Novel Mutations in KCNQ4, LHFPL5 and COCH Genes in Iranian Families with Hearing Impairment

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Abstract

Background: Hearing loss (HL) is the most common sensory deficit in humans, and genetic factors contribute to about half of the cases. With 112 causative genes identified so far and a disproportionate share of the genes within different ethnic groups, HL has proven to be quite heterogeneous.

Methods: Twenty Iranian families having at least 2 children with hereditary HL were initially verified to be GJB2-negative and were then subjected to whole exome sequencing (WES). Sanger sequencing was used to confirm segregation of the variant identified in each family.

Results: In 3 families, WES revealed 3 novel variants in KCNQ4, LHFPL5 and COCH genes. The KCNQ4 gene (DFNA2A) encodes a potassium channel (K,7.4) and the heterozygous variant identified (c.1647C>G, p.F549L) resulted in the substitution of Phe549 residing in the K,7.4 cytoplasmic region. The homozygous variant (c.34A>T, p.K12X) was identified in the LHFPL5 gene (DFNB67) which encodes a transmembrane protein, and another variant in a homozygous state (c.116T>A, p.L39X) was identified in the COCH gene which encodes a secretory protein. Pathogenic variants in the COCH gene are associated with late onset autosomal dominant hearing loss (DFNA9) but the affected individuals displayed early onset HL with a recessive mode of inheritance.

Conclusions: The 16% contribution of GJB2 to HL in the Iranian population necessitates the discovery of the remaining causal factors. This study is the first to report KCNQ4 and COCH related HL in the Iranian population and the second study, globally, to report HL due to biallelic inactivation of the COCH gene.

Keywords: COCH, Hearing loss, Iran, KCNQ4, LHFPL5, Whole exome sequencing


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Introduction

Affecting over 5% of the world population, hearing impairment is regarded as the most prevalent sensory deficit in humans and it continues to mount as the population grows (http://www.who.int/). Nearly 50% of the reported impairments are due to genetic factors which, in the majority of cases, impact solely the auditory sense without causing additional perturbations; these are referred to as “nonsyndromic hearing loss” (NSHL) and constitute 70% of all inherited cases. At least 500 genes are estimated to be responsible for the auditory sense to work flawlessly1 which accounts for the heterogeneity of hearing impairment and necessitates adopting more efficient approaches for gene discovery and diagnosis. The development and implications of next generation sequencing (NGS) including whole exome sequencing (WES) have dramatically accelerated the discovery of the causative genes; since the identification of the first NSHL gene (TPRN) using the NGS technology,2 more than 40 novel genes have been reported which altogether make up nearly one-third of the 112 NSHL causative genes identified (https://hereditaryhearingloss.org/).

Hereditary hearing loss (HHL) is of particular importance in societies with a high rate of consanguinity which clears the way for rare pathogenic variants to appear, and Iran with nearly 40% consanguinity3 is no exception. HL ranks as the second most prevalent disability in Iran4 and the rate of consanguineous marriage among the Iranian deaf population is estimated to reach up to 65%.5 Regarding the major contribution of GJB2 mutations in autosomal recessive nonsyndromic hearing loss (ARNSHL)6 preliminary studies on HHL in the Iranian population were accordingly aimed at this gene and the later extensive investigations yielded an average of 16% of GJB2 related hearing impairment.7-10 Before NGS, studies to identify the remaining causal factors were mainly based on linkage analysis and homozygosity mapping.11,14 Keeping pace with the growing number of studies utilizing WES, a study using a custom targeted genomic enrichment (TGE) panel revealed the underlying genetic cause in 67% of the probands in whom variants in 26 out of 40 causative genes were reported for the first time in the Iranian population.15

Here, we report 3 novel variants in KCNQ4, LHFPL5
and COCH genes which were identified by WES in 3 Iranian families. It is further argued how the variant identified in each gene might impact the protein function, thereby leading to HL.

Materials and Methods
Patients
Twenty-four families, having at least 2 affected children with unknown HHL, were recruited to the Genetics Research Center at the University of Social Welfare and Rehabilitation Sciences in Tehran. The participants’ developmental history and clinical examination did not indicate any syndromic features and HL was the only complaint; diagnosis of sensorineural hearing loss (SNHL) was made based on pure tone audiometry, both air (frequencies ranging from 250 to 8000 Hz) and bone conduction (frequencies ranging from 500 to 4000 Hz). Blood samples were collected after obtaining written informed consent from the participants or legal guardians in case of minors.

