Digenic inheritance in autosomal recessive non-syndromic hearing loss cases carrying GJB2 heterozygote mutations: Assessment of GJB4, GJA1, and GJC3

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Abstract

Objective: Autosomal recessive non-syndromic hearing loss (ARNSHL) can be caused by many genes. However, mutations in the GJB2 gene, which encodes the gap-junction (GJ) protein connexin (Cx) 26, constitute a considerable proportion differing among population. Between 10 and 42 percent of patients with recessive GJB2 mutations carry only one mutant allele. Mutations in GJB4, GJA1, and GJC3 encoding Cx30.3, Cx43, and Cx29, respectively, can lead to HL. Combination of different connexins in heteromeric and heterotypic GJ assemblies is possible. This study aims to determine whether variations in any of the genes GJB4, GJA1 or GJC3 can be the second mutant allele causing the disease in the digenic mode of inheritance in the studied GJB2 heterozygous cases.

Methods: We examined 34 unrelated GJB2 heterozygous ARNSHL subjects from different geographic and ethnic areas in Iran, using polymerase chain reaction (PCR) followed by direct DNA sequencing to identify any sequence variations in these genes. Restriction fragment length polymorphism (RFLP) assays were performed on 400 normal hearing individuals.

Results: Sequence analysis of GJB4 showed five heterozygous variations including c.451C->A, c.219C->T, c.507C->G, c.155_158delTCTG and c.542C->T, with only the latter variation not being detected in any of the control samples. There were three heterozygous variations including c.758C->T, c.717G->A and c.3dupA in GJA1 in four cases. We found no variations in GJC3 gene sequence.

Conclusion: Our data suggest that GJB4 c.542C->T variant and less likely some variations of GJB4 and GJA1, but not possibly GJC3, can be assigned to ARNSHL in GJB2 heterozygous mutation carriers providing clues of the digenic pattern.

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1. Introduction

Hearing loss (HL), with an incidence of about 1 in 1000 neonates, is the most frequent sensorineural disorder. It is extremely heterogeneous and may occur due to genetic or environmental causes or both [1,2]. Despite this genetic heterogeneity, mutations in the GJB2 gene (MIM ID: 121011) which encodes connexin (Cx) 26 protein, are involved in up to 50% of autosomal recessive non-syndromic HL (ARNSHL) cases in many populations [3]. From 10 to 42 percent of patients with GJB2 mutations are heterozygous carriers of a mutant GJB2 alleles [4–10]. Cxs are the building blocks of gap junctions (GJs) that build intercellular channels allowing communication between adjacent cells. The oligomerization of six Cx subunits forms a hemichannel called connexon and assembling of two connexons build up a complete GJ [11]. Mutations in the related genes of some members of the Cx gene family such as Cx26, Cx29, Cx30, Cx31, Cx32, Cx30.3, and Cx43 have been shown to lead to HL [12–14] and can be inherited either in monogenic or digenic mode. Furthermore, Cxs have a great potential to form different combinations of heteromeric and heterotypic GJ assemblies [15]. In Iran 18.29% of ARNSHLs are caused by GJB2 mutations, 22.8% of which, that is 4.17% of total ARNSHL cases, would carry only one mutant allele in GJB2 gene [16]. Digenic inheritance hypothesis in GJB2 heterozygous cases has previously been proved for GJB6 and GJB3 alterations to comprise the second mutant allele [17–21]. A few studies have assessed GJB4 [22–24], GJA1 [23], and GJC3 [23,25] genes in

Abbreviations: Cx, connexin; GJ, gap junction; HL, hearing loss; ARNSHL, autosomal recessive non-syndromic hearing loss.

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ARNSHL cases, though their chief aim was not to appraise the digenic hypothesis. In the present investigation, we have investigated the contribution of GJB4, GJA1, and GJC3 in GJB2-related ARNSHL patients with only one mutant allele.

2. Methods

Subjects and control samples. A total of 34 deaf subjects and 400 normal hearing individuals were included in this study. All deaf

