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GJB2 mutations causing autosomal recessive non-syndromic hearing loss (ARNSHL) in two Iranian populations: Report of two novel variants

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ABSTRACT

Objective: Hereditary hearing loss (HL) is a noticeable concern in medicine all over the world. On average, 1 in 166 babies born are diagnosed with HL in Iran, which makes it a major public health issue. Autosomal recessive non-syndromic HL (ARNSHL) is the most prevalent form of HL. Although over 60 genes have been identified for ARNSHL, *GJB2* mutations are the most prevalent causes of ARNSHL in many populations. Previous studies have estimated the average frequency of *GJB2* mutations to be between 16 and 18% in Iran, but would vary among different ethnic groups. In the present study, we aimed to determine the frequency and mutation profile of 70 deaf patients from two different provinces (center and west) of Iran.

Methods: We enrolled 70 Iranian deaf patients with ARNSHL from Isfahan (40 family) and Hamedan (30 family) provinces. After extraction of genomic DNA, the entire coding region of *GJB2* was directly sequenced in all patients. Multiplex PCR was used for detection of del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) in the *GJB6* gene. *In silico* analyses were also performed by available software tools.

Results: A total of eleven different mutations were detected, nine of which were previously reported and the other two (c.130T > G and c.178T > G) were novel. Homozygous *GJB2* mutations were observed in 22.5% and 20% of all the subjects from Isfahan and Hamedan provinces, respectively. c.35delG was the most frequent mutation. One compound heterozygous genotype (c.358_360delGAG/c.35delG) was observed for c.35delG. Screening for the two *GJB6* deletions did not reveal any positive sample among heterozygous or *GJB2* negative samples.

Conclusions: The present study suggests that mutations in the *GJB2* gene specially c.35delG are important causes of ARNSHL in the center and west of Iran. Totally, 15% of the patients were heterozygous carriers. Further investigation is needed to detect the genetic cause of HL in the patients with monoallelic *GJB2* mutations.

1. Introduction

Hearing loss (HL) is the most frequent sensory impairment. The incidence is 1–2 in 1000 neonates (http://hearing. screening.nhs.uk/nationalprog), with approximately 50–70% of cases being related to genetic causes. It is estimated that 70% of HL includes non-syndromic forms (NSHL), where the hearing deficit is the only sign. Autosomal recessive mode of inheritance (ARNSHL) makes up 80% of the NSHL cases. ARNSHL is highly heterogeneous, for which over 60 mapped loci are known to be involved (http://hereditaryhearingloss.org). Despite this, mutations in one single locus, DFNB1 (13q11-12) which contains *GJB2* (NM_004004.5) and *GJB6* (NM_001110219.2) genes, account for 50% of the etiology in many Western populations. *GJB2* encodes the connexin 26 protein (Cx26), which is a type of gap junction protein

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involved in the inner ear homeostasis through recycling of potassium ions. The gene has a simple genomic structure with only two exons, with exon 1 being untranslated [1]. To date, more than 100 pathogenic mutations in the *GJB2* gene and over 4 pathogenic mutations, including gross del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) in the *GJB6* gene have been identified resulting in ARNSHL (http://davinci.crg.es/deafness). The prevalence of *GJB2* mutations varies among different populations. In Caucasians, c.35delG is the most common mutation with the carrier frequency as high as 2–4% [2]. However, c.167delT, c.235delC and p.Trp24* are the most frequent mutations in the Ashkenazi Jewish [3], Japanese [4] and Indians [5], respectively. Several studies have shown that the contribution of *GJB2* mutations to ARNSHL is about 16–18% in Iran [6,7]. Furthermore, although the next-generation sequencing (NGS) has been developed and extended in medical genetic applications such as molecular diagnostics of HL [8–10], the investigation of *GJB2* mutations is still the primary step before moving on to the NGS.

