Molecular Analysis of the *GJB2* Gene in Iraqi Patients with Sensorineural Non-Syndromic Hearing Loss

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Abstract

Objective: Increasingly, with advanced screening methods, the incidence of congenital deafness at birth is recognized as one of the most common defects worldwide. In over 50% of sensorineural hearing loss, a mutation in the *Gap junction beta 2* (*GJB2*) gene, encoding connexin 26 (Cx26) protein is identified. In this study, we aimed to sequence exon 2 of the *GJB2* gene in sixty-three unrelated Jordanian and Iraqi patients with recessive inheritance deafness.

Methods: DNA from 63 patients and 50 healthy controls was analyzed for mutations in the *GJB2* gene by polymerase chain reaction and DNA sequencing.

Results: Six recurrent mutations were identified in 9 out 53 Iraqi patients (16.9%), included in the study. The c.35delG mutation was found in a homozygous genotype in four unrelated patients. Structural analyses of the Arg98Trp missense mutation which is heterozygous in one patient suggested that a mutation at this residue might potentially impair the permeability of Cx26 gap junctions.

Conclusions: To our knowledge, the present study is the first to screen for mutations in the *GJB2* gene in non-syndromic sensorineural deafness patients of Iraqi origin. Three of the identified mutations (Arg98Trp; glu119lys and His100Argfs*14) in this report are described for the first time in Arabic nationality patients. The overall mutation detection rate in the GJB2 coding region in this study is slightly lower than that of other Mediterranean populations, which suggest other mutated allele may be within other regions of the GJB2 gene, which were not sequenced in this study or in other hearing loss loci.

Keywords: *GJB2*; Cx26; c.35delG; Sensorineural deafness; Jordan; Iraq.

(J Med J 2016; Vol. 50 (3):145- 155)

Received	***Accepted***
Feb 26, 2016	*June 7, 2016*

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Introduction

Hearing loss that is manifested as the only single phenotype in deaf patients is classified as a non-syndromic deafness and accounts for 70% of the total cases, whereas in 30% of the cases, deafness occurs as one of multiple phenotypic syndromic manifestations.[1] In the United States, the incidence of deafness at birth was estimated to be 186 per 100,000 newborns, and in a recent screening 966 Jordanian infants had hearing impairments out of the 63,042 infants screened.[1, 2]

To date, mutations in over 96 autosomal genes have been identified in non-syndromic forms of hearing loss, http://hereditary hearing loss.org; Accessed Nov 14, 2015). Of these genes, 60 are inherited as autosomal recessive, 32 as autosomal dominant and 4 as X-linked.

In 1997, using linkage analysis, several groups successfully linked the non-syndromic sensorineural deafness phenotype in several families, including a family from the Mediterranean, to a region on chromosome 13q11-q12, and subsequently, the *Gap Junction Beta 2* gene (*GJB2*) gene was sequenced as a candidate gene and 6 different mutations were identified.[3,4,5] *GJB2* is the most mutated gene thus far in non-syndromic autosomal recessive deafness, accounts for over 50% of the total recessive cases and occurs in both familial and sporadic cases.[1, 6]

The *GJB2* gene consists of 2 exons, of which exon 1 and the first 22 nucleotides of exon 2 constitute the non-coding region of the gene and encodes the 226 residue connexin 26 (Cx26). Thus far, 302 mutations have been identified in the *GJB2* gene (http://www.hgmd.cf.ac.uk/ac/index.php; Accessed Nov 14, 2015), where missense mutations constitute over 60% of the different mutations types characterized in the gene. Several common mutations in the *GJB2* gene were characterized in specific regions of the world and reviewed by Chan and Chang, including, 35delG, 235delC, V37I and W24X in the Mediterranean, East Asia, Southeast Asia and India, respectively.[6]