DNA Extraction and Whole Exome Sequencing
Before WES, DNA sequencing of both GJB2 exons was performed on the genomic DNA which were extracted from peripheral blood samples using standard salting-out protocols. Four families which were verified to be positive for GJB2 mutations were excluded and proband samples from the remaining 20 families were used to create libraries and capture sequences, according to the SureSelectXT Target Enrichment Preparation Kit for Agilent (Version V6, February 2018). The captured libraries underwent 101 bp paired-end sequencing using the Illumina HiSeq 4000 system (Illumina, Inc., San Diego, CA, USA). Resultant FASTQ files were aligned to the human reference sequence (hg19) by Burrows-Wheeler Aligner (BWA) and SAM files were generated. Further SAM to BAM conversion, BAM file sorting and removal of duplicate reads were carried out by Picard (http://picard.sourceforge.net), followed by local realignment and variant calling by Genome Analysis Tool Kit (GATK) to generate VCF files, and then annotation was performed with Annovar32.

Variant Interpretation and Segregation Analysis
Variant filtering was performed manually and initiated by omitting all variants in non-coding regions as well as synonymous variants in exonic regions. Global minor allele frequency (MAF) ≤ 0.01 was adopted to filter the remaining variants further (including nonsynonymous, indels and splice-site variants) using several databases including 1000 Genomes Project (http://www.1000genomes.org), ESP6500 (http://evs.gs.washington.edu/EVS/), Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/) and Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/). Variants consistent with the mode of inheritance in each pedigree were prioritized based on the scores provided by in silico prediction tools for conservation (GERP and SiPhy) and pathogenicity (PolyPhen2, SIFT, MutationTaster and LRT). Truncating (nonsense, splice-site, and indels) as well as missense variants with a high pathogenicity score (predicted by at least four of the 6 tools mentioned above) were considered to be probably pathogenic. To ensure the absence of candidate variants in an ethnically matched normal population, all variants were further checked against the source of WES data for 800 Iranians (http://www.iranome.com/). For candidates meeting the mentioned criteria, Sanger sequencing was subsequently performed to validate segregation in the family.

Multiple Sequence Alignment and 3D Modeling
For residues predicted to be altered due to an identified missense variant, assessment of the conservation rate across species was performed by multiple sequence alignment of the orthologous sequences, using ClustalOmega (https://www.ebi.ac.uk/Tools/msa/clustalo/). To study the 3D structure of the mutation bearing domain, the corresponding PDB (Protein Data Bank) file (if available) was obtained from RCSB (https://www.rcsb.org/) and visualized with PyMOL (http://www.pymol.org).

Results
Three consanguineous families, 9400034, 9600226 and 9600236 were found to harbor mutations in previously known HL genes KCNQ4, LHFPL5 and COCH, respectively (Table 1).

The proband (V:2, shown with an arrow in Figure 1a) in family 9400034 was a 26-year-old male, born to a 3-generation family with hearing loss, and his mother as well as his 2 sisters were also affected (Figure 1a). Clinical history and audiological assessment of the affected individuals were indicative of progressive, bilateral and autosomal dominant non-syndromic hearing impairment (ADNSHL) with the age of onset ranging from 17 to 24 years. The audiogram pattern for the proband showed moderate to severe HL (Figure 1b), with the initial involvement of high frequencies and a gradual progression towards including lower frequencies (Figure 1c). The WES result identified a heterozygous variant (c.1647 C>G) (Figure 1d) in exon 12 of the KCNQ4 gene (NM_004700) which is located at 1p34 and encodes an ion channel of 695 aa (K7.4) that belongs to the family of voltage-gated K+ channels (K7.1–K7.5). The protein structure in this family is composed of 4 transmembrane subunits followed by a cytoplasmic C-terminal domain (Figure 2a). Each subunit consists of 6 helices forming the voltage sensor domain (S1-S4) and 2 other helices (S5-S6) which constitute the pore domain. The C-terminal domain is composed of 4 segments denoted as A to D and plays a vital role in channel assembly and gating. The variant identified in this family led to a substitution of
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The hydrophobic and aromatic Phe549 by the non-polar aliphatic Leu (p.F549L). This residue which is located in the B helix of the cytoplasmic region of the protein (Figure 2a) is highly conserved, not only in the $K_{v}7.4$ channel across species (Figure 2b) but also among all 5 known members of the $K_{v}7$ family (Figure 2c).

