Mutation analysis of the GJB2 gene of patients with non-syndromic hearing impairment in the Kurdish population in Sulaimani province, Iraq

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ABSTRACT. Approximately 60% of all cases of congenital bilateral sensorineural hearing impairment are due to genetic factors, and about 50% of hearing impairment cases at a later stage are caused by a mutation in a single gene. Because of the high frequency of gap junction beta-2 protein gene (GJB2) mutations, mutation analysis of this gene is widely used in hearing impairment research and diagnosis. This study aimed to determine the prevalence of common GJB2 mutations in patients with profound non-syndromic sensorineural hearing impairment. Sixty-one patients (32 male and 29 female) included in this study had above 90 decibels of bilateral sensorineural hearing impairment. Patient DNA was isolated from buccal cells. The 1st and 2nd exons of the GJB2 gene were amplified with specific primers after gel purification of both regions. Sanger DNA sequencing analysis was used for investigation of changes in these gene regions. The pathological variant was found in nine patients (15%). This variation involved a frameshift mutation in GJB2 (homozygous 35delG) of the 2nd exon; no mutation was detected in the 1st exon. This study is the first report of a genetic investigation of hearing impairment in the Kurdish population in Sulaimani province, northeastern Iraq, near the Iraq-Iran border. The
results show that 35delG mutation has a high prevalence in patients with non-syndromic sensorineural hearing impairment.

Key words: Non-syndromic hearing impairment; GJB2 gene; Gap junction proteins; Connexin 26; Mutation

INTRODUCTION

Hearing impairment is defined as the loss of hearing; the pathology of hearing impairment originates from the outer, middle, or inner parts of the ear, or the pathology of the auditory vestibular nerve or the auditory cortex of the brain (Michels et al., 2019). The incidence of hearing impairment is about 1-2/1000 live births and about 70,000,000 people are affected worldwide (Schmuziger et al., 2008). Additionally, the predominant type of hearing impairment is prelingual sensorineural hearing impairment, affecting about 1/650 of newborns (Tamayo et al., 2009). The causes of hearing impairment are environmental and/or genetic (Sataloff, 1983); more than 130 genes are related, including 54 autosomal dominant, 67 autosomal recessive, eight X-linked, one Y-linked, and two mitochondrial genes (Silan et al., 2017). The hearing impairment origin may be environmental about 30% or genetics 40-60%, the account off non-syndromic hearing impairment is about 70% of the genetics causes, about 80% of non-syndromic hearing impairment is prelingual sensorineural recessive, 20% is dominant and 2-3% is X-linked or mitochondrial causes (Uchida et al., 2011). For identities of genetic hearing impairment, various guidelines are used. The DFN symbol is used for the genetics of non-syndromic hearing impairment, this symbol is derivative from "DeafNess". The prefixes “DFNA” and “DFNB” are used for autosomal dominants and autosomal recessive respectively. The symbol for X-linked and mitochondrial inheritance is “DFN” (Lalwani and Castelein, 1999). the inherited non-syndromic hearing impairment is mostly of sensorineural origin, mutations of the GJB2 gene is the first rank among genes related to non-syndromic sensorineural hearing impairment in many population word wide (Zelante et al., 1997). The GJB2 gene serves as a training ground for cell signaling beta-protein synthesis. This gap junction beta protein, also known as connexin 26, is a species of the connexin gene family that makes gap junctions, thereby enabling nutrients, ions, and signaling substances to flow between cells. Specific connexin proteins, which create channel gap junctions formed by this protein that ferries potassium ions and numerous tiny molecules, are used to identify the length and shape of the particles circulating in it. Connexin 26 may be detected in the human body's cells, including the inner ear (Wingard and Zhao, 2015), at any time. It has the form of a snail and is named the cochlea because it is within; this protein characteristic of the ear, particularly hearing, has piqued the curiosity of researchers. The transformation of acoustic energy into electrical nerve signals is known as hearing. This conversion entails keeping potassium ions in the inner ear at normal levels. According to specialists, roughly 100 GJB2 genetic variants have been discovered to cause non-symptomatic impairment. They hear problems that aren't linked to any other signs. The non-symptomatic hearing disorder is characterized by mutations in the DFNB1 and DFNA3 genes. Because DFNB1 is autosomal recessive, it affects all forms of the GJB2 gene in all cells. Approximately half of all autosomal recessive non-symptomatic hearing impairment occurs in this way (Farjami et al., 2020). It is defined by a degree of deafness that is slightly extreme. This deafness turns out to be an
alteration that affects the infant before it begins to talk and will not be severe in the long run. Mutations in the GJB2 gene cause DFNB1 by eliminating or inserting DNA essential components (base pairs) inside or across the gene. The elimination of a base pair at position 35 of the GJB2 gene is perhaps the most prevalent mutation among diverse ethnicities, notably in Scandinavians. Mutations in Asians are known to destroy a base pair at position 235 (235delC) (Posukh et al., 2019). A base-pair substitution at position 167 (167delT) is a frequent mutation in Eastern European (Ashkenazi) Jewish children (Morell et al., 1998). DFNB1 is caused by mutations in the GJB2 gene, which frequently exchange base pairs with faulty ones or destroy sections of the DNA around the gene. The gene mutation that causes DFNB1 has been described as a loss of function resulting in a non-functional connexin 26 that impairs the function of gap junctions. A lack of gap junctions in the inner ear can affect potassium ion levels, leading to hearing aid cell dysfunction (Jagger and Forge, 2015). Since DFNA3 is inherited autosomal dominant, only one mutant copy of the GJB2 gene is required for each cell. This type of deafness can occur (linguistically) before or after the child begins speaking. Deafness ranges from moderate to severe and worsens over time, affecting the ability to hear high-frequency sounds. Since DFNA3 is autosomal dominant, only one mutant copy of the GJB2 gene in each cell is obtained to cause the disease. This type of deafness can occur (linguistically) before or after the child begins speaking. Deafness ranges from mild to severe and can worsen over time and affect the ability to hear high-frequency sounds (Moore, 2016). The GJB2 gene mutation that causes DFNA3 replaces the defective amino acid with the amino acid of connexin. These mutations, known as "dominant negatives," cause the development of an aberrant version of connexin 26 that interferes with the formation of functional gap junctions.