Each of the six Cx26 molecules assembles to form a single connexon in each cochlea cell where Cx26 is predominantly express.[7,8] Consequently, two connexons of the neighboring cochlea cells make a gap junction between these cells, which serves as a route to maintain the potassium ion concentration balance between the different fluid compartments of the inner ear. Mutations in Cx26 disrupting these cell-to-cell communication channels result in an imbalance of the potassium ion exchange and consequently, impaired hearing function.[7,8] The three-dimensional crystallographic structure of the Cx26 at 3.5 Å resolution revealed that the topological component of each Cx26 monomer crosses the plasma membrane and sheds light on the interactions of the Cx26 monomers in the connexon assembly.[7] The three-dimensional structure aided in the analysis of the consequence of many missense mutations of the connexin proteins in correlation to several phenotypes in defenses, skin diseases and neurological disorders.[7,9]

We sequenced the *GJB2* gene in 63 patients with non-syndromic prelingual hearing loss and described 6 different mutations in Iraqi patients, which, to our knowledge, is the first study to include this nationality.

Methods

Patients

Sixty-three children (53 Iraqis and 10 Jordanians) who were eligible for cochlear implants during the period of 2008 to 2010 were included in this study. The patients were numbered from 1-63 and all had a clinical history consistent with congenital hearing loss without any other syndromic features and encountered a severe to profound bilateral sensorineural hearing loss. There were no other predisposing factors contributing to their condition apart from a history of gentamycin exposure in patient numbers 9 and 17. The ethical and study protocol was approved by the Institutional Review Board of the Jordan University of Science and Technology and the King Abdullah University Hospital. Consent was obtained from the patient parents.

Mutation analysis

Genomic DNA was extracted from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The coding exon 2 of the *GJB2* gene was amplified using three different primer pairs spanning intron 1 to the 3'-UTR regions of the gene. The primers were designed using the Primer3 algorithm (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) according to genomic GenBank accession number NC_000013.11 to amplify a 446, 436, and 421 bp overlapping fragments A, B and C, respectively. The primers had the following sequences: forward-A, [5'-TGCTTGCTTACCCAGACTCA-3'] and reverse-A, [5'-TCACTCTTTATCTCCCCCTTGA-3']; forward-B, [5'-CTGCAAGAACGTGTGCTACG-3'] and reverse-B, [5'-CAGGATGCAAATTCCAGACA -3']; forward-C, [5'-TGGACCTACACAAGCAGCAT -3'] and reverse-C, [5'-TACAGGGGTTTCAAATGGTTG-3']. Briefly, the PCR was performed in a final volume of 25 µl in an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). Each reaction contained 200 ng of genomic DNA, 10 pmol of each primer and 2X GoTaq® Green Master Mix (Promega, USA). The reaction mixtures were initially denatured for 10 min at 95 °C and then cycled 35× at 95°C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. Polymerase chain reaction (PCR) products were purified using the EZ-10 Spin Column PCR Products Purification Kit (Bio Basic, Markham, ON, Canada) and were sequenced in both directions using the Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequencing products were cleaned up with NucleoSEQ spin columns (Macherey-Nagel, Düren, Germany), separated on a 3130xl Genetic Analyzer (Applied Biosystems), and compared to reference sequences (GenBank accession number: NM_004004.5) using ChromasPro 1.34 (Technelysium Pty. Ltd., Australia). The sequence nomenclatures for the variations are described in accordance with the Human Genome Variation Society Nomenclature standards (http://www.hgvs.org/).

Phylogenetic conservation and structural analyses of the Arg98 missense mutation

Multiple Cx26 and other connexin amino acid sequence alignments were generated using Clustal Omega (http://www.clustal.org/omega/). The structural analyses were carried out based on the crystal structure of human Cx26 (PDB ID: 2ZW3) using PYMOL (http://www.pymol.org).

Results

All of the patients experienced severe to profound bilateral sensorineural hearing loss without any other syndromic phenotype. Eighty-three percent of the patients were products of consanguineous marriages, and 35% of the patients had at least one sibling with hearing loss. The patients ages ranged from 1 to 16 years at the time of their referral to the audiologist clinic for cochlear implant eligibility. All of the patients received a complete audiological assessment, radiological examinations and neurological and psychological evaluations.

