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Original Article

Gjb3 Gene Mutations in Non-Syndromic Hearing Loss of Bloch, Kurd, and Turkmen Ethnicities in Iran

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Abstract

Background: Hearing loss (HL) is one of the most common heterogeneous congenital disabilities worldwide. Gap junction protein β-3 (*GJB3*) gene encodes Connexin31 protein (Cx31). The hereditary type of hearing impairment in this gene are known to cause both autosomal recessive and autosomal dominant form. In addition, *GJB3* mutations have been involved in sensorineural deafness, erythrokeratodermia variabilis (EKV), and neuropathy diseases. We aimed to investigate *GJB3* mutations in people suffering from HL among three different ethnicities of Iranian population (Baloch, Kurd, and Turkmen).

Methods: In this descriptive study, 50 *GJB2*-negative non-syndromic hearing loss (NSHL) Iranian individuals from 3 ethnic groups of Baloch (n=17), Kurd (n =15) and Turkmen (n=18) were enrolled. DNA extractions, PCR, and mutation detection was carried out for the two large deletions of the *GJB6*, del (GJB6 -D13S1830,) and del (*GJB6* -D13S1854) followed by direct DNA sequencing method for the *GJB3*.

Results: DNA sequencing of *GJB3* was shown a missense heterozygous mutation rs199689484 (NM_024009.3) GJB3: c.340G>A (p.Ala114Thr) in a Baloch patient, and a polymorphism rs35983826 (NM_024009.3) *GJB3*: c.798C>T (p.Asn266=) in a Turkman patient, in coding region of the *GJB3*. We did not detect del (GJB6 -D13S1830) and del (GJB6 -D13S1854) among these three ethnicities in Iran.

Conclusion: Deafness is a heterogeneous disorder. Specific genes and mutations contribute to hearing loss that varies from locus to locus as well as from population to population.

Keywords: Non-syndromic hearing loss (NSHL); Ethnicity; Iran; Connexin31 (Cx31)

Introduction

Hearing Loss (HL) is an extremely heterogeneous condition, which is one of the most common features of birth defects (1, 2). The prevalence of hereditary bilateral permanent hearing loss is 1 in 500 neonates in developed countries prevalence has increased to 3.5 per 1000 (3). Hearing loss can be resulted from environmental factors (acquired) or have a Genetic base (hereditary) (4). Hearing loss can be classified into sensorineural (inner ear anomalies), conductive (middle ear malfunction) or a mixture of both (5). Genetically based hearing loss is either syndromic or non-syndromic. It can also be classified based on its onset, pre-lingual or post-lingual. Other categories associated with the various types of non-syndromic are DFNA (DFN: deafness; A: dominant), or DFNB (B: recessive) or DFNX (X: X-linked) or mitochondrial (6).

More than 160 loci, around 119 genes, have been known in non-syndromic hearing loss (7). Gap junctions (GJs) are intercellular channels that allow small molecules of the cytoplasm of a cell to be directed to the adjacent cell, including ions such as K^+, Na^+, and Ca^{++}. A gap junctional channel is made by two hemichannels. Each hemichannel is formed of six subunits, which have compounded of connexons (8, 9). Connexins (Cx) are arranged to Gap junction alpha (GJA) protein and Gap junction beta (GJB) protein. Connexins GJB contains 21 isoforms in humans, such as *GJB2* (Cx26), *GJB6* (Cx30), *GJB3* (Cx31) (10). One of the most common mutations is in the *GJB2* (Cx26) gene which are known to be the typical cause of both autosomal non- syndromic hearing loss (ADNSHL), and recessive non- syndromic (ARNSHL) hearing loss in the world, as well as in Iran (11). *GJB6* (MIM604418) locality is the same as *GJB2* and positions on 13q12, which encodes connexin 30 kDa (Cx30). It has 76% homology with connexin26 (12). *GJB6* has four large deletions including 150kb deletion, 140kb deletion, del(*GJB6*-D13S1854) 232 kb, del(*GJB6*-D13S1830), 342 kb, 920kb deletion, and del(chr13:19,837,344–19,968,698) (13-17).