Since the last decade, a series of studies have been conducted on the Iranian population in order to identify the mutation spectrum and prevalence of *GJB2* mutations. The diverse ethnicities, coupled with the high rate of consanguinity rates (38% in average) [11] tend to change mutation frequencies among ethnic groups. Therefore, for accurate and effective genetic counselling, studying certain ethnic groups is of high importance [12,13]. The present study was launched to compare the prevalence and spectrum of *GJB2* mutations in two different provinces of Iran including Isfahan and Hamedan.

2. Materials and methods

2.1. Families and clinical evaluations

We enrolled 70 consanguineous families with multiple hearing impaired individuals. The Ethics Committee of the Isfahan University of Medical Sciences approved this project (code: IR.MUI.REC.1392.3.010). All the family members signed an informed written consent prior to recruitment. They met the following criteria: (1) confirmation of HL by pure tone audiometry (PTA) from 250 to 8000 Hz (2) the autosomal recessive inheritance through pedigree analysis (3) existence of three or more affected members within the pedigree. A complete clinical evaluation including audiological, ophthalmological, and physical examinations was performed to exclude environmental exposures and to determine the presence of syndromic findings in each family.

2.2. Molecular genetic testing

Genomic DNA of patients was extracted from peripheral blood lymphocytes using the standard salting out procedure [18]. *GJB2* was screened for the coding region mutations (exon 2) using direct sequencing. Primers and PCR conditions were selected as previously described by Tabatabaiefar et al. [14]. The following primers were used for amplification of exon 2 of the *GJB2* gene: F1 (5′-GCTTACCCAGAC TCAGAGAAG-3′) and R1 (5′-CTACAGGGGTTTCAAATGGTTGC-3′). The automated Genetic Analyzer ABI 3130 XL (Applied Biosystems 3130, Foster City, California, USA) was applied to bidirectionally sequence PCR products using The BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems 3130, Foster City, California, USA).

Patients with heterozygous variants in the *GJB2* gene were screened for pathogenic variants in exon 1 of *GJB2* and the presence of the del (*GJB6*-D13S1830) and del(*GJB6*-D13S1854) in the *GJB6* gene, as described by del Castillo et al. [15]. The *GJB2* exon 1 was amplified using the following primers: F1: (5′-TCCGTAACTTTCCCAGTCTCCGAGGGA AGAGG- 3′) and R1: (5′-CCCAAGGACGTGTGTTGGTCCAGCCCC- 3′). For the examination of the *GJB6* gene deletions, the breakpoint junctions of del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) were investigated using multiplex with the following primers: del(*GJB6*-D13S1830) breakpoint junction: F1 (5′-CACCATGCGTAGCCTTAACCA TTTT-3′) and R1 (5′-TTTAGGGCATGATTGGGGTGATTT-3′); del(*GJB6*-D13S1854) breakpoint junction: F2 (5′- CAGCGGCTACCCTAGTTGT GGT-3′) and R2 (5′-TCATAGTGAAGAACTCGATGCTGTTT-3′); *GJB6* (exon 1): F3 (5′-CATGAAGAGGGCGTACAAGT TAGAA-3′) and R3 (5′-CGTCTTTGGGGGTGTTGCTT 3′).

2.3. Computational analyses

We used Bioinformatics predictive tools including MutationTaster [16], PolyPhen [17], SIFT and PROVEAN to assess possible effects of mutations on the protein structure. Multiple sequence amino acid alignment of the Cx26 protein and visualization of conserved amino acids was performed using the Mutation@A Glance software [18]. Databases including: Connexin-deafness homepage (http://davinci.crg.es/deafness), the Single Nucleotide Polymorphism database (dbSNP) (http://www.ncbi.nlm.nih.gov/project/SNP), The NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/) and the 1000 Genomes Project (http://browser.1000genomes.org) were investigated for novel variants.