We sequenced the coding and the flanking regions of exon number 2 of the *GJP* gene for all 63 patients. We identified 2 frame-shifts and 4 missense mutations in 9 Iraqi patients, but no mutations were identified in any of the 10 Jordanian patients included in the study. The mutations are: c.35delG; p.Gly12Valfs*2 (rs80338939) (Figure 1A, B), which is found in the homozygous in patients numbered 10, 34, 41 and 63; c.223C>T; p.Arg75Trp (rs104894402) (Figure 1C, D), which is heterozygous in patient 47; c.299T>C; p.Trp77Arg (rs104894397) (Figure 1E, F), which is homozygous in patient 53; the c.292C>T; p.Arg98Trp **(**rs529440698) (Figure 2A, B), which is heterozygous in patient 46; c.355G>A; p.glu119lys (rs150529554) (Figure 2C, D),which, in patient 49, was heterozygous; c. 299_ 300delAT; p.His100Argfs* 14 (rs111033204) (Figure 2E, F), which was homozygous in patient 51. We also identified two missense variants, c.79G>A; p.Val27Ile (rs2274084**)** and c.368C>A; p.Thr123Asn (rs111033188), in patient number 35. An additional missense c.457G>A; p.Val153Ile (rs111033186) and a synonymous substitution c.15G>A; Thr5Thr (rs757226502) variant was characterized in patients 47 and 3, respectively. Three 3'-UTR polymorphisms were also characterized, including c.*84T>C (rs3751385 SNP), c.*104A>T (rs7337074 SNP) and c.*111C>T (rs7329857 SNP).

Similar to both Arg75 and Trp77residues, the Arg98 amino acid are evolutionary conserved across orthologous connexin proteins (Figure 3). Mapping the Arg98 residue on the human crystallographic Cx26 structure, which has been resolved at 3.5 Å resolution, showed that the Arg98 amino acid is located in the interface between the intracellular channel entrance and the membrane of the Cx26 gap junction channel (Figure 4).[7]

Discussion

Thus far, mutations in over 40 autosomal recessive genes have been identified in Middle-Eastern Populations, and the mutation list is reviewed by Najmabadi et al.[10] Mutations in the *GJB2* gene in Arabic people accounts for 33.3% in Lebanon, 23.0% in Palestine, 17.0% Tunisia, 40% in Algeria and 16.2% in Jordan.[11-16] To the best of our knowledge, this is the first study to screen for mutations in the *GJB2* gene in Iraqis patients with hearing lost. Nine out of 53 patients (16.9%) were either heterozygous or homozygous for the 6 different mutations identified in this study.

The c.35delG frame-shift mutation was characterized as homozygous in four unrelated patients. The *GJB2* mRNA transcript of the c.35delG allele harbored an earlier premature translation termination codon (TGA) at Val13, resulting in the truncation of the Cx26 protein by 213 residues. The c.35delG was characterized originally in Mediterranean

families with an allele frequency over 63%.[4] The mutation resulted in the complete loss of function of the Cx26 protein and that was evident from the complete absence of protein expression in the patients homozygous for the mutation.[16] The c.35delG mutation was described in all of the different Arabic nationalities screened for the *GJB2* gene with a variable allele frequency, and notably, it is the only mutation found thus far in Jordanians.[11-17]