The proband (IV:2, Figure 3a) in the second family, 9600226, was a 38-year-old male who, along with his affected sister and all his nephews, suffered from prelingual, bilateral ARNSHL (Figure 3a). Audiological assessment of the proband indicated profound HL(Figure 3b) and the ABR results for his 2 nephews (aged 4 and 11 months) were consistent with bilateral severe to profound hearing loss. Analysis of WES results disclosed a homozygous variant (c.34A>T) (Figure 3c) in the first exon of the $LHFPL5$ gene (NM_182548) which is expressed in cochlear and vestibular hair cells where it modulates the channel conductance of the mechanotransduction machinery. Human $LHFPL5$ (lipoma HMGIC fusion partner-like 5) gene, which is composed of 4 exons, is located at 6p21.31 and encodes a transmembrane protein of 219 aa, also known as Tmhs (tetraspan membrane protein of hair-cell stereocilia). The novel variant detected in this family led Lys12 to be replaced by a premature stop codon (p.K12X) in the N-terminal cytoplasmic region of the protein.

The proband (IV:1; Figure 4a) in the third family, 9600236, was a 35-year-old male who had 3 affected sisters (Figure 4a) and the hearing impairment in Table 1. Pathogenic Variants Identified in This Study

<table>
<thead>
<tr>
<th>Family Code</th>
<th>Origin</th>
<th>Onset</th>
<th>Severity</th>
<th>Gene</th>
<th>N. Change</th>
<th>AA Change</th>
<th>Zygosity</th>
<th>In Silico Prediction</th>
<th>MAF in ExAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>9400034</td>
<td>Ahvaz (South Iran)</td>
<td>Post</td>
<td>Mild to severe</td>
<td>KCNQ4</td>
<td>c.1647C&gt;G</td>
<td>p.F549L</td>
<td>Hetero</td>
<td>S</td>
<td>PP2</td>
</tr>
<tr>
<td>9600226</td>
<td>Torbat-jam (West Iran)</td>
<td>Pre.</td>
<td>Profound</td>
<td>LHFPL5</td>
<td>c.34A&gt;T</td>
<td>p.K12X</td>
<td>Homo</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td>9600236</td>
<td>Najafabad (Central Iran)</td>
<td>Post.</td>
<td>Moderate to profound</td>
<td>COCH</td>
<td>c.116T&gt;A</td>
<td>p.L39X</td>
<td>Homo</td>
<td>-</td>
<td>A</td>
</tr>
</tbody>
</table>

Table 1. Pathogenic Variants Identified in This Study

Severe hearing loss at high frequencies.

Profound hearing loss at high frequencies.

Figure 1. Clinical Features and Segregation Analysis of a Family with Mutation in the KCNQ4 Gene. (a) Pedigree of family 9400034 is consistent with an autosomal dominant mode of inheritance; Open circle, female; filled circle, affected female; open square, male; filled square, affected male; strikethrough, deceased. (b) Pure-tone audiogram for the proband. Frequency in hertz (Hz) and hearing level in decibels (dBHL) are plotted on the x and y-axis respectively; L: left, R: right. Audiogram indicates bilateral sensorineural hearing loss which is moderate at low frequencies and progresses to severe level at high frequencies. (c) The audiometric graph of the proband over a 2-year course is indicative of progressive hearing loss, initially involving high frequencies. (d) Sanger sequencing chromatogram exhibits segregation of the identified variant (c.1647 C>G; p.F549L); +/- and -/-, show the heterozygous (mutant) and homozygous genotypes for the wild-type (WT) allele, respectively.
all affected members was noticed at school age. The inheritance pattern and audiological evaluation of the proband were indicative of bilateral AR ted in cochlear and vestibular labyrinths, and is composed of a signal peptide (SP), the LCCL domain (initially designated as FCH (factor C homology) domain) encoded by exons 4-6, and 2 von Willebrand factor type A (vWFA) homology domains which are encoded by exons 8-10 and exons 11-12, respectively. The LCCL domain has a significant homology with a clotting factor (known as “factor C”) in Limulus (horseshoe crabs) which is activated upon binding to lipopolysaccharide and initiates a coagulation cascade, thereby playing a role in the innate defense mechanism. The vWFAs are interacting domains which are found in many secreted proteins of the immune system, hemostasis, cell adhesion and predominantly the components of the extracellular matrix (ECM) and bind fibrillar collagens, glycoproteins and proteoglycans. The variant identified in this family resulted in the substitution of Leu 39 by a
premature stop codon (p.L39X) in the initial part of the LCCL domain.