Table 1

<table>
<thead>
<tr>
<th>Heterozygous GJB2 variants</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>35delG/WT</td>
<td>30/34</td>
<td>88.23</td>
</tr>
<tr>
<td>E47X/WT</td>
<td>1/34</td>
<td>2.94</td>
</tr>
<tr>
<td>235delC/WT</td>
<td>1/34</td>
<td>2.94</td>
</tr>
<tr>
<td>W244X/WT</td>
<td>1/34</td>
<td>2.94</td>
</tr>
<tr>
<td>R184P/WT</td>
<td>1/34</td>
<td>2.94</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 1. Sequencing electropherogram of detected variations. (A)–(D) GJB4 PCR products, below: WT allele. Above: heterozygous alleles (indicated by arrows): (A) 451C>A, (B) 219C>T, (C) 507C>G, (D) 542C>T. (E) and (F) GJA1 PCR product, below: WT allele. Above: heterozygous alleles: (E) 758C>T, (F) 717G>A. (G) GJB4 PCR product, above: WT allele, middle and below: 155_158delTCTG heterozygous allele sequenced in two directions. (H) GJA1 PCR product, above: WT allele, middle and below: c.3*dupA heterozygous allele sequenced in two directions.
subjects were ARMSH4 cases with only one mutant GJB2 allele (Table 1) identified in 890 affected families with mild to profound sensorineural HL in our previous study [16]. We collected the control samples from 4 provinces (100 for each region) based on the geographic region from where patients with new allelic variants were detected. Informed consent was obtained from all deaf subjects and the controls. The study protocol was approved by the Ethics Committee of Shahrekord University of Medical Science, Iran.

Molecular analysis. Genomic DNA was extracted from peripheral blood of the deaf subjects and control individuals, using a standard phenol–chloroform procedure. Polymerase chain reaction (PCR) reactions were performed to amplify the entire coding sequence of GJB4 (RefSeq ID: NM_153212), GJA1 (RefSeq ID: NM_000165), GJC3 (RefSeq ID: NM_181538), and GJB2 (RefSeq ID: NM_004004), plus sequences of about 50 bp up/down stream. Except GJB2 primer sequences which we reported in our previous study [26], other primers were designed using Primer3 (v. 0.4.0) web-based software1 [27] (Table 2) and were blasted (NCBI/Primer-BLAST2) in order to check their specificity. The Quality of PCR products were verified on poly-acrylamide gel electrophoresis (PAGE). DNA sequencing of the PCR-amplified products was carried out bi-directionally on an ABI 3130 automated sequencer (Applied Biosystems, Macrogen, South Korea) using the same primers.

Sequence data were then analyzed comparing with published (UCSC Genome Browser3) sequences. The found variants were investigated in 100 geographically and ethnically matched control samples by subjecting the related PCR product to the procedure of restriction fragment length polymorphism (RFLP). All products with positive RFLP results were sequenced. The GJB2 gene was sequenced in all control individuals with variants in any of the three genes. Conservation scores of the amino acids, which were predicted to change, were acquired from the ConSurf Server (the online server for the identification of functional regions in proteins4) [28].

## 3. Results and discussion

Altogether, eight different heterozygous allelic variants were identified in the three genes in 9 of 34 (26.47%) deaf subjects studied (Table 3) (Fig. 1). Any variants found in the control individuals for GJB4 or GJA1 using RFLP, were confirmed by DNA sequencing. The GJB2 gene sequence was normal in all the control individuals carrying variants in GJB4 or GJA1.

Analyzing GJB4 gene, we detected five different variants in 5/34 (14.70%) of the patients and 13/200 (6.5%) of the controls. Out of the 5 patients, 4 were heterozygote for 35delG GJB2 mutation and one carried R184P. The first variant c.451C–A (Arg151Ser) was found in a patient from Golestan province in north of Iran. A semi-nested PCR was performed using F2B4 primer with a mismatch nucleotide (Table 2) to produce a restriction site for BsiEI enzyme. We found one heterozygote in 100 controls from the corresponding province. This allelic variant results in a substitution of arginine to serine at codon 151 of Cx30.3 within the second extracellular loop domain affecting a residue with a low conservation score of 2.

### Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer directions</th>
<th>5′–3′ primer sequences</th>
<th>Product size (bp)</th>
<th>Region</th>
<th>Annealing for PCR (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GJB4</td>
<td>Forward</td>
<td>F1B4: TCAATGCCACACCATTAAG</td>
<td>964</td>
<td>Exon 2</td>
<td>Touch down 61 to 56</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>F2B4: GCATCCCTTATATCTCCGA</td>
<td>407</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GJA1</td>
<td>Forward</td>
<td>R1B4: GGGGAGCTTGTGATCATCTTC</td>
<td>1281</td>
<td>Exon 2</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>R1A1: CTTGGGTCAGCTTCTACAGGCT</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GJC3</td>
<td>Forward</td>
<td>F1C3: GCCTCCCTGGAAGGACAGT</td>
<td>896</td>
<td>Exon 1</td>
<td>Touch down 65 to 58</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>R1C3: GGGAGGAGCATCAGACGAGA</td>
<td>190</td>
<td>Exon 2</td>
<td>60</td>
</tr>
<tr>
<td>GJB2</td>
<td>Forward</td>
<td>F1B2: CTTCCTTGTCTGTCCTAGCT</td>
<td>809</td>
<td>Exon 2</td>
<td>Touch down 62 to 58</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>R1B2: CTATCCCTCTATCTGCT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Semi-nested PCR primers. Mismatch nucleotides are given in italics.