3. Results

A total of 70 Iranian families segregating pre-lingual ARNSHL were recruited from two provinces (40 family from Isfahan and 30 family from Hamedan) in the center and west of Iran. In 7 patients, PTA was consistent with severe HL (61–80 dB) and the remaining had profound HL (≥80 dB). Among 70 probands, there were 41 males and 29 females, with ages ranging from 2 to 42 years. In one family, both parents were hearing impaired, suggesting the presence of the same identical-by-descent mutation.

GJB2 mutations were detected in 9 (22.5%) out of the 40 families from Isfahan province and 6 (20%) out of the 30 families from Hamedan. *GJB2* variants are shown in Table 1. Totally, 11 different variants were identified, 9 of which were previously reported as pathogenic. These include: c.35delG, c.358_360delGAG, c.95G > A, c.36T > G, c. 23_24ins A, c.163A > G, c.79G > A, c.314A > G, c.100A > G. The two remaining variants including c.130T > G and c.178T > G, from Isfahan province, are novel. Fig. 1 shows the distribution of the identified mutations in the schematic structure of Cx26. All the *in silico* programs predicted c.130T > G and c.178T > G variants to have damaging effects (Table 1). The homozygous c.35delG mutation was observed in 6 families from Isfahan, accounting for 71.0% of the *GJB2* mutations and in 6 families from Hamedan, accounting for 100.0% of the *GJB2* mutations. Thus, in both of the studied populations, c.35delG was the most frequent mutation. One compound heterozygous genotype (c.358_360delGAG/c.35delG) for c.35delG was observed in Isfahan province. Other identified homozygous mutations included: c.358_360delGAG and c.95G > A, each in a single family. The p.Val27Ile variant was found in one patient in the *cis* state with p.Glu114Gly (p.Val27Ile; p.Glu114Gly/wt). Our study included one non-consanguineous deaf-to-deaf marriage. Interestingly, both of the couples were found to be homozygous for c.35delG.

We did not find any correlation between the severity and progressivity of HL and the presence of the identified variants in the study groups. In all of the patients with *GJB2* mutations, PTA was consistent with profound HL (≥80 dB).

The two novel *GJB2* variants including p.Trp44Gly and p.Cys60Gly appeared in the heterozygous state.The p.Trp44Gly variant is the result of c.130T > G transition, changing a TGG codon for Trp residue to a GGG codon for Gly. The pedigree and electropherogram results of the c.130T > G heterozygous allele are shown in Fig. 2-A. The second novel variant, p.Cys60Gly is the result of c.178T > G transition. This T to G nucleotide change at position 178 of the *GJB2* gene converts the amino acid Trp at codon 44 to glycine. The p.Cys60Gly/wt genotype was found in the pedigree ISF-10 (Fig. 2-B). In both related families (ISF-10 and ISF-18), PTA was consistent with profound HL (≥80 dB)

Table 1
Identified *GJB2* mutations, their frequencies and *in silico* analyses.

Mutations	No(%)		mutation type	classification	Functional effect					
	Isfahan	Hamedan			PolyPhen Prediction	Mutation Taster	SIFT	SIFT Score	PROVEAN	PROVEAN Score
c.35delG	13 (16.25)	12(20)	Deletion/ Nonsense	T	NA	Disease causing	NA		NA	NA
c. 23_24insA	0	1(1.67)	Frame Shift	T	NA	Disease causing	Damaging	0.008	NA	NA
c.358_360delGAG	3(3.75)	0	In Frame Deletion	NT	NA	Disease causing	Damaging	0	NA	NA
c.95G > A	2 (2.5)	0	Missense	NT	probably damaging	Disease causing	Damaging	0	Deleterious	−4.88
c.130T > G	1(1.25)	0	Missense	NT	probably damaging	Disease causing	Damaging	0.008	Deleterious	−12.81
c.100A > G	1(1.25)	0	Missense	NT	probably damaging	Disease causing	Damaging	0.008	Neutral	−2.09
c.163A > G	1(1.25)	0	Missense	NT	benign	Disease causing	Damaging	0.09	Deleterious	−4.58
c.178T > G	1(1.25)	0	Missense	NT	probably damaging	Disease causing	Damaging	0	Deleterious	−11.93
c.79G > A	3(3.75)	0	Missense	NT	probably damaging	polymorphism	Tolerated	0.193	Neutral	−0.66
c.314A > G	2(2.5)	0	Missense	NT	benign	polymorphism	Tolerated	0.227	Neutral	0.08
Normal	56	47								
Total	80	60								

NA: not available.