GJB3 has also been linked to non-syndromic hearing loss (NSHI) (18). Moreover, two different *GJB3* mutations (N166S and A194T) are occurring in compound heterozygosity with the 235delC and 299delAT of *GJB2* were identified in three unrelated families (19). *GJB3* (NM 024009) gene's locus DFNA2B is on chromosome 1p35.1 by fluorescence in situ hybridization (9). Besides, *GJB3* gene (OMIM #605608) has two exons, 810 nucleotides, 270 amino acids, and its molecular mass is 30.8 kDa (10). Structure of Cx31 in UniProtKB/Swiss-ProtO75712 (*GJB3*_HUMAN) database contains N-cytoplasmic termini (NT; amino acids 1–20), four transmembrane segments(TMSs), TM1 (amino acids 21–40), TM2 (amino acids 76–98), cytoplasmic loop (CL: amino acids 99–126), TM3 (amino acids 127–149), TM4 (amino acids 188–210), two extracellular loops, (E1; amino acids 41–75), and (E2; amino acids 150–187) and C-terminal domain (CT; amino acids 211–270) (20). Previous study on mouse have shown that Cx31 is expressed in the auditory nerve, supporting cells, cells at the tip of the spiral limbus and also in the spiral ligament of the cochlear lateral wall of the inner ear (21). Age-Related Hearing Loss (ARHL) is the most prevalent sensorineural deficit in the aging and Cx31 was known to be involved age-related hearing impairment (22, 23). *GJB3* as intercellular channels plays an essential role in ion homeostasis, K^+ recycling circulation in the hearing process, also, have an essential second messengers role between the nonsensory cells (24). If K^+ recycling pathway is blocked, this issue can be a factor in hearing inability (25). Molecular details of Cx31 mutations have not been identified yet (26). *GJB3* gene mutations have been known in both autosomal dominant and autosomal recessive deafness and EKV sicknesses (27, 28).

Several ethnicity groups and consanguineous marriages in Iran is the cause of different heterogeneous pattern in hearing loss among Iranian population (29). We decided to investigate whether *GJB3* might be involved in hearing loss etiology in Iran

that lead this research to be designed to study the associated mutations in Connexin31 with NSHI in three different ethnicities of Iranian population Kurd in the west, Baloch in the south- east, and Turkmen in the north- east of Iran (Fig. 1).

Fig. 1: Distribution of ethnic groups in Iran (https://en.wikipedia.org/wiki/Ethnicities_in_Iran)

Materials and Methods

In this descriptive study, 50 Non-syndromic hearing loss (NSHL) and *GJB2* negative individuals were enrolled. Seventeen individuals were originating from Baloch, 15 individuals were Kurd and 18 individuals were Turkmen. All the patients included in this descriptive study signed an informed consent.

All DNA samples were extracted from 5ml EDTA whole blood with Salting-out/Proteinasek^{+} method. The primers were designed for *GJB2*, *GJB6*, and *GJB3* gene by Gene Runner (Version 3.05). GJB2- negative Samples were given further testing for the two large deletions of the *GJB6* gene, del (GJB6-D13S1830) and del (*GJB6* - D13S1854), using gap-PCR mutation detection method. PCR amplification of Cx26, Cx30, and Cx31 was performed in a PeQlab PCR (peqSTAR thermocycler, peqSTAR, Erlangen, Germany) using the forward and reverse primers that are shown in Table 1.

Table 1: primer sequences of *GJB2, GJB3*, and *GJB6* genes

	Forward	***Reverse***
GJB2	F:5′GTAGCGCGAGGCCATGTCTCCCTGTTC TGTCCTA-3′	R:5′CAGGGCCAGCGATGACTCTAACAACTG GGCAATG-3′
GJB6	F:5′TTAGGCATGATTGGGGTGATTT-3′	R1: 5′CACCATGCGTAGCCTTAACCATTTT-3′ R2: 5′-TCATCGGGGGTGTCAACAAACA-3′
GJB3	F: 5′TGCAGCTTGGGAGGAATAAC-3′	R: 5′CCCCTGTAGGACCTCTCCAC -3′

PCR conditions were as follow; initial denaturation at 95 °C for 2 min, denaturation at 95°C for 20 sec, annealing at 40 sec (Touch down PCR), extension at 72 °C for 1 min, final extension at 72 °C for 5 min. PCR reaction including, 10µl PCR Master Mix, 1µl of each primer forward and reverse (10pm/µl), 50ng DNA, 7µl DW with final valium 20µl. The amplified fragments of the *GJB2*, *GJB6*,

and the *GJB3* gene were electrophorese on 1% agarose gel. For mutation detection of *GJB6*, Gap PCR was performed. Subsequently, DNA sequencing of the *GJB3*, and *GJB2* PCR products were performed in ABI 3130xI DNA sequencer (Applied BioSystems, Foster City, CA, USA DNA). Sequencing results were analyzed by chromas software, which were compared with the reference Human Genome Database and GenBank at the NCBI interface. http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). These variants were cheeked from the Deafness Variation Database (http://deafnessvariationdatabase.org/gene_page/GJB3).

