Evaluation of \textit{GJB2} and \textit{GJB6} Mutations in Patients Afflicted with Non-syndromic Hearing Loss

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Abstract

Background

Non-syndromic hearing loss (NSHL) is assumed as one of the highly prevalent congenital defects in the world. In this regard, \textit{gap junction protein beta 2}(\textit{GJB2}), and \textit{gap junction protein beta 6}(\textit{GJB6}) mutations are considered as the leading congenital causes of deafness. The present study aimed to assess the prevalence of \textit{GJB2} and \textit{GJB6} mutations in NSHL cases.

Materials and Methods

This cross-sectional study was implemented from Jan. 2015 to Sep. 2017 at Alzahra Hospital (Isfahan, Iran). 46 patients afflicted with NSHL were recognized and recruited by physicians. Heparinized blood was collected and DNA of each participant was extracted. Genetic analysis of \textit{GJB2} and \textit{GJB6} genes was performed using PCR and GAP-PCR methods respectively.

Results: 35delG mutation had the highest prevalence with allelic frequency of 6.12%. The allelic frequencies of 35delG, and delE120 were 6(6.12%), and 3(3.06%), respectively. Allelic frequency of W77R, Y65H, G160, and R127H was 2(2.04%) for each of them. In addition, 2 patients were heterozygous for p.V153I rare polymorphism (2.04%).

Conclusion

Overall, the present study indicated that 35delG mutation could be considered as the foremost causative factor of NHCL. \textit{GJB2} mutations were highly prevalent among NSHL cases (23.9%). As a result, the mutation analysis of this gene could be appropriately used for prevention and early diagnosis of NSHL.

Key Words: Hearing Loss, \textit{GJB2}, \textit{GJB6}, Mutation.


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1- INTRODUCTION

Hearing loss is one the most prevalent forms of the sensorineural and congenital defects (1, 2). World Health Organization (WHO) states that about 360 million individuals around the world, have been afflicted with mild to severe hearing loss (3). This impairment impedes speech progression, causing many social and vocational implications (2). Hearing loss is a heterogeneous disorder since it could be induced by both environmental factors and genetic defects (2). The role of the genetic factors, however, surpasses the others (3). The non-syndromic hearing loss (NSHL) may be induced by 150 loci mutations and it could have autosomal recessive (80%), autosomal dominant (10%), X-linked (<1%), Y-linked (<1%), and mitochondrial (<1%) basis. Most of the genetic defects are induced by DFNB1 locus mutation (MIM#220290), including two genes: gap junction protein beta 2 (GJB2), and gap junction protein beta 6 (GJB6) (1-5).

GJB2 (MIM 121011, GeneID 2706) comprises two exons, one of which can be translated. This gene encodes connexin-26 protein (26 kDa), constructed from 226 amino acids (4-6). This protein has a four-transmembrane spanning structure and is expressed in hair cells (5, 7). Connexin-26 is involved in potassium recycling of endolymph fluid; hence, GJB2 mutations impede K+ recycling and lead to necrosis of hair cells (5, 8). Moreover, connexin 26 expression is crucial for cochlear development (5, 9). In fact, abnormal GJB2 causes cochlear amplification impairment, endolymphatic potential reduction, and hair cell degeneration (5, 10). Some forms of hearing loss, however, are caused by monoallelic mutations of GJB2 accompanied with a second mutation of GJB6 (MIM 604418, GeneID 10804) (11, 12). This gene encodes a protein, called connexin-30 (30kDa), comprised of 261 amino acids (11, 13).

Connexin 30 constitutes gap junction between the hair cells (11, 14). The gap junction, also known hemiconnexon structure, allows the transmission of ions and some metabolites (11, 15). The first and conformational structures of connexin 30 and 26 closely resemble each other (11, 16). Moreover, these proteins are expressed simultaneously (11, 15). There is no question that consanguinity is a risk factor of autosomal recessive hearing loss in different populations (5). Besides, previous studies have reported high levels of hearing loss (3 in 1000 newborns) and consanguinity (36.8%) in Iran (2, 4, 5). The current study aimed to assess GJB2 and GJB6 mutations in NSHL cases living in Isfahan, a metropolis in the center of Iran.

2- MATERIALS AND METHODS

2-1. Study design and population

This was a cross-sectional study carried out at Alzahra Hospital (Isfahan University of Medical Sciences (IUMS), Isfahan, Iran from Jan. 2015 to Sept. 2017. Sample size was estimated according to the previous studies (n=46) (17, 18).

2-2. Ethical considerations

This study was approved by the Ethical Committee of IUMS (Project Code: 395819). All parts of the study protocol and its aims were clearly described for the patients and their guardians, and informed consent was acquired. The participants were assured that their information would be kept confidential.