**Discussion**

In the current study on 3 consanguineous Iranian families with hearing loss, WES revealed 3 novel mutations in KCNQ4, LHFPL5 and COCH genes to be the underlying causes of the impairment (Table 1). Mutations in the KCNQ4 gene are known to cause DFNA2A nonsyndromic hearing loss (OMIM #600101) and are among the most commonly reported causes of ADNSHL. Hearing impairment is manifested by the initial loss of hearing at high frequencies which gradually deteriorates and progresses to involve all frequencies; this phenotype is compatible with the predominant expression of KCNQ4 in the basal turns of the cochlea where high frequencies are sensed. It is speculated that lack of native K+ currents due to impaired Kv7.4 channels in the sensory outer hair cells (OHCs) of these regions might lead to an overload of K+ and result in gradual cell degeneration. To date, 27 mutations in this gene have been reported in individuals mainly from East Asia and America, with the age of onset ranging from the first to the fifth decade, and to the best of our knowledge, this study is the first report of KCNQ4-related hearing impairment in a family of Iranian origin (for an overview of the DFNA2A related mutations reported in KCNQ4, see Huang et al). The variant identified in this study (c.1647C>G, p.F549L) leads to the replacement of Phe549 residing in the B helix of the cytoplasmic domain which plays a central role in channel assembly and gating. The B helix (Pro528-Arg554) together with the adjacent A helix (His330-Met357) form an antiparallel helical pair which are separated by a short AB linker (Ala364-Met527). This structure, which is close to the channel pore, has been shown to be wrapped around by the Ca2+-binding protein calmodulin (CaM) in both Apo-CaM and Ca2+-CaM forms (Figure 5a). CaM is a strong modulator of Kv7 function and studies have shown that disruption of

![Figure 4](image_url)

**Figure 4.** Clinical Features and Segregation Analysis of a Family with Mutation in the COCH Gene. (a) Pedigree of family 9600236 displays an autosomal recessive mode of inheritance; (b) Pure-tone audiogram for the proband is indicative of moderate hearing loss at low frequencies sloping down to profound level at high frequencies. (c) Sanger sequencing chromatogram confirms segregation of the identified variant (c.116T>A, p.L39X); homozygous (mutant) and heterozygous (carrier) genotypes for the wild-type (WT) allele are indicated by -/- and +/-, respectively. For symbols and graph description, see Figure 1.

![Figure 5](image_url)

**Figure 5.** 3D Structure of the AB Domain of Kv7.4 Interacting with CaM. (a) The A and B helices of the Kv7.4 cytoplasmic domain form an antiparallel helical pair separated by a short helix (AB linker). X-ray crystallography of Ca2+-CaM (PDB code: 6B8N), visualized by PyMOL, depicts how CaM embraces both the A and B helices by its C and N-lobes, respectively. (b) Phe549 is located close to the end of the B helix and its replacement by Leu is speculated to disrupt the interactions in the surrounding microenvironment, thereby interfering with proper CaM:AB association, leading to a malfunctioning channel.
CaM interaction with K,7 interferes with proper channel assembly and trafficking.\(^{31}\) Based on structural studies, the A and B helices of K,7 are in contact with the CaM C-lobe and N-lobe, respectively and mutations of some residues critical for Apo/CalM/K,7 are shown to impair channel trafficking.\(^{40}\) The highly conserved Phe549 is located close to the end of the B helix (Figure 5b) and is part of a pair of overlapping 1–5–10 motifs, previously proposed as one of the CaM binding features.\(^{32}\) How substitution of Phe549 by Leu (p.F549L) (Figure 5c) interferes with protein function and contributes to HL has yet to be determined. Further studies are needed to elucidate whether this alteration directly impairs B-helix:CaM interactions or it disturbs the proper conformation of the AB domain and subsequently disrupts its association with CaM.

