FREQUENCY OF GJB2 MUTATIONS IN FAMILIES WITH AUTOSOMAL RECESSIVE NON-SYNDROMIC HEARING LOSS IN KHUZESTAN PROVINCE

Parisa TAHMASEBI1,2, Morteza Hashemzadeh CHALESHTORI1, Fatemeh ABDOLLAHNEJAD3, Zahra ALAVI4, Ladan SADEGHIAN3, Farah TALEBI5, Javad MOHAMMADI-ASL6, Nader SAKI7, Seyed Reza Kazemi NEZHAD1, Mohammad Amin TABATABAIEFAR8,9*

1Department of Genetics, Faculty of Science, Shahid Chamran University of Ahvaz, Ahvaz, Iran
2Department of Biology, Faculty of Science, Ilam University, Ilam, Iran
3Cellular and Molecular Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran
4Department of Molecular Genetics, Faculty of Science, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran
5Genetic Counseling Center, Welfare Organization of Ahvaz, Ahvaz, Iran
6Department of Medical Genetics, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
7Department of Otolaryngology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
8Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
9Genetics Department, Erythron Pathobiology and Genetics lab, Isfahan, Iran


Hearing loss is caused both by genetic and environmental factors. In this sense, more than half of the cases are genetic. Hereditary hearing loss is divided into syndromic and non-syndromic cases. Main pattern of inheritance (80%) in non-syndromic cases is autosomal recessive, which is known as autosomal recessive non-syndromic hearing loss (ARNSHL). Although the disease is very genetically heterogeneous, the GJB2 gene has

*Corresponding author: Mohammad Amin Tabatabaiefar, Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Hezarjarib St., Isfahan 81746-73461, Iran, Tel: (098) (0)31 3792 2487, E-mail: tabatabaiefar@med.mui.ac.ir
highest effect. The aim of the present research is to determine the role of \textit{GJB2} mutations and DFNB1 locus in a group of families with ARNSHL in Khuzestan province of Iran. This study was conducted on 50 large families with ARNSHL (with the priority of 4 patients) in Khuzestan province, southwest Iran. First, in order to study \textit{GJB2} gene mutations, direct sequencing was conducted in all families. Besides, in the families with negative or heterozygous mutations in \textit{GJB2}, linkage analysis was done using STR (Short Tandem Repeat) markers related to DFNB1 locus. Genotypes, related to each family, were determined using PCR-PAGE method. From 50 families with ARNSHL, after direct sequencing, six families (12\%) showed homozygote mutations in the \textit{GJB2} gene. Accordingly, it was observed that c.35delG was the most frequent mutation (50\%) in the population. Moreover, c.358-360delGAG, c.506G>A, c.71G>A mutations were identified in 3 different families. In addition, no linkage was observed in DFNB1 locus for the remaining families. Low frequency (12\%) of \textit{GJB2} mutations in our study suggests that other loci and genes should be addressed to elucidate the etiology of ARNSHL in this province.

\textbf{Keywords:} RNSHL, \textit{GJB2}, DFNB1 locus, Iran.

\textbf{INTRODUCTION}

Hearing loss (HL), which is the most common sensory impairment in humans, affects millions of people. More than half of the HL cases are genetic. Hereditary HL is divided into syndromic and non-syndromic in which autosomal recessive non-syndromic HL (ARNSHL) is the most common form. Autosomal recessive genes with more than 55 different genes (http://hereditaryhearingloss.org) have been identified so far and they are responsible for about 80\% of non-syndromic HL cases (CRYNS and VAN CAMP, 2004; MAEDA et al., 2009). However, in some populations, mutations in the \textit{GJB2} (Gap junction protein, \textit{GJB2}, 26kDa) gene, which is located in DFNB1 locus, are responsible for half of the ARNSHL cases (BIRKENHÄGER et al., 2014). The \textit{GJB2} gene encodes connexin26 (Cx26) protein. This gene, which is 5.5 kb long, is located on the long arm of chromosome 13 and is made up of two exons and one intron. It is worth mentioning that Exon 2 is the only sequence of the \textit{GJB2} gene which codes for the Cx26 protein (COYLE et al., 1998; TEKIN et al., 2001). In humans, the connexin gene family has 21 members. Connexins, as the building blocks of gap junctions, form intercellular channels and allow for the passage of molecules smaller than 1000 Daltons such as ions and some metabolites (ALBERT et al., 2006). The Cx26 protein is found in the cochlea of the inner ear and plays a role in potassium homeostasis (AZAIEZ et al., 2007).

