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A N   I N T E R N A T I O N A L   J O U R N A L

# Investigation of the *GJB6* Deletion Mutations Del (GJB6-D13s1830) and Del (GJB6-D13s1854) in Iranian Patients with Autosomal-Recessive Non-Syndromic Hearing Loss (ARNSHL)

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## ABSTRACT

*Hearing loss (HL) is the most common inherited sensory disorder affecting about 1 in 1000 births. The first locus for nonsyndromic autosomal recessive HL is on chromosome 13q11–22. The two genes, GJB2 and GJB6, are closely located on chromosome and are known to be co-expressed in the embryonic cochlea. Deletion mutations involving GJB6 were associated with autosomal-recessive nonsyndromic hearing loss (ARNSHL) and in combination with a GJB2 mutation with digenic ARNSHL. The objective of this study was to screen for the del (GJB6-D13S1830) and del (GJB6-D13s1854) mutations in GJB6 gene in patients with ARNSHL from Iran, using multiplex PCR and direct sequencing methods. Agarose gel electrophoresis and DNA sequencing of amplified fragment of the PCR reaction showed none of the patients was found to carry deletion in GJB6 gene which indicates that these deletions are restricted to certain populations and indicating a founder effect regarding these deletions.*

**Key words:** Hearing loss, GJB6, GJB2, Deletion Mutation, Iran.

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## INTRODUCTION

Hearing loss (HL) is a common sensory deficit in humans and most auditory system dysfunctions resulting in HL are genetically inherited (Wang et al. 2011). The incidence of congenital HL is about 1 in 1000 births. It is estimated that over 100 genes may be involved in nonsyndromic genetic deafness, and of these, approximately 80% are associated with autosomal recessive, 15% autosomal dominant, 1 to 3% chromosome X, and 0.5 to 1% mitochondrial mutations (Moriera et al. 2015).

In many populations, up to 50% of all cases of ARNSHL are caused by mutations in the DFNB1 locus (MIM 220290) on 13q12.4. This locus contains the GJB2 gene (MIM 121011), encoding connexin-26 (Cx26), which belongs to a family of transmembrane proteins with about 20 members in humans (Kelsell et al. 1997).

A large number of affected subjects with only one GJB2 mutant allele complicate the molecular diagnosis of DFNB1 deafness. There are other mutations in the DFNB1 locus but outside the GJB2 gene. Deletions in the DFNB1 locus outside GJB2, but truncating the neighbouring GJB6 gene (MIM 604418), which encodes connexin-30 (Cx30), another component of the gap junction networks of the cochlea (Lerer et al. 2001).

Gap junction channels intercede direct cell-to-cell communication by enabling intracellular transport of small biological molecules, including electrolytes, second messengers and metabolites. The connexins are a family of proteins that form GJ channels in vertebrates (Chang et al. 2003; Beyer et al. 2009). Intercellular communication via gap junctions is critical for auditory function. The important role of intercellular communication, particularly that between GJB2 and GJB6 (encoding CX26 and CX30), has been established by evidence that certain connexin gene mutations cause sensorineural HL (Yung et al. 2010).

The GJB6 gene (AJ005585) encodes a 261-amino acid protein. In some populations, GJB6 mutations, including DFNB1 and DFNA3, reportedly cause HL. The GJB6 deletion mutations del (GJB6-D13S1830) and del (GJB6-D13s1854) have been associated with HL in some countries (Batissoco et al. 2009; Wang et al. 2011; del Castillo et al. 2003).

The previous study, the occurrence of 35delG and other point mutations in the GJB2 gene in patients with ARNSHL in Marand in the Northwest of Iran were identified. Six different mutations in the GJB2 gene including 35delG, R184P, R216K, 363delC, C202R and V84M were identified in the subjects. Therefore, mutations in the CX26 gene were found in 20% of the patients. Among these mutations, the 35delG was the most common mutation found in 5 out of 50 cases with 6% allelic frequency (Onsori 2015).

Given the high prevalence of deafness in Marand, Iran, this study investigates the GJB6 deletion mutations del(GJB6-D13S1830) and del(GJB6-D13s1854) in ARNSHL patients with no mutations in GJB2 gene or were heterozygous.

## MATERIALS AND METHODS

### Subjects

To meet the purpose of the study, 50 unrelated patients with ARNSHL (either heterozygous or negative for a GJB2 mutation) were selected. This research project has been approved by ethical committee.

### Genomic DNA Extraction and PCR

After taking written informed consent, blood samples were obtained. Genomic DNA was extracted from 1ml of EDTA anticoagulated peripheral blood by rapid genomic DNA extraction (RGDE) method (Onsori et al. 2014).

For analyzing the deletion mutations in the *GJB6* gene, multiplex PCR used based on del Castillo’s definithion (del Castillo et al. 2005). The primers used in the multiplex PCR assay were GJB6-1R, 5′-TTTAGGGCATGATTGGGGTGATTT-3′, and BKR-1, 5′-CACCATGCGTAGCCTTAACCATTTT-3′ (for amplification of the del(GJB6-D13S1830) breakpoint junction); DelBK1, 5′-TCATAGTGAAGAACTCGATGCTGTTT-3′, and DelBK2, 5′-CAGCGGCTACCCTAGTTGTGGT-3′ (for amplification of the del(GJB6-D13S1854) breakpoint junction); Cx30Ex1A, 5′-CGTCTTTGGGGGTGTTGCTT-3′, and Cx30Ex1B, 5′-

CATGAAGAGGGCGTACAAGTTAGAA-3′ (to amplify GJB6 exon 1).