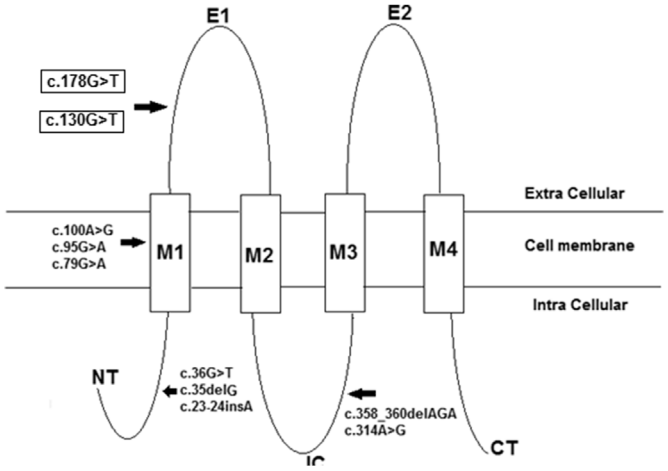

Fig. 1. Schematic structure, domains and distribution of mutations of the Cx-26 protein in this study. Novel mutations reported for the first time are indicated in the box. M1-M4 denote transmembrane domains, E1-E2 denote extracellular domain, IC denotes cytoplasmic domain, NT denotes amino (NH2) terminus and CT denotes carboxyl (COOH) terminus.

(Fig. 2-C).

For these heterozygous patients, no mutation was detected in exon 1 of the *GJB2*. Screening for the two *GJB6* deletions did not reveal any positive sample among *GJB2* negative or heterozygous cases.

4. Discussion

Hearing loss (HL) related to *GJB2* is the most frequent cause of autosomal recessive non-syndromic HL (ARNSHL) [19]. Differences exist as to the spectrum of *GJB2* mutations in different populations; it ranges from 86.7% in Finland [20] to 57.5%, 33.3% and 25% in Lithuania [21], Croatia [22] and Turkey [23], respectively to very low frequencies in the populations such as Pakistan 3.7% [24] and Oman 0% [25]. Previous studies on the *GJB2* mutations in Iran have shown that the mutation frequency of *GJB2* varies between 0 and 35% among different regions of this country [26].

A study performed by Chaleshtori et al. [27] on different Iranian populations with hearing loss showed that the frequency of *GJB2* mutations to be 27.5% in the North and Northwest of Iran, while it was less than 4% in the Southeast region. This finding is comparable to those obtained previously by Najmabadi et al. [7]. They reported the highest percentage of *GJB2*-related HL in the Northwest and West provinces of Iran to be 38.3% and 22.2%, respectively, highlighting a non-homogeneous distribution in the frequency of *GJB2* HL throughout Iran. The observed Northwest-to-Southeast *GJB2* HL gradient is further supported by data specific to the Southeast and Northwest Iran, where the populations are related to the neighboring Pakistan and Turkey. Naghavi et al. [28] screened 100 ARNSHL families from Sistan & Baluchistan province in Southeast Iran for *GJB2* mutations. They reported that *GJB2* mutations were found in 7% of the ARNSHL families studied. Interestingly, p.Trp24* was the most frequent *GJB2* mutation (80% of the identified *GJB2* mutations), while c.35delG was absent in this ethnicity. This finding was similar to the presented data from the Pakistan population [24]. On the other hand, Davarnia et al. [29] presented that *GJB2* mutations were responsible for about 26% of ARNSHL in the Iranian Azeri patients (Northwest Iran) and the allele frequency of the c.35delG mutation was reported to be 62% of *GJB2* mutations, which is similar to the reported results of the Turkish population [23].