So far, more than 370 different mutations in the \textit{GJB2} gene have been reported (http://www.hgmd.org). However, prevalence of \textit{GJB2} mutations is different in various geographical regions or ethnicities. For example, a frame-shift mutation c.35delG mutation is the most common one in Caucasians, c.235delC in East Asians, c. 167delT in Ashkenazi Jews and c.71G>A in Indians (AZAIEZ et al., 2007; BIRKENHÄGER et al., 2014; DENOYELLE et al., 1999; MANI et al., 2009). In addition, to \textit{GJB2} coding region mutations (exon 2), two pathogenic mutations in the noncoding first exon (c.-23G>T) and donor splice site (c.IVS1 + 1G > A) have been identified (DENOYELLE et al., 1999; MANI et al., 2009). Also, the studies showed that common del (\textit{GJB6}-D13S1830) (LERER et al., 2001) and del (\textit{GJB6}-D13S1854) (DEL CASTILLO F. et al., 2005) gene deletions in the \textit{GJB6} gene (Connexin 30, Cx30), which is located in the vicinity of \textit{GJB2} in DFNB1 locus, would also cause HL. Homozygous deletions of \textit{GJB6} along
with mutations in the \textit{GJB2} gene have been reported as the causes of HL in some European countries, United States, Brazil and Australia (DEL CASTILLO, L. \textit{et al.}, 2003; ERBE \textit{et al.}, 2004; PANDYA \textit{et al.}, 2003).

Based on the studies conducted so far, mutations in \textit{GJB2} are the primary causes of HL in Iran and on the average, 17\% of ARNSHL cases are caused by this gene (BAZAZZADEGAN \textit{et al.}, 2012; DENOYELLE \textit{et al.}, 1999; KURIMA \textit{et al.}, 2002; TABATABAIEFAR \textit{et al.}, 2011). Studies show that the frequency of \textit{GJB2} mutations are different in various parts of Iran due to racial diversity and geographical area, in a way that \textit{GJB2}-related deafness in the north-western areas of Iran is approximately 2 to 4 times more than the south-eastern parts (KURIMA \textit{et al.}, 2002), Given the different frequencies of mutations of this gene in various parts of Iran, it is necessary to study each of them separately. Primary studies indicate that the rate of consanguineous marriages in Khuzestan is higher than the average rate of it in Iran (MANI \textit{et al.}, 2009). Consequently, prevalence of autosomal recessive diseases, such as ARNSHL, is high in this province. The aim of the present research is to study the role of the DFNB1 locus in causing ARNSHL in Khuzestan province located in the south-western part of Iran.

\textbf{MATERIALS AND METHODS}

\textit{Sample collection}

In this study, we analyzed 50 large unrelated consanguineous families with ARNSHL from Khuzestan province, southwest Iran. All samples were collected from different cities of Khuzestan province and various ethnic groups such as Arab, Bakhtiari, Fars, Lur. Medical histories were taken for all the patients to exclude suspected forms of syndromic and environmental causes for HL.

An informational questionnaire was completed for each family and the pedigrees were drawn for all families. Each family had at least 4 affected individuals who had no other associated clinical findings. Also, HL was pre-lingual and non-progressive. After obtaining informed consent from all individuals in families, blood samples were collected from them. Moreover, pure tone audiometry tests were down on all the patients.

