFNB24, DFNB29, and DFNB42 loci with ARNSHL in South of Iran. To illuminate the link between these loci and ARNSHL, which will be useful in genetic counseling for such families, contribute to reduce the incidence of HL and decrease socioeconomic burden, we aimed to analyze *GJB2* and *GJB4* genes mutations and evaluate the contribution of these loci in ARNSHL families in Southern Iran.

MATERIALS AND METHODS

Study subjects
A total of 36 large ARNSHL pedigrees with at least two affected subjects were included in this descriptive study from Hormozgan province of Iran. HL screening for members of the families was performed by pure tone audiometric test, enabling determination of the degree, type and configuration of a HL and clinical examination, with special emphasis on recognizing potential environmental causes of hearing impairment such as infections, trauma, and information on exposure to known or possible ototoxic drugs or evidence of syndromic forms of deafness. HL informational questionnaires were obtained from all members of families, and the pedigrees were drawn based on the filled-out questionnaires and interview with families by genetic counselors. Based on their pedigree, 36 families (167 individuals) were finally screened as ARNSHL. This study was approved by the Institutional Review Board and Ethics Committee. All Participants signed an informed written consent form, and filled-out demographic characteristics form before enrollment.

DNA extraction and gap junction beta-2 gene mutation screening
Genomic DNA was extracted from EDTA-containing peripheral blood of all members of the families using commercial kit (Genet Bio, South Korea) according to the manufacturer's instructions. The quality and quantity of extracted DNA were confirmed by agarose gel electrophoresis and NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA), respectively. All affected subjects were screened for mutations in exon 2 of the *GJB2* gene using direct sequencing technique. To amplify the exon 2 of the *GJB2* gene, the primers F: 5'-CGTCTTTTCCAGAGCAAACCG-3' and R: 5'-AGCTCCATTGTGGCATCTGG-3' were used

in the polymerase chain reaction (PCR) procedure. PCR thermal conditions were as follows initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. The amplified fragments of *GJB2* gene (809 bp in length) were detected on 1% agarose gel. Subsequently, the PCR-amplified products were bidirectionally sequenced using Sanger sequencing (Macrogen, South Korea).

Gap junction beta-4 gene mutation screening

Negative and heterozygous samples for *GJB2* gene mutations were tested for *GJB4*. PCR was performed to amplify the entire coding sequence of *GJB4* with F primer: 5' TCAATCGCACCAGCATTAAG 3' and R primer: 5' GGGGGA CCTGTTGATCTTATC 3'. PCR thermal conditions were as follows initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, final extension at 72°C for 5 min. Finally, the PCR products were separated by electrophoresis procedure on 1% agarose gel. DNA sequencing of the PCR-amplified products was carried out directionally (Macrogen, South Korea), using the same primer. *GJB2* and *GJB4* Sequencing data were analyzed with CLC genomic workbench 4 software and compared with the published sequences of Human Genome Database and GenBank (*GJB2*: NG_008358.1; *GJB4*: NG_016243.1).

STR markers genotyping and linkage analysis

Negative samples for *GJB2* gene mutations were tested for linkage analysis of DFNB21, DFNB24, DFNB29, and DFNB42 loci using STR markers which are shown in Table 1. Based on STRs physical distance in NCBI Map Viewer and NCBI UniSTS, we selected the STRs and their primers. A panel of 15 different STR markers was genotyped for DFNB21, DFNB24, DFNB29, and DFNB42 loci, according to the selection criteria including greater heterozygosity

values, shorter amplicon, and locating near the known locus. The PCR solutions contained 100 ng genomic DNA, 10X PCR buffer, 10 pmol of each primers, 10 nmol of each deoxyribonucleotide triphosphates, 1.5 mmolMgCl2 and 1 U Taq polymerase in a final volume of 25 μl. A two-step touchdown thermal cycling was designed for each STR markers based on the Tm of both primers. PCR was started with an initial denaturation step 94°C for 5 min was followed by 7 cycles of denaturation (94°C, 15 s), annealing (60°C, 40 s) and extension annealing (72°C, 40 s) followed by a final 25 cycles of denaturation (96°C, 40 s), annealing (58°C, 40 s), and extension (72°C, 40 s) and final extension at 72°C for 10 min. Finally, the PCR products were separated by electrophoresis procedure on 10% polyacrylamide gels stained by silver nitrate.