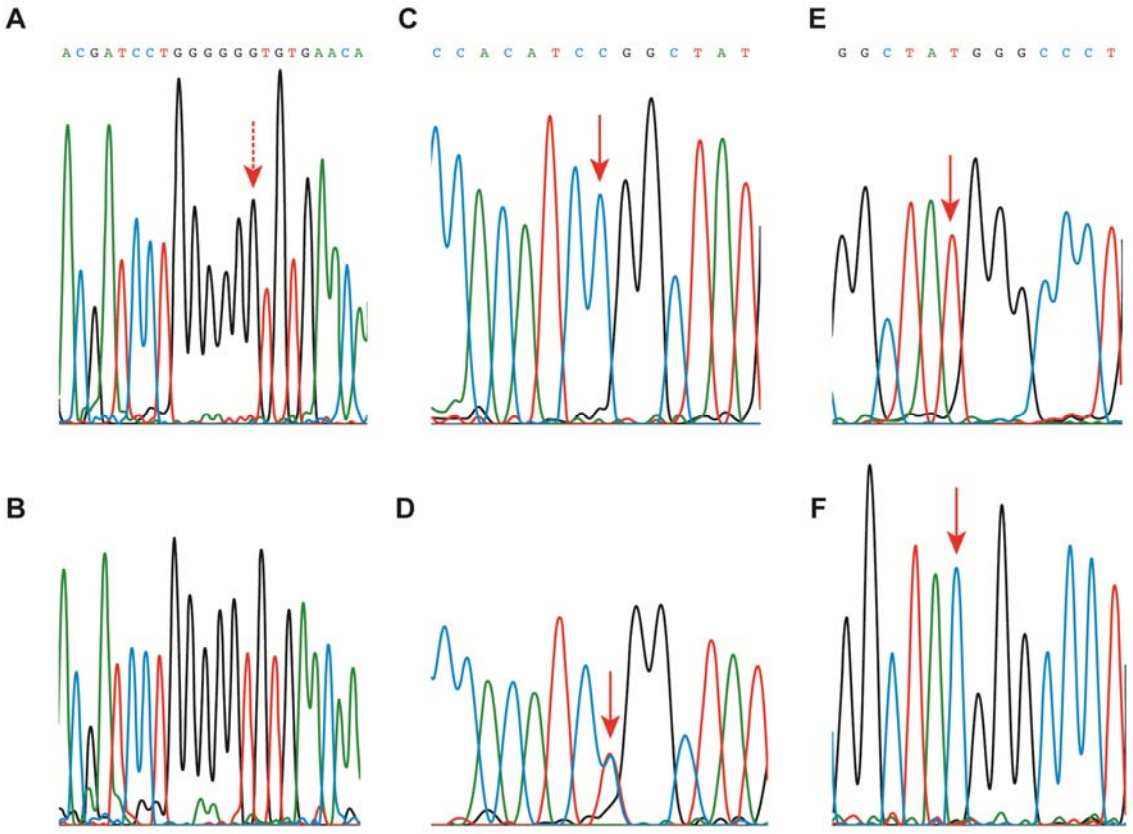

Figure 1. Sequencing chromatograms encompassing the *GJB2* mutations. c.35delG; p.Gly12Valfs*2 (A) homozygous wild type; (B) homozygous mutant type; c.223C>T; p.Arg75Trp (C) homozygous wild type; (D) heterozygous genotype; c.299T>C; p. Trp77Arg; (E) homozygous wild type; (F) homozygous mutant type. The dashed arrow indicates the location of the G nucleotide deletion, and the solid arrows indicate the locations of the substitution mutation

The c.299_300delAT frame-shift mutation that introduced a premature stop codon at residue 113 (Ser295X) and resulted in an amino acid substitution for residue His100 was characterized at a low allele frequency as heterozygous and homozygous in Asian and Mediterranean populations, respectively.[18,19] In this study, we identified this mutation for the first time in Arabic patients with profound hearing loss.

One patient was compound heterozygous for the Val153Ile polymorphism and the Arg75Trp mutation. The Ile153 is found in trans with the Trp75 in normal hearing parents, which confirms the polymorphic nature of this

variant.[20] The Arg75Trp mutation was characterized initially as a heterozygous genotype in an Egyptian family with sensorineural deafness and palmoplantar keratoderma syndrome.[21] Both the Cx26 Trp75 mutant and the wild type Cx26 coimmunoprecipitated from HeLa cells extracts, suggesting dominant negative effects for this mutation.[22] Interestingly, the variable phenotypic expressivity for the Arg75Trp and Arg75Gln mutations, which affect the same residue, have been observed.[23]