### Table 3

Full report of detected variations.

<table>
<thead>
<tr>
<th>Variant genotype</th>
<th>Amino acid change</th>
<th>Primary GJB2 genotype</th>
<th>Protein domain</th>
<th>Predicted effect</th>
<th>Patients freq. (%)</th>
<th>Control Freq.</th>
<th>SNP</th>
<th>RFLP enzyme</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GJB4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.451C&gt;T/Wt</td>
<td>(Arg151Ser)</td>
<td>35delE/G/Wt</td>
<td>E2</td>
<td>Missense</td>
<td>1/34 (2.94)</td>
<td>1/100</td>
<td>rs78499418</td>
<td>BsiI</td>
<td>This study</td>
</tr>
<tr>
<td>c.219C&gt;T/Wt</td>
<td>(p.+)</td>
<td>35delE/G/Wt</td>
<td>M2</td>
<td>Silent</td>
<td>1/34 (2.94)</td>
<td>–</td>
<td>rs143547547</td>
<td>–</td>
<td>This study</td>
</tr>
<tr>
<td>c.507C&gt;G/Wt</td>
<td>(p.Cys169Trp)</td>
<td>35delE/G/Wt</td>
<td>E2</td>
<td>Missense</td>
<td>1/34 (2.94)</td>
<td>7/100</td>
<td>rs79193415</td>
<td>StyI</td>
<td>[22]</td>
</tr>
<tr>
<td>c.155_158delTCTG/Wt</td>
<td>(p.Ala52ValfsX55)</td>
<td>35delE/G/Wt</td>
<td>E2</td>
<td>Frameshift</td>
<td>1/34 (2.94)</td>
<td>5/100</td>
<td>NR</td>
<td>Thbl1111</td>
<td>[22]</td>
</tr>
<tr>
<td>c.542C&gt;T/Wt</td>
<td>(p.Thr181Met)</td>
<td>R184P/Wt</td>
<td>E2</td>
<td>Missense</td>
<td>1/34 (2.94)</td>
<td>0/100</td>
<td>rs142410428</td>
<td>Ncol</td>
<td>This study</td>
</tr>
</tbody>
</table>

### M.

- **M. transmembrane domains**: E, extracellular loop domains; C, C-terminal domain; NR, not reported.
- **HGVS (Human Genome Variation Society) nomenclature.**
- **Reported in database of Single Nucleotide Polymorphisms (dbSNP).**

3. [http://genome.ucsc.edu/cgi-bin/hgGateway](http://genome.ucsc.edu/cgi-bin/hgGateway) Created by the Genome Bioinformatics Group of UC Santa Cruz, ©The Regents of the University of California (last accessed 2.23.2012).
(Table 4). This domain is crucial for docking of GJ hemichannels and is responsible for the compatibility between different connexin proteins to form heterotypic functional GJ channels [29]. However, considering that the altered arginine was a non-conserved amino acid with possibly low functional property in the protein, and observing the variation among healthy control samples, there would be a low possibility of its contribution to the disease.

Another variation was c.219C>T, found in one of the deaf subjects from Gilan in north of Iran, accompanying GJB2 35delG allele. It does not change the highly conserved histidine codon in position 73 in the second transmembrane domain and is considered a silent (synonymous) variation. Although the integrity of this domain is essential for the accurate transport of connexin into plasma membrane, it seemed fairly improbable for a silent variation to play a role in disease pathogenesis. No control samples were screened for that. More studies are necessary to specify any possible role of this variation on protein levels or conformation [30].

The variant c.507C>G (p.Cys169Trp), found in a patient from Azerbaijan Sharqi, Northwest Iran, was in double heterozygosity with GJB2 35delG. This patient had two other siblings with HL. The altered cysteine is a residue with a high conservation score of 9 (Table 4) substituted by tryptophan at codon 169 of Cx30.3 in the second extra cellular domain. Therefore, this alteration may result in incompatible forming of heterotypic functional channels. As the variation generated an additional restriction site for StyI, using the restriction enzyme, we detected seven heterozygotes carrying this variant in 100 control samples from the same region. As the GJB2 sequence of all the healthy control individuals was normal, it can be regarded as the evidence of possible digenic effect between GJB4 and the mutant GJB2. Cys169Trp has previously been suggested to contribute to HL in two studies based on the fact that its observed frequencies in patients were significantly higher than those of the controls [22,23].