\textit{DNA Extraction and DNA sequencing of GJB2}

DNA extraction was carried out using a standard phenol-chloroform method (GRIMBERG \textit{et al.}, 1989). At least, one affected individual from each pedigree was subjected to DNA sequencing. To amplify the coding region (exon 2) of the \textit{GJB2} gene: F (5\' \text{GTCTCCCTGGTCTGCCATG} 3\') and R (5\' \text{TCTACAACTGGGCAATG} 3\') primers were used. PCR mixture (25 \text{μl}) contained: 12.5 \text{μl} Taq 2X master mix RED (Ampliqon, Denmark), 1 \text{μl} (10 pmol) of each primer, 1 \text{μl} (50 ng) DNA template. PCR program was performed in a thermocycler (Applied Biosystems,USA) as follows: 95\(^\circ\)C for 5 min, 95\(^\circ\)C for 40 s, 64\(^\circ\)C for 40 s and 72\(^\circ\)C1 min, for 33 cycles with a final 5 min extension step at 72\(^\circ\)C. PCR products were qualified by electrophoresis on 1.5\% agarose gel, and, then, were sequenced using the same forward and reverse primers. The sequencing results were analyzed by SeqMan\textsuperscript{TM} software version 5.00 (DNA Star Inc., USA), and then were compared to the \textit{GJB2} reference GenBank sequence (NM\_004004).
Linkage analysis for DFNB1

Heterozygotes or negative families for GJB2 mutations were tested for the linkage to DFNB1 using at least 2 informative markers. Forward and Reverse primers sequences, annealing temperature and cycle number for each STR markers are shown in Table 1. After performing PCR for each marker, amplified products were separated by electrophoresis on 8% polyacrylamide gel and, then, were visualized by silver staining. Notably, linkage analysis was conducted as previously described (TABATABAIEFAR et al., 2011, KASHEF et al., 2015).

Table 1. STR markers used in this study for linkage analysis to DFNB1.

<table>
<thead>
<tr>
<th>Marker</th>
<th>UniSTS ID</th>
<th>Forward and Reverse primer (5'→3')</th>
<th>PCR product range(bp)</th>
<th>annealing temperature</th>
<th>cycles number</th>
</tr>
</thead>
<tbody>
<tr>
<td>D13S1236</td>
<td>UniSTS:47290</td>
<td>F:GCACCTGGCCTGGGTAA R:AAGGGCTGGGCTCTTCA</td>
<td>108-132</td>
<td>61° C</td>
<td>26</td>
</tr>
<tr>
<td>D13S1275</td>
<td>UniSTS:18239</td>
<td>F:ATCAGGTGAAATGAGGCTTACGAG T:GCATGACCTTTAACAG</td>
<td>180-214</td>
<td>63° C</td>
<td>26</td>
</tr>
<tr>
<td>D13S175</td>
<td>UniSTS:63643</td>
<td>F:ATGATCATGAAATGAGGCTTACGAG T:GCATGACCTTTAACAG</td>
<td>101-113</td>
<td>61° C</td>
<td>30</td>
</tr>
</tbody>
</table>

RESULTS

In this study, we analyzed the DFNB1 locus in 50 large families from different cities of Khuzestan province with at least four patients. All families in the survey showed ARNSHL, prelingual and non-progressive HL. Totally, 33 families (66%) were of Arab ethnicity, 6 were Bakhtiari (12%), 6 were Lur (12%), 1 family (2%) from Fars and 4 families (8%) were from other ethnic groups.

From the 50 families studied, six families (12%) showed GJB2-related HL (homozygous mutations). In addition, no linkage was observed in DFNB1 locus for families with negative or heterozygous mutations in GJB2. Among the mutations identified in the GJB2 gene, c.35delG was the most commonly observed mutation (3 families, 50%). Also, c.358-360delGAG, c.506G>A, c.71G>A mutations were observed in 3 other families. The c.358-360delGAG mutation leads to the removal of Glu amino acid at position 120. The c.506G>A substitution mutation changes Cys amino acid to Tyr at position 169, the c.71G>A mutation changes Trp amino acid to the termination codon at position 24.