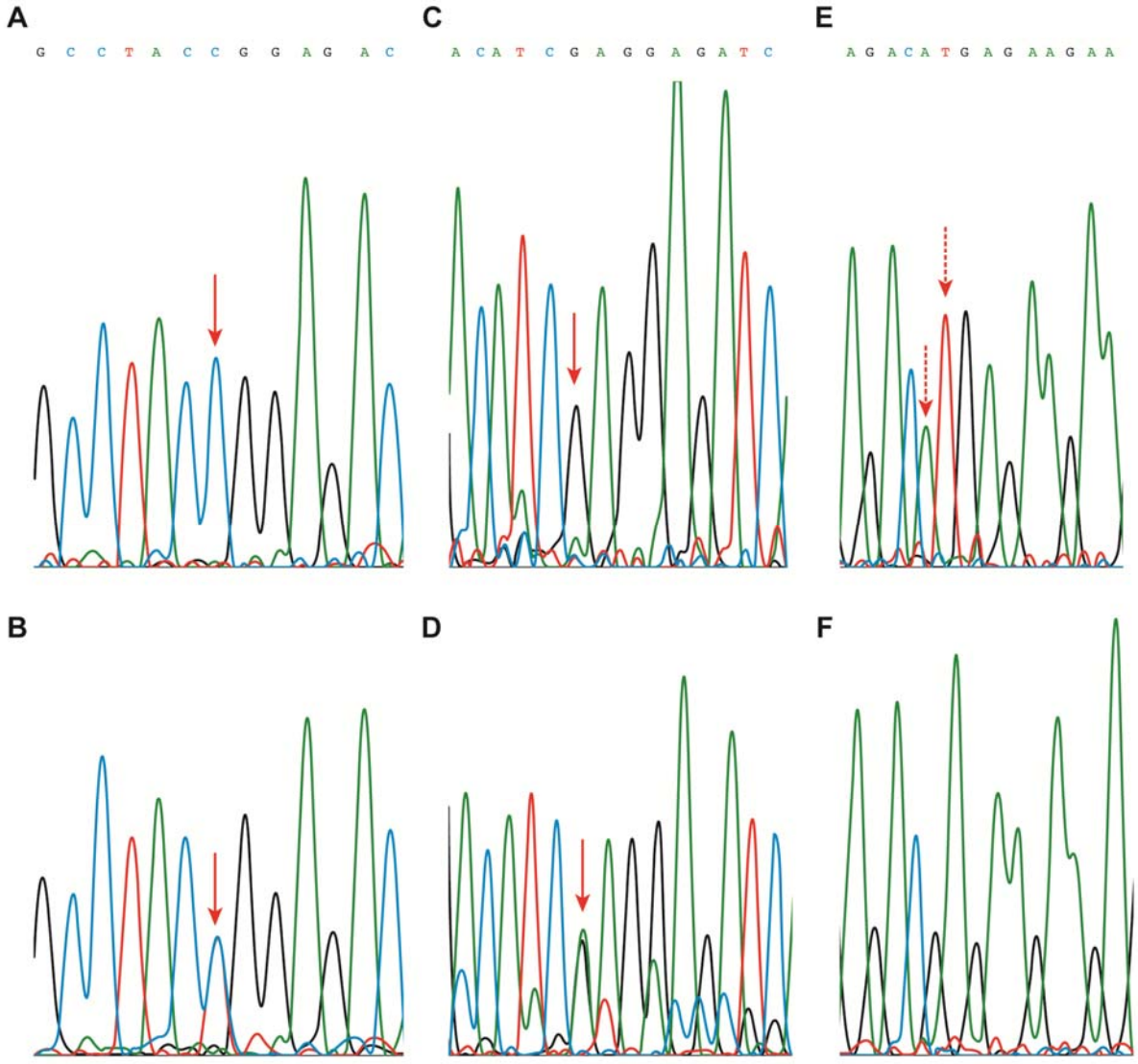

Figure 2. Sequencing chromatograms encompassing the *GJB2* mutations. c.292C>T; p.Arg98Trp (A) homozygous wild type; (B) heterozygous genotype; c.355G>A; p.glu119lys (C) homozygous wild type; (D) heterozygous genotype; c.299_300delAT; p.His100Argfs*14 (E) homozygous wild type; (F) homozygous mutant type. The dashed arrow indicates the location of the AT dinucleotide deletion, and the solid arrows indicate the locations of the substitution mutation