The families presented in this study live in the west and center parts of Iran. According to the previous studies, we expected high frequencies of *GJB2* mutations; the contribution of *GJB2* mutations to ARNSHL was 22.5% and 20% in Isfahan and Hamedan provinces, respectively. This is about three times the frequency of *GJB2* mutations in Southeast Iran (Table 2). In both of the studied populations, the most common mutation was c.35delG, accounting for 100% of the *GJB2* mutations in Hamedan and 75.0% of *GJB2* mutations in Isfahan. The data conform to the Northwest-to-Southeast c.35delG related HL gradient that is suggested across Iran, drawing the movement pathway of the initial founders through the silk route (Table 2). In our study, the homozygous c.358_360delGAG mutation was found in one family from Isfahan (1/40). c.35delG and c.358_360delGAG were the first and second most common mutations in Iran, respectively, which is in agreement with other data [30], Besides, we could identify the homozygous c.95G > A (p.Arg32His) mutation in one family. This mutation has been reported from a Lebanese family for the first time [31]. The Arg32 residue is a highly conserved residue in the *GJB2* protein and *in silico* functional analysis shows that Arg to His substitution is a damaging variant (Table 1). The pathogenic roles of the p.Arg32His mutation has been investigated *in vitro* by Xiao et al. [32]. They inferred that the mutated protein failed to reach the cell membrane and remained localized completely within the endoplasmic reticulum in the cells.

Both p.Val27Ile (c.79G > A) and p.Glu114Gly (c.341G > A) are common in the East Asia [33]. Pandya et al. [34] suggested that when these two variants present together in the *cis* state, homozygous p.Val27Ile; p.Glu114Gly or compound heterozygote with another variant can result in HL. In order to investigate the pathogenesis of p.Val27Ile and p.Glu114Gly variants, Choi et al. used both an *in vitro* assay and population study and revealed that the p.Glu114Gly variant was deleterious but p.Val27Ile; p.Glu114Gly was as a non-pathogenic variant.