The second novel variant reported in the current study (c.34A>T, p.K12X) involves the LHFP5 gene which is associated with autosomal recessive nonsyndromic deafness DFNB67 (OMIM #610265). This gene was initially identified as a causative gene for HL and vestibular dysfunction observed in *hurry-scurry (hscy)* mice; in mutant mice, hair bundles became disorganized and degenerated as the mice grew.\(^{33}\) In the following year, DFNB67 was mapped in 2 consanguineous Pakistani families with NSHL which led to identification of mutations in LHFP5.\(^{34}\) The encoded protein by this gene, Lhfpl5 (or Tmhs), localizes near the lower point of tip links in hair cells and along with 3 other transmembrane proteins Tmc1 (transmembrane channel protein 1), Tmc2 (transmembrane channel protein 2)\(^{35}\) and Tnie (transmembrane protein of inner ear hair cells) are associated with the MET (mechano-electrical transduction) channels.\(^{36}\) These channels are part of the molecular components of MET machinery in the mammalian hair cells that converts sound waves into electrical signals.\(^{37}\) Lhfpl5 is known to interact and bind Protocadherin-15 (Pcdh15) which is the major component of lower tip links and it is also essential for Pcdh15 and Tmc1 to be targeted to their locations.\(^{19,38}\) To date, 12 pathogenic variants in LHFP5 have been reported in ARNSHL families from the Middle East and northern Africa and unlike hscy mice, none of the human LHFP5 mutations are accompanied by vestibular symptoms (Table 2). TGE in a cohort of 302 GJB2-negative families led to the first report of LHFP5-related HL in the Iranian population, including 2 previously reported as well as a novel variant in 3 families\(^{31}\) and recently, in a separate study on 5 Iranian families using NGS, another novel mutation in LHFP5 gene was revealed.\(^{39}\) Unlike the nonsense mutation (p.K12X) detected in the current study, previously reported variants in the Iranian populations include missense mutations as well as an in-frame deletion (Table 2). In mammalian cells the premature termination codons (PTC) which are located more than 50-55 bp upstream of the last exon-exon junction are known to elicit translation-dependent nonsense-mediated decay (NMD)\(^{40}\) and accordingly, the substitution of Lys12 by a PTC is speculated to result in degradation of the transcript harboring the PTC.

The third variant in this study (c.116T>A, p.L39X) resides in the COCH gene, which was first isolated from a cDNA library of human fetal cochlea and was subsequently mapped within the locus for DFNA9.\(^{20}\) The first direct evidence for contribution of COCH mutations to hearing impairment was provided by a study of 3 unrelated families, all harboring missense mutations in the LCCL domain of its encoded protein, Cochlin.\(^{47}\) Since then, 25 mutations have been reported in this gene which are all inherited in a dominant mode and associated with late onset, progressive SNHL with vestibular dysfunction (DFNA9, OMIM #601369).\(^{48}\) Vestibular involvement varies and ranges from asymptomatic individuals to those who suffer from vertigo and vestibular hypofunction.\(^{57,49}\)

Reported mutations thus far are missense variants which predominantly involve LCCL and vWFA2 domains of COCH.