PCR reactions were carried out in 20 μL reaction mixture as final volume containing 0.2 mM dNTP, 10 pmoles of each primer, 1.5 mM MgCl2, 0.5 U
of Taq DNA polymerase, 1× PCR buffer, and about 1μg of genomic DNA on a SENSOQUEST (Labcycler/Germany) Thermal Cycler.

PCR was performed under the following conditions: One cycle of denaturation at 96°C for five minutes; five touchdown cycles of denaturation at 94˚C for 50 s, and annealing for 45 s at 65˚C for the first cycle and a 1˚C reduction per cycle; 25 cycles of denaturation at 94°C for 50 seconds, and annealing at 60°C for 45 s; and a final extension step of 72˚C for seven min. The amplified fragments were run on 1.5% agarose gel by safe dye staining.

**Sequencing and Sequence analyzing**

Expected PCR products [with 333bp length for GJB6 exon1, 460 bp for del(GJB6-D13S1830) and 564 bp for del(GJB6-D13S1854)] were subjected to direct sequencing in both directions. The position of the PCR products on agarose gel corresponds to the deletion breakpoint junctions and to GJB6 exon 1. The sequencing results were analyzed by sequencing-analysis Chromas Lite 2.1 software and were compared with the wild type.

## RESULTS AND DISCUSSION

Out of the 50 unrelated ARNSHL studied samples, no deletions of GJB6 gene were found and all the individuals showed the normal 333bp amplified fragment band on agarose gel (Fig. 1). Direct sequencing of PCR product confirmed the identity of GJB6 exon 1and validated the test (Fig. 2).

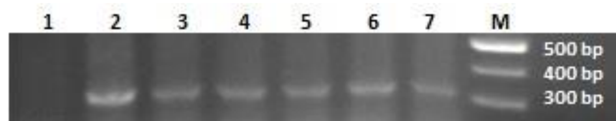


**Figure1-** A part of 1.5% agarose gel electrophoresis showing the position of the PCR products corresponding to the GJB6 exon 1. Lanes from left to right are: 1: Negative control; 2-7 patients and M: Molecular weight standard Marker.

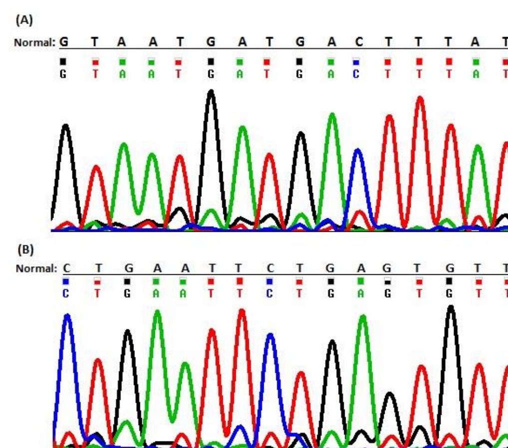


**Figure 2-** A and B two parts of DNA sequence of the GJB6 exon 1 indicating no GJB6 deletion mutations.

Although more than 100 genes involved in the development of HL, *GJB2* gene is listed as main cause of deafness in different populations and 35delG mutation is the most common mutation in this gene in many populations. However, this mutation is rare in some East Asian peoples (Zelante et al. 1997;Kenneson et al. 2002). Connexins 26 and 30 are extremely expressed in epithelial supporting cells of the mammalian cochlea and are said to play a key role in the cycling of potassium from the hair cells back to the endolymph (Batissoco et al. 2009).

Whereas biallelic mutations in the *GJB2* gene account for 50% of ARNSHL, a large number of cases with a single mutation are left mysterious by screening GJB2 alone. The *GJB6 gene* maps adjacent to GJB2 at the DFNB1 locus, and two large deletions [one of 309 kb, del(GJB6-D13S1830) and another of 232 Kb, del(GJB6-D13S1854)] upstream the GJB2 gene are frequently found among deaf individuals in Spain (Del Castillo et al. 2002; 2003).

These deletion mutations in trans with mutations in the *GJB2* gene provide an explanation for HL. Since the frequencies of these deletions varied from 5 to 15% in individuals with only one detected mutation in *GJB2* gene, finding the GJB6 deletion mutations in heterozygous individuals to GJB2 has been and still is the goal of researchers around the world (Del Castillo et al. 2003). As a result, the researcher's central focus in this study is to investigate the common deletion mutations in CX30 gene.

Previous studies in some countries have shown that, the del(GJB6-D13S1830) mutation is

most common in Spain, France, the United Kingdom, Israel, and Brazil but less frequent in the USA, Belgium, and Australia, and very rare in southern Italy (del Castillo et al. 2003). However, recent studies in have found that the deletion is present in northern Italy at frequencies like to those of other European countries (Gualandi et al. 2004). Still, there are other studies showing that this deletion mutation was also found in other countries such as Germany (Bolz 2004) and USA (Pandya 2003), but not in Austria (Gunther et al. 2003), China (Liu et al. 2002), or Turkey (Tekin et al. 2003; Uyguner et al. 2003).

Still, there are some other studies show that the del(GJB6-D13S1830) mutation, found in many populations over the world, is much more frequent than del(GJB6-D13S1854), which is for the present restricted to a few countries (del Castillo et al. 2004).

In addition, several studies conducted in Iran did not reveal any mutation in GJB6 that was associated with ARNSHL (Jabbarpour Bonyadi 2006;Tabataeifar et al. 2010). The absence of GJB6 deletions found in this study strongly corresponds with those of the above.

In conclusion, it can be said that the types and frequencies of mutations are intensely influenced by the ethnic composition of the population. Moreover, hereditary HL is genetically extremely heterogeneous and every year other loci are found to be associated with hereditary HL. The present study suggests massive parallel sequencing (MPS) for HL patients with the use of gene panels that contain HL causal genes.

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**Erratum**

In the 01 page, that read:

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