RESULTS

Families and clinical data

Out of 36 families (167 people) who enrolled in this study, 72 individuals had ARNSHL (43.11%). Twenty out of 36 studied families had consanguineous marriage (55.55%). Hearing is measured in decibels (dBs). The threshold for each frequency (0-dB) indicates the level at which a normal young adult hears a tone burst 50% of the time. It is considered normal hearing if an individual's thresholds are within 25 dB of normal thresholds. The subjects divided into mild (26–40 dB), moderate (41–60 dB), severe (61–80 dB), and profound (≥80 dB) hearing impairment according to World Health Organization classification of HL.[13] The frequencies of the moderate, severe, and profound HL among affected subjects were 11.11% ($n = 8$), 58.33% ($n = 42$), and 30.55% ($n = 22$), respectively.

Screening of gap junction beta-2 and gap junction beta-4

After comparing sequencing data with published (UCSC Genome Browser) sequences, we revealed a homozygous

Table 1: Short Tandem Repeat markers of each locus and their primer sequences

Locus (gene)	Marker	Forward primer (5'→3')	Reverse primer (5'→3')
DFNB29 (CLDN 14)	D21S1440	GAGTTTGAAAATAAAGTGTTCTGC	CCCCACCCCTTTTAGTTTTA
	D21S1252	TCTGTCTTTGTCTCACTATCTG	GCAATGCTCTGTGGCT
	D21S270	GAAATGTTTTAATAAATGGTGGTTA	ACAAAGTTATGGTCAAGGGG
DFNB24 (RDX)	D11S927	AGTGAGCCGAGTTCGC	ACCAAAAGCCTGGAATG
	D11S1391	TGCATGCATACATACATACATACA	CATCCATCCCTCTGTCTCTG
	D11S1778	TAACCTNCTACACAGTGTCGTT	GCTTCAGCAGAGAAGCCAT
	D11S4206	ATGTGAATTGCTCTCTGTGTCACTC	TGGAAACCTAATACCTAGCCTTTTG
DFNB21 (TECTA)	D11S4089	ATTCCTAGTTCCCTCATAAACACTG	TAATCAAAGGCTGTAGTGAATTGG
	D11S4107	TCATTCTACAAGACTAGCATTACC	GCTTGATCATGGTGTATTATCTT
	D11S925	AGAACCAAGGTCGTAAGTCCTG	TTAGACCATTATGGGGGCAA
	D11S4144	AGACAGGTCTTCACCACAGC	ATGGCTTCCAGGTTAGTGC
DFNB42 (ILDR2)	D3S3720	AGCAATCCTCCAGCCT	GCTGTATCTGAATCTAAAGCCT
	D3S3709	TGCATGTGCGTGTATATTTG	ACAGTTGTGAGACACCACCTATT
	D3S3576	GATCTAGTTGGCAAGGCGT	TTGGACTCGAGCAATCC
	D3S4009	TGCCCTAACACATCATGTTG	GGGCACGTGTAACCTTATGT

nonsense mutation W77X (231 G>A) and a homozygous missense mutation C169W (507 C>T) in 2/36 (5.55%) of studied families in the coding sequence of *GJB2* and *GJB4* genes, respectively. Furthermore, DNA screenings identified other three allelic variants in *GJB2* gene in 7/72 (9.72%) of subjects [Table 2]. Out of the 7 patients, 4 for V63G (188T>G), 2 for A78T (232 G>A), and 1 for R127H (380 A>G) were heterozygote in coding region of *GJB2* gene [Table 2 and Figure 1]. Moreover, we found two additional heterozygous variants including R103C (307 C>T) and R227W (679 C>T) in the coding region of *GJB4* gene in 2/72 (2.78%) of patients [Table 2 and Figure 2]. No evidence of digenic inheritance mode was observed between *GJB2* and the *GJB4* mutations in patients who carried heterozygous mutations.
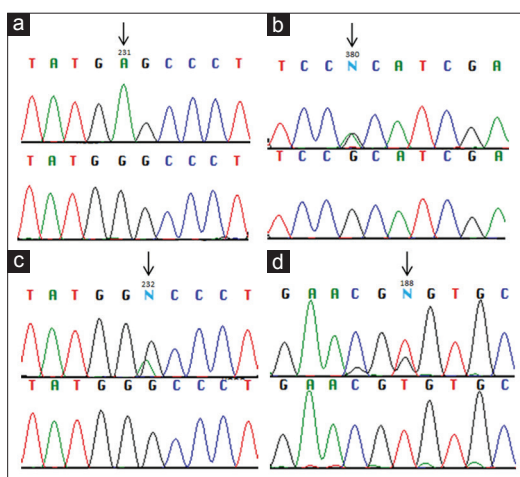
In silico analysis of new nonsynonymous variation *c.188T>G* in gap junction beta-2 and *c.679 C>T* in gap junction beta-4 genes