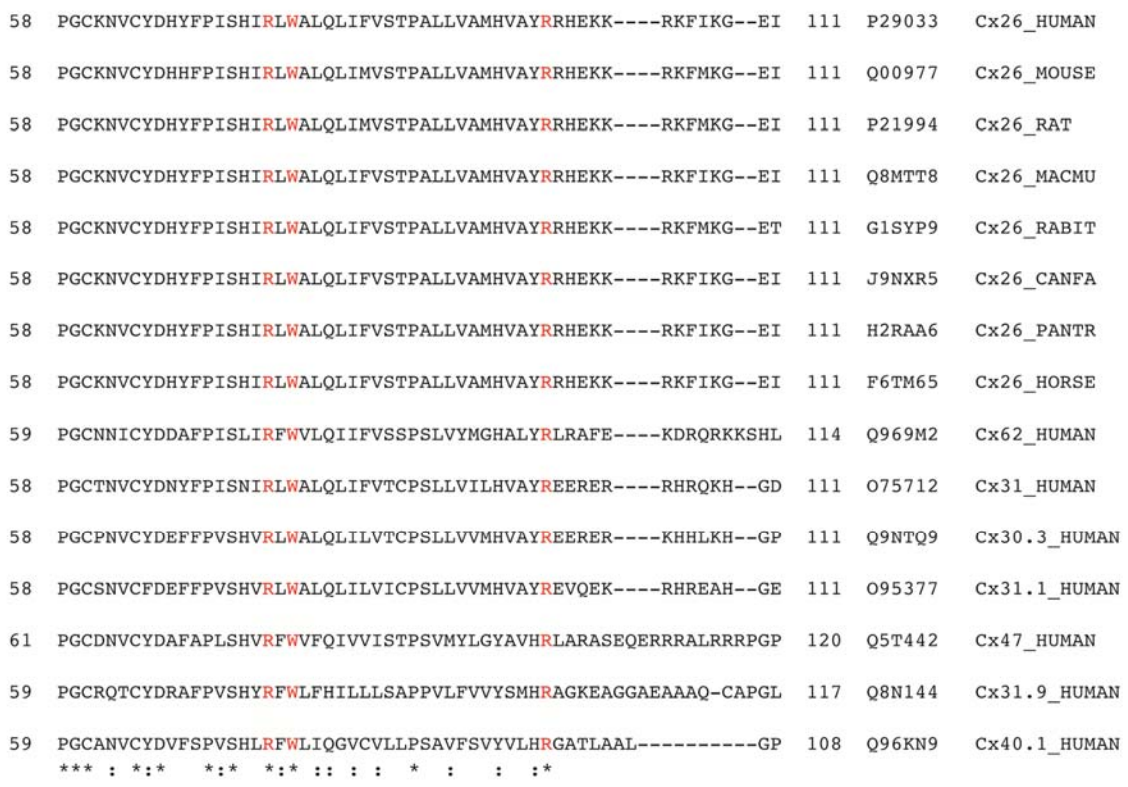

Figure 3. Phylogenetic conservation analysis of the connexin 26 (Cx26) Arg75, Trp77 and Arg98 residues. The mutant amino acids are highlighted in red.

The Trp77Arg missense mutation occurred as homozygous in one patient and was originally characterized in a large Arabic family. [5] The consequence of the replacement of the non-polar residue (Trp) by a positively charged amino acid (Arg) in the second transmembrane domain (TM2) of Cx26 was the complete loss of the capability of Cx26 to form functional gap junction channels.[24] This is in agreement with finding this mutation in the severely deaf patient number 53.