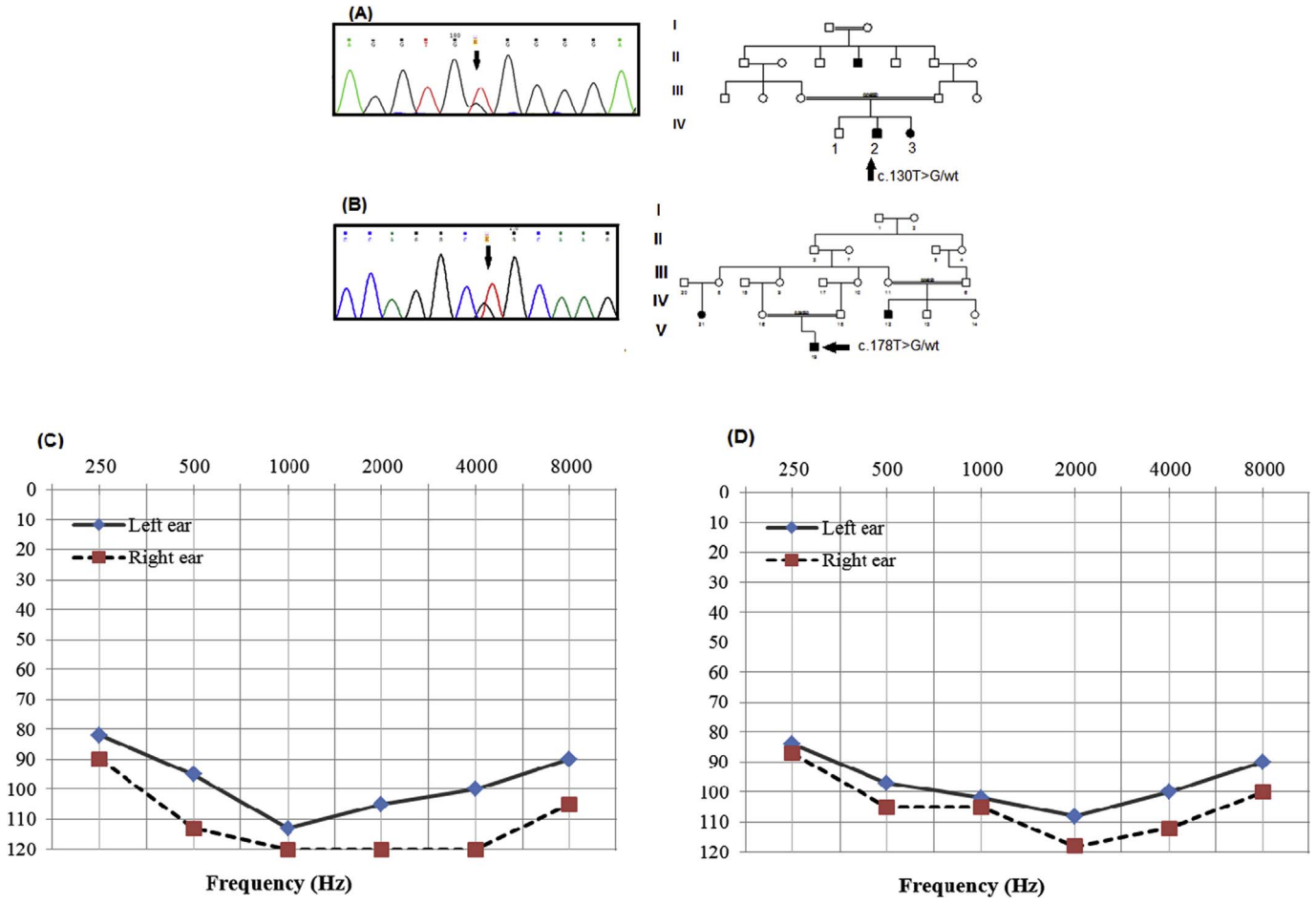

Fig. 2. A: Pedigree diagram and electropherogram of the patient (IV-2) from ISF-18 family shows the (c.130T > G/wt) variant of *GJB2*. B: Pedigree and electropherogram of the patient (V-1) from ISF-10 family shows the (c.178T > G/wt) variant. C, D: Right and left ear audiograms in the (IV-2) and (V-1) patients from ISF-18 and ISF-10, respectively.

They reported that only the homozygous p.Glu114Gly or its compound heterozygote with other mutations may cause HL [35]. Based on the above finding, the p.Val27Ile; p.Glu114Gly/wt genotype found in our study is not related to the HL pathogenesis.

The c.100A > G (p.Met34Val) mutation has been reported from Turkish families for the first time [36]. The p.Met34Val variant has also been reported from several other populations [37]. The Met34 residue is a conserved residue in the *GJB2* protein and computational analyses

Table 2
GJB2 mutations in different provinces of Iran.