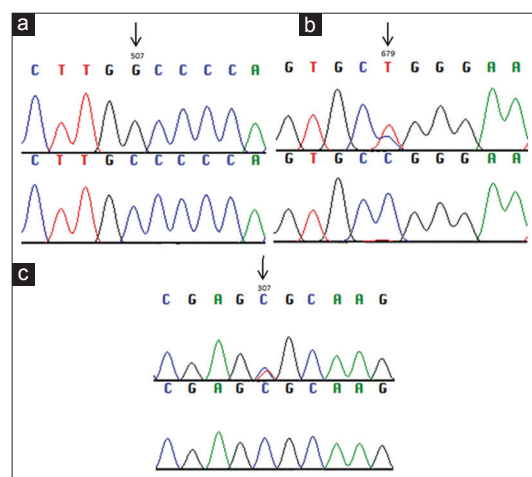Among the detected variation, two variation including c.188T>G in *GJB2* and c.679 C>T in GJB4 genes have not been previously reported. To predict the putative effects of amino acid substitutions at codon positions 63 and 227 on the structure and function of Cx26 and Cx30.3 proteins, respectively, we used PolyPhen-2 online software (http://genetics.bwh.harvard.edu/pph2/index.shtml). The substitution of valine by glycine at codon 63 of Cx26 and arginine by tryptophan at codon 227 of Cx30.3 were predicted to be deleterious.(sensitivity: 0.00; specificity: 1.00).

Linkage analysis of DFNB21, DFNB24, DFNB29, and DFNB42 loci

We failed to identify linkage of the DFNB21, DFNB24, DFNB29, and DFNB42 loci among thirty-six *GJB2* negative families. If at least three STR markers (fully informative) of a DFNB locus did not show homozygosity among the affected individuals of a family, then the locus would be determined unlinked. All members of *GJB2* negative families, including parents, deaf, and healthy siblings were heterozygous for all studied STR markers.

DISCUSSION

Owing to high genetic heterogeneity in ARNSHL, designing a general efficient genetic test for diagnosis of the precise molecular basis and genetic counseling is complicated.[14] It becomes more and more complicated because the type and frequency of pathogenic mutations are notably dependent on either ethnic or geographic origins of the populations.[15] The best approaches to overcome these obstacles are the identifying common pathogenic mutations and determining significantly associated loci in ARNSHL patients for carrier screening.[16] Hence, we designed this study to screen *GJB2* and *GJB4* mutations and to evaluate the contribution of the DFNB21, DFNB24, DFNB29, and DFNB42 loci in ARNSHL families in Southern Iran.

Table 2: Gap junction beta-2 and gap junction beta-4 gene mutations found in Iranian families

Gene	Nucleotide change	Protein change	Mutation type	Number of found in participants (based on family)	
				Heterozygous	Homozypous
GJB2	188 T > G	V63G	Missense	4	-
GJB2	232 G > A	A78T	Missense	2	-
GJB2	380 A > G	R127H	Missense	1	-
GJB2	231 G > A	W77X	Nonsense	-	1
GJB4	307 C > T	R103C	Missense	1	-
GJB4	679 C > T	R227W	Missense	1	-
GJB4	507 C > T	C169W	Missense	-	1