The evolutionarily conserved amino acid Arg98 is reported to be mutated to Gln, Pro and Trp residues. Interestingly, the mutations Arg98Pro and Arg98Gln occurred as heterozygous in the hearing loss patients, whereas the Arg98Trp mutation occurred as compound heterozygous in a Greek proband.[25,26,27] In this study, the c.292C>T; p.Arg98Trp mutation was characterized as heterozygous in two sisters with bilateral, prelingual, sensorineural profound deafness, whereas the Greek patient manifested postlingual hearing loss. [27] The mutation was not found in 100 control chromosomes, confirming the pathogenicity of this mutation. The Arg98 residue mapped to the interface between the intracellular channel entrance, which is an important component of the permeation pathway of the Cx26 gap junction channel and the membrane.[7] The intracellular channel entrance is formed by the intracellular parts of the transmembrane helices 2 and 3 (TM2 and TM3) of Cx26 where the positively charged residues in both TM2 and TM3 generate a positively charged environment at the channel entrance. The positive atmosphere

around the intracellular channel entrance is favorable for concentrating and increasing the absolute permeability of negatively charged molecules. A mutation from the positively charged arginine to the large hydrophobic tryptophan in this location might interfere with the permeability of these molecules. In addition, one cannot exclude the fact that such a mutation also might cause some conformational changes in this area.[9] Further functional studies are needed to evaluate the pathogenicity and inheritance pattern (dominant or recessive) of this mutation.

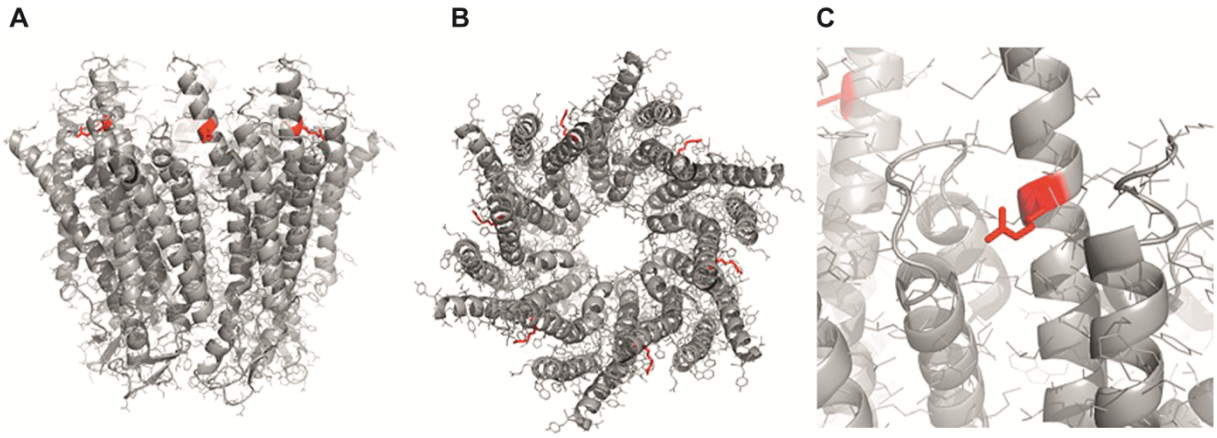

Figure 4. Structural analyses of the Arg98 mutation based on the crystal structure of the human Cx26 gap junction channel. The amino acid Arg 98 (depicted in red) is located in the positively charged intracellular channel region (PDB ID: 2ZW3; Maeda et al, Nature. 2009 Apr 2;458(7238):597-602). (A) Side view of the connexon with the intracellular channel region on top. (B) Top view of the connexon looking down the intracellular region. (C) Close-up view of Arg 98. The figures were prepared with PYMOL (http://www.pymol.org)

The c.355G>A; p.glu119lys mutation was found as heterozygous in one patient and was originally described in a four-year-old male with severe to profound hearing lost. [28] The mutation occurred in the cytoplasmic loop (CL; residues 110–124) of the Cx26 protein and replaced a negatively charged residue by a positively charge amino acid. Glu119 is not involved in gap junction formation; however, it might affect the permeation of the connexin channels by affecting the voltage-dependent gating mechanism. [29] The second mutated allele in this patient might be within the *GJB2* 5'-regulatory region, which was not sequenced in this study, or in other connexin genes considering the digenetic inheritance of hearing impairment.