2-3. Inclusion and exclusion criteria

The inclusion criteria for patient recruitment were: (1) participants afflicted by moderate (40-70 decibel (dB)), severe (70-95 dB), or profound (>95 dB) NSHL, (2) patients having autosomal recessive hearing loss. Exclusion criteria were deafness caused by: (1) conductive hearing loss, (2) toxic drugs used during the
pregnancy, (3) septicemia and related antibiotic therapies in neonates, (4) trauma and other environmental factors.

2-4. Laboratory assessment

Heparinized peripheral blood (5 ml) was collected from every patient and DNA was extracted, using a DNA extraction kit (Qiagen, Germany Cat. No 51304) according to the manufacturer’s instructions. DNA concentration and quality were assessed by determination of 260A/280A using a NonoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel (2%) electrophoresis.

The coding exon of $GJB2$ gene was amplified by PCR method. For DNA amplification the forward (CTCCCTGTTCTGTCCTAGCT) and reverse (CTCATCCCTCTCATGCTGTC) primers (Cox26E2) that were applied yielded 809 bp band (19). Standard PCR was done in a solution comprised of 40 ng genomic DNA (1.5μl), 100 pml of each primer (0.2μl), 10mM deoxyribonucleotide triphosphates (dNTP) (0.5μl), 50mM MgCl$_2$ (1μl), 10 X PCR buffer (2.5μl), 5 U/ml Taq polymerase(0.2μl). This multiplex PCR condition was set up as follows: denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, extension at 72 °C for 1 min, and finally 7 min at 72 °C.

2-5. Data Analyses

The Sanger sequencing was carried out on an ABI 3130 automated sequencer (XL genetic analyser) using the BigDye terminator V.3.1. All of the results were aligned with human $GJB2$ sequences presented in Human Genome Database and GenBank. The common deletions of $GJB6$ were assessed through GAP-PCR method.

2-6. Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

3- RESULTS

In this study, 46 unrelated patients (30 males and 16 females) aged from 2 to 36 years were analyzed (Table.1). All of them were from consanguineous marriage (mostly first cousins), and 89% of the patients had a history of hearing loss in their family members (more than one affected patient in a pedigree). Partial or total hearing loss in all of them was not associated with other signs and symptoms. Coding exon of $GJB2$ was amplified and sequenced with specific primers.
Sequencing results revealed that 2 patients had homozygote 35delG mutation and one of them had heterozygote 35delG. c.229 T>C (p.Trp77Arg) and c.195C>A (p.Tyr65His) mutations were found in homozygote status. Two patients had c.358-360delGAG (p.Glu120del) mutation (one homozygote and one heterozygote). c.478G>A (p.Gly160Ser) and p.R127H mutations were found in heterozygote forms in 2 separate patients. The allelic frequencies of 35delG, and del E120 were 6(6.12%) and 3(3.06%) respectively. Allelic frequency of W77R, Y65H, G160 and R127H was 2(2.04%) for each of them. In addition, 2 patients were heterozygous for p.V153I rare polymorphism (2.04%) (Figure.1). Clinical data, family characterization and genotype of patients bearing specific GJB2 variants are listed in Table.2. D13S1830 deletion of GJB6 gene was also analyzed by multiplex gap PCR and as was expected no deletion was shown in our 46 deaf patients.

Table-1: Baseline characteristic of the patients affricted by non-syndromic hearing loss

<table>
<thead>
<tr>
<th>Patient’s Characteristic</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year mean (range)</td>
<td>13 (2-36)</td>
</tr>
<tr>
<td>Gender: Number (%)</td>
<td>Male</td>
</tr>
<tr>
<td>Male</td>
<td>30 (65.21)</td>
</tr>
<tr>
<td>Nationality: Number (%)</td>
<td>Iranian</td>
</tr>
<tr>
<td>Iranian</td>
<td>46 (100)</td>
</tr>
<tr>
<td>History of hearing loss in family members</td>
<td></td>
</tr>
<tr>
<td>41 (89)</td>
<td></td>
</tr>
<tr>
<td>Level of consanguinity</td>
<td></td>
</tr>
<tr>
<td>46 (100)</td>
<td></td>
</tr>
</tbody>
</table>

Fig.1: Mutation found in gap junction protein beta 2 (GJB2) gene.
Table-2: The clinical data and family history of deaf patients bearing specific GJB2 variants