Results

Not all patients with negative *GJB2* had any mutation in *GJB6* gene. The sequencing results of the coding region of the *GJB3* gene (exon2) showed a missense heterozygous mutation substitution NM_024009.3(*GJB3*):c.340G>A (p.Ala114Thr), rs199689484, that leads to the replacement of Alanine amino acid with Threonine amino acid, among Bloch ethnicity (Fig. 2). We found a polymorphism NM_024009.3 (*GJB3*):c.798C>T (p.Asn266=), rs35983826 in a Turkmen patient that not affects the amino acid sequence (benign). We did not find any mutation in Kurd ethnicity.

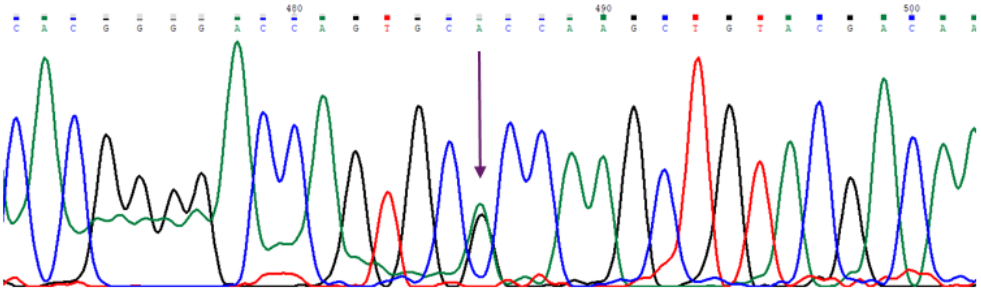

Fig. 2: A heterozygote mutation c.340G>A (p.Ala114Thr) in *GJB3* gene in one patient with Baloch ethnicity

Discussion

The mutation NM_024009.3 (*GJB3*): c.340G>A (p.Ala114Thr), rs199689484, in a Baloch patient and polymorphism NM_024009.3 (*GJB3*): c.798C>T (p.Asn266=), rs35983826 in a Turkman patient in coding region of *GJB3* gene was found. There could not identify any large deletions of the *GJB6* gene, del (*GJB6* -D13S1830) and del (*GJB6* -D13S1854) in three ethnicities in Iran.

Iran has a different gene pool in its population comparing with other populations. It has different ethnic groups and consanguineous marriage, which is a crucial element that have increased the risk of hearing loss in these ethnicities (29, 30).

According to the studies, the most frequent mutation is del (*GJB6*-D13S1830), in United Kingdom, France, Spain and Brazil, but these deletions have not been reported in Iran, Turkey, India and China. In our study, we did not find any large deletions of the *GJB6* gene, del (GJB6 -D13S1830) and del (*GJB6* -D13S1854) in three ethnicities in Iran (31-33).

Since *GJB2* (OMIM# 121011) is typically caused the most common NSHL in many populations, in previous studies, its allele frequency was studied in eight provinces of Iran. The reported result was as follow; Azerbaijan Sharqi in the northwest (22–27%), Gilan and Golestan in the north (27–38%), Kordestan in the west (15–16%), Khoozestan and Chaharmahal and Bakhtiari in the southwest (6–15%), Hormozgan and Sistan va Balouchestan in the south (0–4%) (31-34). The most common mutations of *GJB2* related to deafness that have reported in Iranian populations were 35delG, R127H, V27I+ E114G,235delC, R184P, W24X, V37I, R143W (32, 35-37). It was reported that around 13% of all the hereditary hearing loss is

caused by *GJB2* mutation in Iranian population, which is less than other populations, such as USA and Europe (38, 39). Therefore, we hypothesized that different isoform members of the connexin protein family might know of hereditary sensorineural hearing loss other genes similar to *GJB2* may be responsible for hereditary hearing loss among Iranian population. Moreover, *GJB3* has a diegetic pattern with *GJB2* (OMIM# 220290) and has 75.9% homology with *GJB2* in humans (23). Cx31 and Cx26 were responsible for A194T compound heterozygosity in mouse cochlea and co-express in gap junctions in HEK293 cells (19). Additionally, the *GJB3* gene has a compound heterozygote pattern with a recessive mutation (423-425delATT/I141V). This mutation damages the function of the M3 domain segment of Cx31; therefore, it was determined that Cx31, like Cx26 could be responsible for AR/ADNSHL (27).