Also, in two families, heterozygous mutations in GJB2 were observed. These pathogenic mutations included c.71G>A and c.194A>G. In addition, in several families, polymorphisms in GJB2 were observed as presented in Table 2.

Table 2. GJB2 gene polymorphisms observed in the studied families.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Number of family</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.Val27Ile(c.79G&gt;A)</td>
<td>3</td>
</tr>
<tr>
<td>c.*84T&gt;C</td>
<td>13</td>
</tr>
<tr>
<td>p.Phe31Lue(c.93T&gt;G)</td>
<td>3</td>
</tr>
<tr>
<td>p.Glu114Gly(c.341A&gt;G)</td>
<td>1</td>
</tr>
<tr>
<td>p.Arg127His(c.380G&gt;A)</td>
<td>1</td>
</tr>
</tbody>
</table>
DISCUSSION

In the present research, out of 50 families with ARNSHL, 6 families (12%) had homozygous mutations in the GJB2 gene. This rate is lower than the average rate in the country (17%) (DENOYELLE et al., 1999; KURIMA et al., 2002; TABATABAEIFAR et al., 2011) for GJB2 gene mutations in causing ARNSHL in Iran. Frequency of mutations in the GJB2 gene is different in various parts of Iran and a downward slope is observed regarding the involvement of GJB2 mutations from the north-western to the south-eastern parts of the country. These mutations show the highest rate in the north-western parts of Iran, that is 22-38%, and reach approximately 9% in the south-eastern part of the country, near Pakistan (KURIMA et al., 2002). In many populations, mutations in this gene are the main cause of nonsyndromic and pre-lingual HL, and even in some populations (North America, Mediterranean region and most of Europe), 50% of ARNSHL cases and more likely in Spain and Italy, 79% of ARNSHL cases are related to mutations in this gene (CRYNS and VAN CAMP, 2004; DENOYELLE et al., 1997; FREI et al., 2005; MARLIN et al., 2001; SABAG et al., 2005; ZELANTE et al., 1997). However, studies in Pakistan, Turkey, India, Palestine, Sudan and Kenya have shown that involvement of this gene in HL is less as compared to American and European populations (NAJMABADI and KAHRIZI, 2014).

So far, two separate studies have been conducted on the contribution of GJB2 mutations in causing ARNSHL in Khuzestan province. In a study conducted by HOSSEINPOUR et al. (2005), 4.1% of the studied population had homozygous mutations in this gene (HOSSEINPOUR et al., 2005). On the other hand, in a study conducted by GALEHDARI et al. (2009) on 61 deaf individuals of Arab ethnicity in Khuzestan province, complete lack of mutations in GJB2 was reported (GALEHDARI et al., 2009). However, in our study a higher percentage (12%) was obtained. On the other hand, in this study 66% (4 families out of 6 families) of the families found to be positive for GJB2 mutations were from Arab ethnicity. The difference between our study and the previous mentioned studies may be due to our selection and proper distribution of the samples across Khuzestan province. The obtained frequency (12%) confirms the downward slope of the percentage GJB2 involvement in the southern areas as compared to the northern and north-western areas of Iran, such as Gilan (27.6%) (CHALESHTORI et al., 2004) and Azerbaijan (31%) (BONYADI et al., 2014). In Khuzestan’s neighboring provinces such as Ilam, Lorestan, Chaharmahal and Bakhtiari, studies on the contribution of GJB2 to causing HL have also been conducted. In a study conducted by MAHDIEH et al. (2016), 11.29% of the cause of HL in 62 deaf individuals in Ilam province was associated with homozygous mutations in this gene. In another study conducted by SEPAHVAND et al. (2006) on 53 nonsyndromic HL individuals in Lorestan province, 17% of the patients had mutations in GJB2. In a study conducted by HASHEMZADEH et al. (2005) on GJB2-related HL in 79 families with deaf members in Chaharmahal and Bakhtiari province, the frequency of mutations in GJB2 was obtained to be 7.8% (CHALESHTORI et al., 2006). Also, in a study conducted by NAJMABADI et al. (2005) the frequency of GJB2-related HL in the south-western part of the country (several provinces in the south-western part of the country) was reported to be 15.2% (KURIMA et al., 2002). These investigations as well as the results of our study suggest that in this province and the neighboring areas, other loci may have higher frequencies and even due to the large number of genes involved in HL, new loci may be responsible for this disease.