GJB = Gap junction beta

Figure 1: Sequence chromatographs of the mutated part of the gap junction beta-2 gene in proband of each family as compared to wild type. Arrows denote the mutations. (a) 231 G>A nonsense mutation. (b) 380 A>G missense mutation. (c) 232 G>A missense mutation. (d) 188 T>G missense mutation

Figure 2: Sequence chromatographs of the mutated part of the gap junction beta-4 gene in proband of each family as compared to wild type. Arrows denote the mutations. (a) 507 C>T missense mutation. (b) 679 C>T missense mutation. (c) 307 C>T missense mutation

Analysis of the coding sequence of the *GJB2* gene was revealed four different allelic variants including W77X (231 G>A), V63G (188T>G), A78T (232 G>A), and R127H (380 A>G) in subjects. The pathogenic role of W77X (231 G>A), A78T (232 G>A), and R127H (380 A>G) *GJB2* mutations was previously described in various papulations[5,17] while V63G (188T>G) novel sequence variation was not reported. The W77X (231 G>A) homozygous mutation was found in one subject of studied family. This nonsense mutation occurs at the transmembrane region of Cx26, associated with moderate-to-severe HL and have been reported commonly in Indian and Pakistani families[17-19] the A78T (232 G>A) mutation which occurs in the second transmembrane domain of Cx26 was rarely detected in Indonesian and Chinese subjects.[20] The pathogenic role of R127H (380 A>G) is controversial, and several investigations have concluded that R127H most likely represents a polymorphism.[21] A novel V63G (188T>G) sequence variation was predicted as a deleterious mutation using PolyPhen software. The mutated residue lies in the first extracellular loop of Cx26 which serves as the key docking site to hemichannels at the extracellular medium.[22] According to the previous studies, it has been demonstrated that mutations in lining residue of this domain lead to disruption of voltage properties of gap junction pore.[23]

We detected a homozygous missense mutation C169W (507 C>T) in coding region of *GJB4* in one patient with severe HL phenotype. This mutation results in a substitution of cysteine to tryptophan at codon 169 of Cx30.3 protein within the second extracellular loop domain which play a fundamental role in docking of gap junction hemichannels.[24] The C169W mutation has previously been reported in Taiwanese and Iranian families.[25,26] In addition, the sequencing of *GJB4* revealed two allelic variants R103C (307 C>T) and R227W (679 C>T) variants. The association of the first variant with ARNSHL have previously been founded.[27] *In silico* analysis, demonstrated that the second variation, R227W (679 C>T), is a deleterious mutation.

We did not unveil any linkage of the DFNB21, DFNB24, DFNB29, and DFNB42 loci among *GJB2* and *GJB4* negative families. In accordance with our findings, Sadeghi *et al*. were not found evidence of linkage for DFNB21 locus in ARNSHL patients in Markazi and Qom provinces of Iran.[28] However, several investigations have reported different frequencies of DFNB21 mutations (2.7%–6.6%) in various ethnics of Iran.[29] Data from the previous studies have been indicated that mutations in *RDX* gene (DFNB24) are less common in ARNSHL cases of Middle Eastern countries such as Iran, India, and Pakistan.[9] These data are in harmony with our results. Although the mutations of *CLDN14* gene (DFNB29) were identified in Pakistani and Grecian subjects with ARNSHL, it seems that its mutations are not

linked to profound deafness in Iran, Tunisia, and Turkey.[30] Up to now, more than fifteen different mutations have been detected in Pakistani, Saudi Arabian, and Czech as well as in Iranian populations.[31-33] However, we failed to find linkage of the DFNB42 locus among studied families.

CONCLUSION

In this study, we observed seven mutations in the coding region of *GJB2* and *GJB4* genes that three of them (W77X, A78T, and R127H) in *GJB2* and two mutations (R103C and C169W) in *GJB4* have been reported worldwide. We also found two novel allelic variations including V63G (188T>G) and R227W (679 C>T) in coding region of *GJB2* and *GJB4* genes, respectively. *In silico* analysis predicted that both novel variations are deleterious mutation. In contrast to several publications from Iran, we did not unveil any linkage of the studied loci among *GJB2* and *GJB4* negative families. Observed disagreement between the reports, highlights the diversity of the Iranian population and the fact that the contribution of other loci should be investigated in South of Iran.

Conflicts of interest
There are no conflicts of interest.

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