Based on previous study, *SLC26A4* has high frequency after *GJB2* (40). In addition, in a Chinese family, there was a combined heterozygous mutation in *SLC26A4* and *GJB3* gene as follows: SLC26A4 IVS-2 A>G, SLC26A4 c.2168 A>G and *GJB3* c.538 C>T that may be responsible for hearing loss (41). Previously in Hormozgan Province of Iran Cx31 mutations (788G/A, 284C/T and 973G/C) was found (42). Five mutations were reported {c.53C> T (P18S), c.250G> A (V84I), c.520G> A (V174M), c.547G> A (E183K), and c.580G> A} in Taiwanese patients in Cx31 (43). That study indicated that *Cx31* protein plays the main role in the normal function of cochlea in the inner ear and suggested *GJB3* may be responsible for high-frequency non-syndrome hearing loss for auditory neuropathy (43). Recently two amino acid variants were reported (G12R and R32W) associated with *GJB3* gene mutation and EKV (44).

In this study, this mutation NM_024009.3 (*GJB3*): c.340G>A (p.Ala114Thr), rs199689484 in exon 2 of the *GJB3* which was found in a Baloch patient that damages the function of cytoplasmic loop (CL) domain segment of CX31. In addition, this missense variant is associated with tree phenotypes, non-syndromic hearing loss dominant, erythrokeratodermia variables, and a phenotype that is not specified (https://ensembl.org/Homo_sapiens/Variation/Mappings). Interpretation was reported likely benign based on https://www.ncbi.nlm.nih.gov/clinvar/variation/46083/. Moreover, Illumina Clinical Services Laboratory, Illumina submitted interpretations and pieces of evidence that is likely benign in 2016 that is associated with two phenotypes, non-syndromic hearing loss dominant and erythrokeratodermia varibilis (https://www.ncbi.nlm.nih.gov/clinvar/submitters/504895/).

In this study, the Polymorphism NM_024009.3 (*GJB3*): c.798C>T (p.Asn266=), rs35983826 in exon 2 of the *GJB3* have been know the C-terminal domain (CT) domain segment of Cx31. Interpretation was described benign that not altered the amino acid (https://www.ncbi.nlm.nih.gov/clinvar/variation/46087/). Moreover, Laboratory for Molecular Medicine, Partners HealthCare Personalized Medicine reported this variant had been known in 10.1% of European, American by the NHLBI Exome Sequence ng Project (http://evs.gs.washington.edu/EVS; dbSNP rs35983826). In addition, screened 47 Hungarian with *GJB2*-heterozygous was reported the SNP c.798C>T as factor 6% of patients with polymorphisms (45).

Up to now the mutation NM_024009.3 (*GJB3*):c.497A>G (p.Asn166Ser), which is digenic between *GJB2*/*GJB3* and these mutations NM_024009.3 (*GJB3*):c.421A>G (p.Ile141Val), NM_024009.3 (*GJB3*):c.421_423del (p.Ile141del) have been reported as pathogenic (19, 27).

However, it seems that the mutations in the *GJB3* are different in Iranian population compared to the other populations.

Conclusion

A missense heterozygous mutation in Baloch ethnicity, and a Polymorphism in Turkman in the coding region of the *GJB3* gene was identified, this study was not comprehensive and limited only to three different ethnicities. It was the first time *GJB3* gene was studied in these ethnicities. More

studies in a large sample size and a broad study of other Hearing Loss related genes in Iran using more sophisticated techniques such as Next Generation Sequencing (NGS) is recommended.

Ethical consideration

The authors have observed ethical issues (Including plagiarism, misconduct, informed consent, data fabrication and double publication and submission, falsification, redundancy, etc.).

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Conflict of interest

The authors declare that there is no conflict of interest.

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