No molecular study of the connexin 26 (GJB2) genes had been reported in patients with non-syndromic sensorineural hearing impairment in Sulaimani province. Therefore, this study aimed to investigate the common mutations in the connexin 26 (GJB2) genes in Sulaimani deafness patients.

MATERIAL AND METHODS

Before we started working, we received consent from all the patients who participated in this research, as well as themselves or their families who signed the participation form. This study was approved by the Ethics Committee of University of Sulaimani, college of science, with reference (A-E4, 5/July 2022) and considering the Declaration of Helsinki of 1964.

Subjects

This study was performed in the Sulaimani province located in northeastern Iraq, where the main ethnic group is Kurdish (Figure 1). Hearing impairment of our patients was diagnosed by audiologist specialists from Hiwa institution for deaf and mute, Sulaimani, Iraq, after a medical history, physical examination, audiology evaluation, radiological imaging and laboratory findings. The medical history included the age of patients, the number of family members, of which members have hearing impairment if in the family any other individuals have hearing impairment including closer relatives, the severity of hearing impairment, and consanguinity of parents were questioned, The hearing disability
of patients was started before 2.5 years of age, Buccal cells were collected from (61) (32 male and 29 female) patients aged between 8-25 years old. Both exons of the GJB2 gene were sequenced after being subjected to PCR and gel purification.

Figure 1. Sulimani Province location in Iraq (created depending on the Iraq map available at Global Security .org, 2000) (Ghafur, 2021).

**DNA isolation**

Patient DNA was isolated from buccal cell samples taken by swab from the mouth, and the swabs were stored at -20°C until DNA extraction. DNA extraction and molecular analysis were carried out at Kurdistan Institution for Strategic Studies and Scientific Research (KISSR. HigherPurity™ Buccal Swab Genomic DNA Extraction Kit was used for DNA extraction according to the kit protocol of the manufacturer and the spin column-based purification method (Canvax, Spain).

**PCR**

The HotBegan™ Hot Start Taq DNA Polymerase Master Mix was used for amplification of the exon 1 and exon 2 of the Connexin 26 gene from genomic DNA isolated from buccal cells. The two suitable sets of primers were used for amplification of the 2 exons, the exon 1 PCR was performed using the forward (F1-CCCTCCGTAACTTTCCAGT) and reverse (R1-CCAAGGACGTGTTTGGTC) and the exon 2 using forward (F2-CCCCTGAGTGCCTTTCAGCTAACGA) and reverse (R2-GCGGCTTCGAAGATGACCCGGAAGA) primers. The total volume of the reaction was
15 μL, 7.5 μL of Master Mix, 1 μL of each forward and reverse primers, 3 μL of gDNA template (75ng/ μL), 2.5 μL of nuclease-free water were mixed in a 0.2mL sterile tubes. The mixture was thoroughly homogenized. PCR was performed on an (Applied Biosystems VeritiPro Thermal Cycler), The PCR was performed under cycling conditions of initial denaturation of 10 min. at 94°C, followed by 35 cycles of 45 sec. at 94°C, 45 sec. at 57°C and 1.5 min. at 72°C. The PCR fragments were loaded on 1% agarose gel electrophoresis and compare to LD 100bp ladder plus (DS bio, China).