In a study conducted by BAZAZADEGAN et al. (2012) on the population of several provinces, the level of involvement of GJB2 mutations in nonsyndromic HL in Khuzestan
province was reported to be 10%, which is close to the percentage obtained in the present study (12%).

In the neighboring Persian Gulf countries, the contribution of GJB2 mutations is also lower than European countries. In two separate studies in Saudi Arabia, the level of involvement was reported to be 10.09% and 3% (AL-QAHTANI et al., 2010; IMTIAZ et al., 2011), respectively. Therefore, in this country, like our country and the results of the present study, a high level of heterogeneity is observed and the low frequency of mutations in GJB2 is probably due to the fact that other genes play a role in causing HL.

In the present research, c.35delG mutation had the highest frequency and each of the c.358-360delGAG, c.506G>A, c.71G>A mutations had equal roles in causing the disease. c.358-360delGAG, c.506G>A, c.71G>A mutations are of high frequencies among Iranian ARNSHL population. Besides, c.35delG is the most prevalent mutation in this population (BAZAZZADEGAN et al., 2012; MAHDIJEH et al., 2010). In the present study, similar to other studies conducted on the population of this province, c.35delG mutation was reported to be the most frequent mutation (HOSSEINIPOUR et al., 2005). The c.506G>A mutation changes Cys to Tyr at position169. This change was first considered as polymorphism, but further studies confirmed its pathogenic effect. The presence of this amino acid is essential for the formation of disulfide bonds within the three-dimensional structure of the extracellular domain of Cx26 and, therefore, for the interaction between the two Connexons. The presence of this cysteine, in addition to creating proper folding, is essential for proper performance of the ionic channel. Mutation in one of the three Cys residues in the extracellular domain of the Cx26 protein disturbs the flow of K+ ions, hence ionic homeostasis, in the inner ear (BIRKENHÄGER et al., 2014; MAEDA et al., 2009).

In some cases, mutations in GJB6 (homozygous mutations along with heterozygous mutations in GJB2) (DEL CASTILLO, I. et al., 2003; ERBE et al., 2004; PANDYA et al., 2003) or homozygous mutations in the first exon of GJB2 can cause HL (DENOYELLE et al., 1999; KASHEF et al., 2015; MANT et al., 2009). Therefore, in the remaining studied families (44 families) with or without heterozygous mutations in GJB2, the DFNB1 locus linkage analysis was conducted for the related markers and no linkage was observed. Therefore, the possibility of mutations in this locus was ruled out. Notably, so far, none of the GJB6 mutations has ever been reported in Iran and it appears that these mutations do not play a role in causing HL in Iran (BEHESHTIAN and DANESHI, 2016; KHURSHEED and RAJ, 2016).

Since the families participating in the present research were part of extensive kinships, the results obtained for GJB2- positive families can be used in genetic counseling for all the people in these pedigrees. The presence of various ethnic groups in Khuzestan province and high rate of consanguineous marriages and HL cases make this province a rich source for studying autosomal recessive HL. This further shows the necessity of studying the main loci contributing to HL and determining the contribution of each of them in causing this disease in Khuzestan province. It should be noted that most of the studies in Iran have been conducted on the combination of the population of several provinces (BABANEJAD et al., 2012; BAZAZZADEGAN et al., 2012; KASHEF et al., 2015; TABATABAIEFAR et al., 2011).