The two variants, p.Val27Ile and p.Thr123Asn were originally described as benign polymorphisms.[30] However, in other study, including 324 cases of deafness and 100 control samples, both variants were found in the patients but not in the controls. Thus, the pathogenicity of these two variants is still uncertain.[28]

In summary, we identified six mutations in the *GJB2* gene in 9 Iraqi patients with congenital sensorineural deafness. Three of these mutations (Arg98Trp; glu119lys and His100Argfs*14) are described for the first time in Arabic nationality patients. The lower

mutation rate in the *GJB2* coding exon in this study group suggested other mutated allele may be within the untranslated regions of the gene, which were not sequenced in this study, or in other recessive hearing impairment loci. Thus, these findings emphasize the need for high-throughput molecular diagnostic methods, such as a next generation sequencing platform, to allow for the screening of more deafness genes in the patients.

Acknowledgements

We are thankful to the patients and their family members for their participation in the study. We are grateful for the contribution of the Princess Haya Biotechnology Centre staff at the Jordan University of Science and Technology (JUST) for their technical help in sequencing the patient *GJB2* genes. This work was supported by a grant from the Deanship of Research at JUST.

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دراسة الطفرات الوراثية لجين الـ GJB2 المسببة للصمم الخلقي غير المتلازم عند الأطفال العراقيين

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الملخص

الهدف: بازدياد وتطور طرق الكشف المتقدمة، يتم التعرف إلى حالات الصمم الخلقي عند الولادة باعتبارها واحدة من أكثر العيوب شيوعاً في جميع بلدان العالم. في نحو أكثر من 50% من مرضى فقدان السمع الحسي العصبي الخلقي غير المتلازم تكون نتيجة لطفرة في جين الفجوة تقاطع بروتين بيتا 2 (*GJB2*) والمسؤول عن إنتاج بروتين الكونكسن 26 (Cx26). تهدف هذه الدراسة التعرف إلى الطفرات في جين الـ *GJB2* في ثلاث وستين مريضاً من الأردنيين والعراقيين مصابين بفقدان السمع الخلقي المتنحي.

الطريقة: تم استخلاص الحمض النووي (DNA) من 63 مريضاً وتم مضاعفة الـ (DNA) عن طريق تقنية البلمرة المتسلسل (PCR) والتعرف إلى تسلسل الحمض النووي للجين باستعمال تقنية الـ DNA-Sequencing .

النتائج: تم تحديد ستة طفرات في 9 من 53 مريضاً عراقياً (16.9%) شملتهم الدراسة وكانت الطفرة (c.35delG) هي الأكثر تكراراً حيث وجدت في أربعة مرضى. وأشارت التحليلات الهيكلية للطفرة Arg98Trp والتي وجدت في مريض واحد لاحتمالية أن تضعف هذه الطفرة نفاذية قناوات الفجوة بين الخلايا.

الاستنتاجات: هذه الدراسة هي الأولى للمتغيرات الوراثية لجين *GJB2* في مرضى عراقيين مصابين بفقدان السمع الخلقي. ثلاثة من الطفرات التي تم تحديدها في هذه الدراسة تم وصفها لأول مرة في مرضى من أصول عربية. نسبة اكتشاف الطفرات في جين *GJB2* في مجموعة المرضى التي شملتهم الدراسة هي أقل من شعوب المنطقة المتوسطية التي درست لهذا الجين، وهذا دليل على وجود طفرات أخرى في جينات أخرى مسببة لفقدان السمع في مجموعة مرضى الدراسة.

الكلمات الدالة: الفجوة تقاطع بروتين بيتا 2، كونكسن 26، c.35delG، فقدان السمع الخلقي، الأردن، العراق.