Genotype	Description	Gilan	Ardebile	Hamedan	Isfahan	Sistan & Baluchestan	Ahvaz
		(North)	(Northwest)	(West)	(Center)	(South-East)	(South)
studied subjects		75	50	30	40	100	73
c.35delG	p.Gly12Valfs*2	47/150(31.3)	18/100 (18)	12/60 (20)	16/80 (20)	0	9/146 (6.2)
c.23_24insA	p.Ile9Aspfs*39	0	0	1/60 (1.6)	0	0	0
c.358_360delGAG	p.delGlu120	0	2/100 (2)	0	2/80 (2.5)	0	0
c.95G > A	p.Arg32His	0	0	0	2/80 (2.5)	0	0
c.130T > G (NEW)	p.Trp44Gly	0	0	0	1/80 (1.25)	0	0
c.100A > G	p.Met34Val	0	0	0	1/1/80 (1.25)	0	0
c.163A > G	p.Thr55Ala	0	0	0	1/1/80 (1.25)	0	0
c.178T > G (NEW)	p.Cys60Gly	0	0	0	1/80 (1.25)	0	0
c.463-464delT	p.Tyr155Metfs*13	0	4/100 (4)	0	0	0	0
c.299-300delAT	p. His100Argfs*14	0	2/100 (2)	0	0	0	0
c.79G > A	p.Val27Ile	1/(1.2)	0	0	3/80 (3.75)	0	0
c.457G > A	p.Va153Ile	4 (4.6)	0	0	0	0	0
c.314A > G	p.Glu114Gly	0	1/100 (1)	0	2/80(2.5)	0	1/146 (0.64)
c.511G > A	p.Ala171Thr	0	1/100 (1)	0	0	0	0
c.475G > C	p.Asp159His	0	0	0	0	0	1/146 (0.64)
c.71G > A	p.Trp24fs*	0	0	0	0	10/200 (5)	0
c.380G > A	p.Arg127His	0	0	0	0	4/200 (2)	0
c.167delT	p.Lys56Argfs*26	0	0	0	0	2/200 (1)	0
Reference		Hashemzadeh et al. [43]	Davarnia et al. [29]	present study	present study	Naghavi et al. [28]	Hosseinipouret et al. [13]