**Sanger direct sequencing**

The PCR products about (15 μL) were loaded on a 2% agarose gel after the gel purification process was loaded on the automated DNA sequencer (3100 Genetic Analyzer/ Applied Biosystems™ 3130xl System) with a capillary electrophoresis system to determine the exone1 and 2 of connexin 26 gene. The DNA sequences were read with the (KB™ Basecaller Software v1.4.1) and analyzed with Molecular Evolutionary Genetics Analysis (MEGA 11) and Chromas version 2.6.6.

**RESULTS**

In this research 61 (32 male and 29 female) Kurdish samples were selected to determine the genetic and molecular basis of hearing impairment. After amplification of both exons of GJB2 gene and loaded the samples on 1% of agarose gel, we obtained two DNA bands of exons 1 and 2 were (363) bps (Figure 2) and (803) bps (Figure 3) respectively for 11 samples as example, in compare to the 100bp ladder plus, after gel purification and Sanger direct sequencing, the results were screened for mutations with MEGA11 and Chromas 2.6.6. Homozygous c.35delGt; p.Gly12Val (rs80338939) was detected in (9) patients (35delG homozygous mutant) no variation was found in 52 patients (Wild type), an example of both homozygous mutant and wild type show in (Figure 4).The result was uploaded to the National Center for Biotechnology Information genebank accession number (OP557972).

![Figure 2](image_url). PCR products of GJB2 gene, exon 1 on (1%) agarose gel electrophoresis. Lane M: 100bp ladder plus; Lanes 1 to 11: PCR products of eleven samples.
DISCUSSION

The most common mutation of Cx26 in humans is 35delG; it is about half of all pathological mutations in the Cx26 (Qiu et al., 2022). It is the deletion of the last guanine of 6 guanine residue starting from 30 to 35 of the 2nd exon is error point of alpha DNA polymerase. This mutation causes a conversion of glycine to valine in the 12th codon, which leads to the formation of a premature stop codon (Silan et al., 2017; Mishra et al., 2018). Those who are homozygous for this mutation can range from moderate to severe hearing impairment (Azadegan-Dehkordi et al., 2019).

In this study, we identified the 35delG mutation in the GJB2 gene in nine probands with pre-lingual hearing impairment of Kurdish population in Sulaimani province-Iraq. This mutation accounts for 85% of the mutations in GJB2 gene; various studies had determined that the 35delG mutation is the most common in many ethnic groups, (Bahrami et al.,...
GJB2 study in Sulaimani, Iraq

The 35delG mutation originated in ancient Greece and was subsequently propagated in the Mediterranean region. This is the most common mutation in the Mediterranean (Duman and Tekin, 2012) and in Caucasian regions (Elsayed and Al-Shamsi, 2022). According to our results, 35delG is the most frequent GJB2 mutation among the Kurdish population, which is similar to previous studies on the Kurdish in Iran, which found 17.7% (Azadegan-Dehkordi et al., 2019), 13.33% (Biyachal et al., 2021) and 20% (Bahrami et al., 2017). Similarly, several studies indicated that the 35delG variant was the most common mutation in the GJB2 gene among Arab, Fars and Turkish ethnicities. In the Iraq population, this mutation is not fully studied except the studies of Jarada et al. and Al-janabi et al.: they found 16.9% (Jaradat et al., 2016) and 42.1% (Al-Janabi et al., 2021), respectively; our result is similar to the first but far from second one. This mutation was the most common compared to other GJB2 mutations in other Arabic groups, such as Algerians, Lebanon, Palestine, Tunisia, and Jordanians, which gave counts of 81, 33.3, 23, 17 and 16.2%, respectively (Mustapha et al., 2001; Talbi et al., 2019; Al-Janabi et al., 2021); but in Omanian people, it was 0% (Koohiyan, 2019). Our results are close to Jordanian result but far from the result for other Arabic populations. In Iranian population this mutation accounting maximum in northwest is about 22-27%, (Najmabadi et al., 2005; Chaleshtori et al., 2007) north is 20.7% (Chaleshtori et al., 2007), central 13-15% (Bazazzadegan et al., 2012), but the minimum cases reported in the south is 0-4% cases. (Najmabadi et al., 2005; Chaleshtori et al., 2007). In the Turkish ethnicity this mutation accounts for 30% (Kalay et al., 2005). In another finding the 35delG mutation was observed most frequently, with about 23.3% (Uyguner et al., 2003); in a study in the Turkish population the heterozygous mutation of 35delG had a frequency of 4.3% (Silan et al., 2017). Also the frequency of this mutation is higher in most European populations than our result, in the Belgian, Italian, Spanish and American populations, it was 73, 66, 55 and 46%, respectively (Cryns et al., 2004).

CONCLUSION

In this study, we could find the genetic basis of hearing loss in only 9 out of 61 patients. GJB2 mutations were the most common cause of hearing loss, accounting for about 15% of the patients. Other loci should also be investigated to clarify the molecular etiology of hearing impairment in Sulaimani province. Our results highlight the heterogeneity of hearing loss and provide information for the development of a different panel of DFNB loci involved in the etiology of hearing impairment in this province.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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