In conclusion, in this study, the cause of HL was identified in 12% of the studied population. Further studies are warranted to determine the cause of HL in the remaining families to determine the role of other genes in causing HL. Undoubtedly, the results of such studies can significantly contribute to proper genetic counseling as well as Preimplantation Genetic Diagnosis (PGD) and future therapeutic intervention to prevent HL in this population.
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FREKVENCIJA GJB2 MUTACIJA KOD FAMILIJA SA AUTOZOMALNIM RECESIVnim NE-SINDROMSKIM GUBITKOM SLUHA U HUZESTAN PROVINCIJI

Parisa TAHMASEBI1,2, Morteza Hashemzadeh CHALESHTORI1, Fatemeh ABDOLLAHNEJAD3, Zahra ALAVI3, Ladan SADEGHIAN3, Farah TALEBI1, Javad MOHAMMADI-ASL5, Nader SAKI1, Seyed Reza Kazemi NEZHAD1, Mohammad Amin TABATABAIEFAR8,9

1Departman za genetiku, Shahid Chamran Univerzitet u Ahvazu, Iran
2Departman za biologiju, Ilam Univerzitet, Ilam, Iran
3Čelijski i molekularni istraživački centar, Shahrekord Univerzitet za medicinske nauke, Shahrekord, Iran
4Departman za molekularnu genetiku, Shahrekord orgranak, Islamski Azad Univerzitet, Shahrekord, Iran
5Genetički savetodavni centar, Organizacija za socijalnu pomoć, Ahvaz, Iran
6Departman za medicinsku genetiku, Medicinska škola, Ahvaz Jundishapur Univerzitet za medicinske nauke, Ahvaz, Iran
7Departman za otolaringologiju, Medicinska škola, Ahvaz Jundishapur Univerzitet za medicinske nauke, Ahvaz, Iran
8Departman za genetiku i molekularnu biologiju, Medicinska škola, Isfahan Univerzitet za medicinske nauke, Isfahan, Iran
9Genetički departman, Erythron Patobiološka i genetička laboratorija, Isfahan, Iran

Izvod
Gubitak sluha je uzrokovan i genetskim i faktorima spoljne sredine. U tom smislu, više od polovine slučajeva je genetski uzrokovan. Nasledni gubitak sluha se deli na sindromske i ne-sindromske slučajeve. Glavni način nasleđivanja (80%) u ne-sindromskim slučajevima je autozomni recesivni, što je poznato kao autozomni recesivni ne-sindromski gubitak sluha (ARNSHL). Iako je bolest veoma genetski heterogen, gen GJB2 ima najveći efekat. Cilj ovog istraživanja je da se utvrdi uloga GJB2 mutacija i DFNB1 lokusa u grupi porodica sa ARNSHLom u provinciji Huzestan u Iranu. Ova studija je sprovedena u 50 velikih porodica sa ARNSHLom (sa prioritetom od 4 pacijenta) u provinciji Huzestan, u jugozapadnom Iranu. Prvo, u cilju proučavanja GJB2 genskih mutacija, izvršeno je direktno sekvenciranje u svim porodicama. Osim toga, u porodicama sa negativnim ili heterozigotnim mutacijama u GJB2, linkage analiza obavljena je pomoću STR (Short Tandem Repeat) koji se odnose na DFNB1 lokus. Genotipovi, vezani za svaku porodicu, određeni su metodom PCR-PAGE. Od 50 porodica sa ARNSHL-om, nakon direktnog sekvenciranja, šest porodica (12%) je pokazalo homozigotne mutacije u GJB2 genu. Shodno tome, primećeno je da je c.35delG najčešća mutacija (50%) u populaciji. Štaviše, c.358-360delGAG, c.506G>A, c.71G> mutacije su identifikovane u 3 različite porodice. Pored toga, kod DFNB1 lokusa nije bilo povezivanja za preostale porodice. Niska frekvencija (12%) mutacija GJB2 u našoj studiji ukazuje na to da se drugi lokusi i geni trebaju proučavati u cilju razjašnjavanja etiologije ARNSHL-a u ovoj provinciji.

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