<table>
<thead>
<tr>
<th>No.</th>
<th>Gender</th>
<th>Age at diagnosis</th>
<th>Hearing loss severity</th>
<th>Consanguinity of parents</th>
<th>Family history</th>
<th>Identified mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>At birth</td>
<td>Sever</td>
<td>No</td>
<td>+</td>
<td>35delG/N rs398123814</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>At birth</td>
<td>Sever</td>
<td>No</td>
<td></td>
<td>c.478G&gt;A (p.Gly160Ser)/N rs34988750</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>At birth</td>
<td>Profound</td>
<td>Yes</td>
<td>+</td>
<td>35delG/35delG rs398123814</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>4 years</td>
<td>Sever</td>
<td>Yes</td>
<td>+</td>
<td>c.195C&gt;A (p.Tyr65His) / c.195C&gt;A (p.Tyr65His) rs763572195</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>2 years</td>
<td>Moderate</td>
<td>Yes</td>
<td>+</td>
<td>c.358-360 delGAG (p.Glu120del) / c.358-360 delGAG (p.Glu120del) rs150529554</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>At birth</td>
<td>Profound</td>
<td>No</td>
<td>+</td>
<td>c.229T&gt;C (p.Trp77Arg) / c.229T&gt;C (p.Trp77Arg) rs104894397</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>At birth</td>
<td>Sever</td>
<td>Yes</td>
<td>+</td>
<td>c.380G&gt;T(R127H)/N rs111033196</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>7 years</td>
<td>Profound</td>
<td>Yes</td>
<td></td>
<td>c.457G&gt;A(V153I)/N rs111033186</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>At birth</td>
<td>Sever</td>
<td>Yes</td>
<td>+</td>
<td>c.358-360 delGAG (p.Glu120del) /N rs150529554</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>At birth</td>
<td>Sever</td>
<td>No</td>
<td></td>
<td>c.457G&gt;A(V153I)/N rs111033186</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>At birth</td>
<td>Profound</td>
<td>No</td>
<td>+</td>
<td>35delG/35delG rs398123814</td>
</tr>
</tbody>
</table>

F: female; M: male.

4- DISCUSSION

In the present study, 16 varied alleles were detected in 11 of 46 patients. In this regard, 35delG mutation had the highest prevalence with allelic frequency of 6.12%. Our findings were in agreement with the previous studies conducted in other provinces of Iran, which reported high prevalence of 35delG mutation (12%) (22-27). Genetic screening of GJB2 gene could be assumed as the first step for molecular analysis of patients afflicted by NSHL. According to the Human Gene Mutation Database (HGMD®) (http://www.hgmd.cf.ac.uk), more than 200 variants in the GJB2 gene have been reported. Among these, variants deletion of guanine in position 30-35 (named 35delG) is the most frequent mutation (roughly 70%) of GJB2 variants (28). The other prevalent mutations of GJB2 found in Iran are R127H, delE120, W24X, R184P, -3170G>A (IVSI-1G>A) and 235delC (29-31). D13S1830 deletion in GJB6 gene has been considered as not only the most frequent deletion but also the second frequent mutation in white populations (32). Del Castillo et al found that more than half of patients with heterozygote variants in GJB2 gene have a large deletion in that gene (33). The D13S1830 deletion is the most frequent deletion of GJB6 in France (33, 34), Spain (33), Brazil (35), Argentina (36), and UK (33). This deletion removes 309 Kb near GJB6 and GJB2 and terminates GJB6 (13). It is still unclear whether mutations in GJB2 gene can be inherited in digenic pattern with D13S1830 deletion or whether the removed region contains common regulatory elements of GJB2 and GJB6 genes (37-39). The frequency of GJB6 deletion varies wildly among different populations (40). This deletion was absent
in the current study and not reported in Turkey (41), India (42) and China (43). The delE120 (deletion of glutamic acid in codon120 mutation) was the other mutation detected in the current study, and found in 3.06% of alleles (44, 45). This deletion is also reported as the second prevalent mutation in some western, and southwestern parts of Iran (18, 45, 46).

Pathogenicity of R127H variant which leads to substitution of arginine for histidine is not confirmed and is controversial among different mutation prediction software. Another common polymorphism in GJB2 gene is V153I, substitution of Valine for Isoleucine due to G to A transition. Since gene polymorphisms could change sequence of coding protein, association of these polymorphisms with other polymorphisms in this gene or another gene may result in some degree of hearing loss. Previous studies showed that variable phenotypic manifestation of mutations, may be due to association of modifier agents (18, 47). In this study, we identified patients with homozygote GJB2 mutations. The patients who have heterozygote GJB2 mutations and GJB2 negative patients were subjected to further clinical and molecular analysis. However, none of the cases were affected by GJB6 mutation. Our findings supported previous results, indicating the absence of GJB6 mutation in Iranian population (48).

4-1. Limitations of the study

There are unknown genetic mutation contributing to NSH, as a result, expanded genetic screening of NSHL cases could be beneficial.

5- CONCLUSION

The results showed that distraction technique had a good effect on the intensity of pain in children. Given the need for pain control and its effects on the course of treatment, further studies are needed to be done.

6- ACKNOWLEDGMENT

We would like to thank the vice chancellor for research at Isfahan University of Medical Sciences (IUMS) for the financial support. This study was funded by IUMS (grant number: 395819).

7- CONFLICT OF INTEREST: None.

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