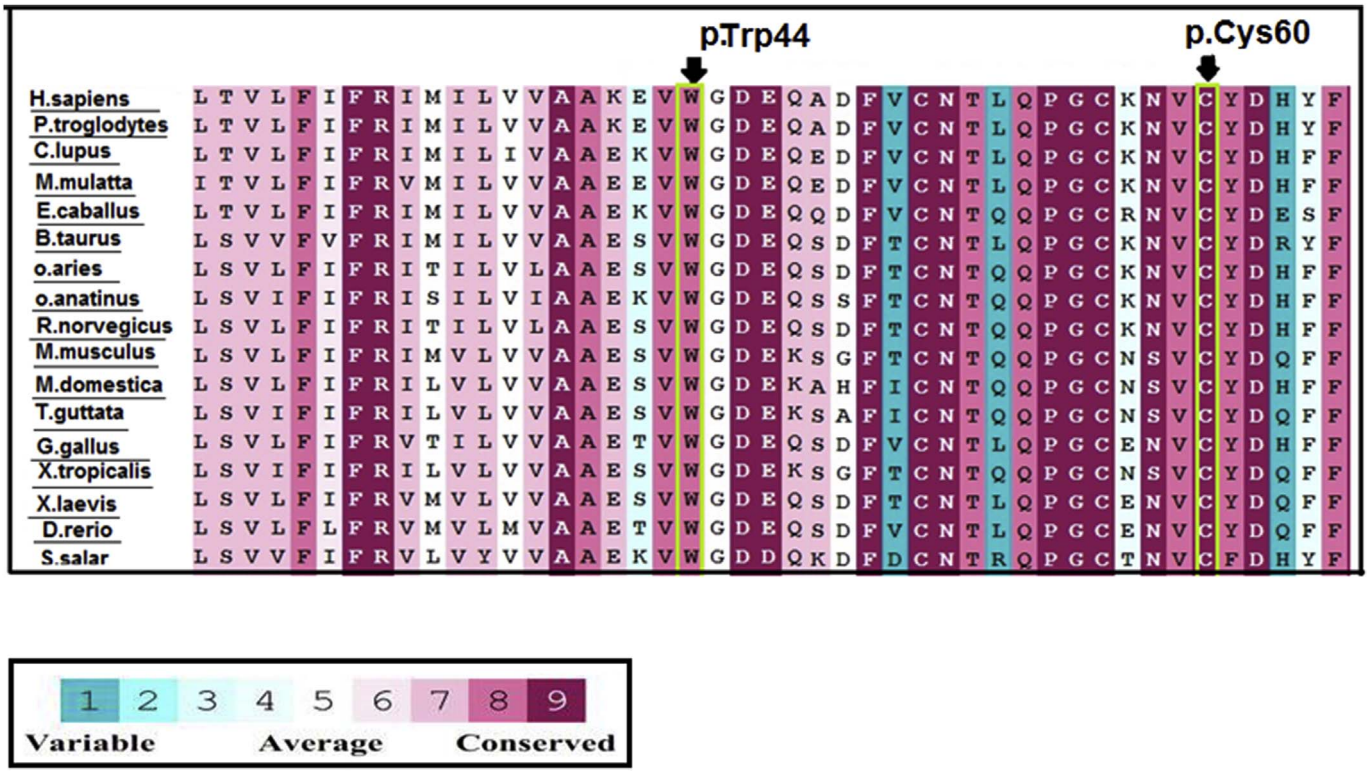

Fig. 3. The identified variants c.130T > G and c.178T > G occur at highly conserved positions (Trp44 and Cys 60) in the Cx26 protein. The calculated score for Trp and Cys amino acid were 8 and 9, respectively.

show that Met to Val substitution (p.Met34Val) is a damaging variant (Table 1). Functional experiments have shown that p.Met34Val is a damaging variant. In addition, as derived from the structure of the Cx26 channel at 3.5 A resolution, Met34 residue is essential for the gating process [38].

The two novel variants we found in this study including c.130T > G and c.178T > G have not been reported in the population databases. The c.130T > G variant in exon 2 of the *GJB2* gene was identified in the heterozygous state in a patient with profound HL. This T to G nucleotide change at position 130 of the *GJB2* gene converts the amino acid Trp at codon 44 to glycine. The Trp44 residue, which is located in the first extracellular domain (E1), is highly conserved in all species studied, and among different connexions (Fig. 3) [39]. The substitution of the residues with a residue which has different physicochemical properties might result in damaging effects. Trp is a highly hydrophobic amino acid with aromatic structure, while Gly is a small and non-charged amino acid [40]. This substitution would affect its contacts with the neighboring residues, thereby influencing the folding of the Cx26 protein with the mutated residue [41]. The MutationTaster and SIFT tools indicates that this variant could be damaging. The other novel variant, c.178T > G changes the Gly to Cys in the N-terminal domain of Cx26. Cys is a hydrophobic amino acid with the nonpolar structure which is highly conserved across species (Fig. 3). Cys60 can be linked by other Cys residue at position 174 of the *GJB2* gene to form a disulphide bond [42]. Its substitution by Gly may influence the formation of this interaction in the protein, resulting in the wrong folding of the protein. Our *in silico* analysis suggests that c.178T > G and c.130T > G variants would probably be damaging to the protein function (Table 1) [16]. The PolyPhen prediction tool indicates that c.178T > G and c.130T > G variants could be probably damaging for the protein conformation, SIFT and PROVEAN software tools identified them as a damaging and deleterious variants (Table 1) [17]. Future *in vitro* functional studies are needed to fully understand the pathogenic mechanism of c.178T > G and c.130T > G in the *GJB2* gene.

As the second allele was not found in exon 1 of *GJB2* or *GJB6* gene for the monoallelic carriers in the related family, it is much likely that another causal gene is responsible for HL in these patients.

5. Conclusions

In the Iranian populations, no del(*GJB6*-D13S1830) or del(*GJB6*-D13S1854) have been detected so far. This is in agreement with the finding of this study and suggests that they probably have no role in etiology of ARNSHL in the Iranian population. Our data support the view that different profile of *GJB2* variants is found in different populations and that c.35delG is the major pathogenic mutation in the *GJB2* gene in the Iranian population.

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