In one patient from Azerbaijan Sharqi, with GJB2 35delG mutation, a 4 bp deletion (c.155_158delTCTG) was detected in GJB4. The patient was the only affected person in his family. This allelic variation causes a frameshift generating a new stop codon at position 55 (counting starts with the changed amino acid). This deletion eliminated a restriction site of Thh111 enzyme. Among 100 control samples from Azerbaijan Sharqi, five had this variant in heterozygote form. López-Bigas et al. [22] proved that this variation is not a cause of HL since they found no significant difference between patient and control groups in this variant. In view of digenic hypothesis, this protein with the premature stop codon that would be exposed to degradation in the cell, would have minimal effect on the HL pathogenesis [31].

The c.542C>T variant was detected to be double heterozygous with GJB2 R184P mutation in one patient from Azerbaijan Sharqi. He had another affected brother. The variation substituted a tryptophan, with the high conservation score of 9 (Table 4), to methionine in second extra cellular loop domain of Cx30.3. While the tryptophan residue is present in the sequences from a diverse set of taxa including fish, Primata and rodent, M residue is only seen in Xenopus sp. based on the multiple sequence alignment involving 150 sequences from different sources. Thus, it is very probable to negatively affect the hemi-channel docking and the co-assemble of GJB4 with different connexins in a heterotypic channel. This variant introduced one restriction site for Ncol. We did not detect this allelic variation in any of 100 control individuals from Azerbaijan Sharqi. Therefore Among our detected GJB4 variations T181M is the most likely variant to contribute to HL in digenic mode together with the GJB2 mutation R184P.

Analysis of GJA1 gene showed 3 variants in 4/34 (11.76%) patients and 7/200 (3.5%) of the controls. They all carried 35delG as the primary GJB2 mutation. One patient from Khuzestan, in southwest Iran, was found to carry c.758C>T in GJA1 along with a 35delG mutation in GJB2. The variant was seen in 1/100 of the controls from Khuzestan province. The variant results in a substitution of an alanine with a low conservation score of 1 (Table 4), to valine in unusually long cytoplasmic C-terminal domain of Cx43. This domain of the protein is the only part of it that includes functional phosphorylation sites for kinases. In the presence of this variation, one of the restriction sites for Hhal was abolished. The variation has been previously reported to contribute to glaucoma [32] and oculodentodigital dysplasia [33]. Taking into account that the altered alanine is a non-conserved amino acid, it is less likely to have a contributory effect on HL pathogenesis in this case.

The c.717G>A was detected in one patient from Khuzestan, in double heterozygosity with GJB2 35delG mutation. It caused no change in arginine residue in cytoplasmic C-terminal domain of Cx43. This variation seems to have no causative effect on protein and/or disease pathogenesis.

A variation of c.3’dupA, in which the third nucleotide downstream of the stop codon was duplicated, was found in one patient from Khorasan Razavi, northeast Iran, and in another case from Azerbaijan-Sharqi, both being in double heterozygosity with GJB2 35delG. A mismatch forward primer (F2A1) was designed (Table 2) to conduct a semi-nested PCR which generated a restrictions site of BsiI. This variant was found in two of 100 control individuals from Khorasan Razavi and 4/100 of controls from Azerbaijan Sharqi. Since this variant is not located in the coding sequence (3’ UTR), the possible outcome is currently unclear and warrants functional studies.

We detected no variations in the two exons of GJC3 gene and all the 34 patients showed normal sequences. In the present study, we tried to clarify the possible contribution of digenic inheritance in ARNSHL in Iran and to identify probable suitable genes to be assessed in this issue. In this study, 26.47% of GJB2 heterozygotes had a second variant in another Cx gene, actually being double
heterozygote. Notably, none of the controls were in double heterozygosity status for the studied genes and GJB2. Thus, a given variant in one of the studiedCx genes might be pathogenic when accompanied by another variant in GJB2 in a digenic pattern. We propose that GJB4 T181M variant and with lower possibility other various alterations of GJB4 and GJA1, but not possibly GJC3, can contribute to ARNSHL in a digenic pattern in GJB2 heterozygous mutation carriers. Further functional and familial studies are required to definitely confirm the role of other variations of GJB4 and GJA1, and to determine whether other members of the Connexin gene family play any role in the digenic hypothesis in HL.

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