**Table 2.** Overview of LHFP5 Mutations Described in DFNB67

<table>
<thead>
<tr>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>HL Severity</th>
<th>Zygosity</th>
<th>Age of Onset</th>
<th>Origin</th>
<th>Author/Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.1A&gt;G</td>
<td>p.Met1Val</td>
<td>Severe</td>
<td>Homozygous</td>
<td>Prelingual</td>
<td>Palestinian</td>
<td>Shahin et al, 2010(^{40})</td>
</tr>
<tr>
<td>c.16+1G&gt;A</td>
<td>-----</td>
<td>Homozygous</td>
<td>Prelingual</td>
<td>Prelingual</td>
<td>Pakistani</td>
<td>Liaqat et al, 2018(^{41})</td>
</tr>
<tr>
<td>c.34A&gt;T</td>
<td>p.Lys12X</td>
<td>Profound</td>
<td>Homozygous</td>
<td>Prelingual</td>
<td>Iranian</td>
<td>This study</td>
</tr>
<tr>
<td>c.89dupG</td>
<td>p. Thr11 Tyr6X41</td>
<td>Profound</td>
<td>Homozygous</td>
<td>ND</td>
<td>Tunisian</td>
<td>Bensaid et al, 2011(^{42})</td>
</tr>
<tr>
<td>c.246delC</td>
<td>p.Leu84X</td>
<td>Profound</td>
<td>Homozygous</td>
<td>Prelingual</td>
<td>Pakistani</td>
<td>Shabbir et al, 2006(^{44})</td>
</tr>
<tr>
<td>c.258,260delCTC</td>
<td>---</td>
<td>Severe to profound</td>
<td>Homozygous</td>
<td>Postlingual</td>
<td>Iranian</td>
<td>Sloan et al, 2015(^{43})</td>
</tr>
<tr>
<td>c.269 C&gt;G</td>
<td>p.Pro90Arg</td>
<td>Profound</td>
<td>Homozygous</td>
<td>Age range</td>
<td>Iranian</td>
<td>Shang et al, 2018(^{45})</td>
</tr>
<tr>
<td>c.380A&gt;G</td>
<td>p.Tyr127Cys</td>
<td>Severe</td>
<td>Homozygous</td>
<td>Prelingual</td>
<td>Pakistani</td>
<td>Shabbir et al, 2006(^{44})</td>
</tr>
<tr>
<td>c.452 G&gt;T</td>
<td>p.Gly151Val</td>
<td>Profound</td>
<td>Homozygous</td>
<td>Prelingual</td>
<td>Pakistani</td>
<td>Liaqat et al, 2018(^{41})</td>
</tr>
<tr>
<td>c.494C&gt;T</td>
<td>p.Thr165Met</td>
<td>Severe to profound</td>
<td>Homozygous</td>
<td>Prelingual</td>
<td>Turkish</td>
<td>kalay et al, 2006(^{46})</td>
</tr>
<tr>
<td>c.518T&gt;A</td>
<td>p.Cys173Ser</td>
<td>Profound</td>
<td>Homozygous</td>
<td>Prelingual</td>
<td>Algerian</td>
<td>Ammar-Khodja et al, 2015(^{44})</td>
</tr>
<tr>
<td>c.649delG</td>
<td>p.Glu216Arg6X26</td>
<td>Severe to profound</td>
<td>Homozygous</td>
<td>Prelingual</td>
<td>Turkish</td>
<td>kalay et al, 2006(^{46})</td>
</tr>
</tbody>
</table>

ND, not defined.

\(^{a}\) Also reported in the Iranian population.
the protein and while vertigo is a prevalent complaint in individuals harboring mutations in the LCCL domain, it is less commonly reported in people with vWFA2 mutations (DNF9 related mutations in the COCH gene with detailed description of clinical features are discussed by Tsukada et al). Recently, a nonsense variant (c.292C>T, p.R98X) in a consanguineous family of Moroccan origin has been reported which subsequently leads to vestibular dysfunction with moderate prelingual sensorineural hearing loss. The novel variant reported in the current study (c.116T>A) is the second report of a mutation in this gene to be recessively inherited and the first report of COCH-related hearing impairment in the Iranian population. The affected individuals display a sloping SNHL toward higher frequencies which is similar to that observed in the Moroccan family, but on the other hand, no symptoms or complaints implying vestibular dysfunction has been reported among the family members. DFNA9 related mutations in the COCH gene are associated with characteristic histopathological findings which are marked by accumulation of cochlin-staining acellular deposits in cochlear and vestibular labyrinths accompanied by loss of cellular composition in the structures of both the inner and middle ear. These pathological aggregations of impaired proteins seem to build up over time, consistent with the late onset of manifestations in DFNA9 mutations. As opposed to the defining characteristics of DFNA9, the variant identified in this study (p.L139X) as well as the one reported in the Moroccan family (p.R98X) are nonsense mutations which display a much earlier age of onset and a recessive mode of inheritance. These newly reported characteristics are probably due to the truncating nature of both variants which introduce PTCs in the initial part of the transcripts probably due to the truncating nature of both variants which would otherwise remain unresolved or hard to be identified.

**Authors’ Contribution**

HM performed WES tertiary analysis, variant interpretation, cosegregation analysis and wrote the manuscript. MM, MA and KJ carried out DNA extraction and library preparation and contributed to the WES pipeline. SA assisted with sample collection, and GJB2 screening was performed with help from NN. KK contributed to patients’ clinical evaluation and verification of the causative variants. The overall framework and direction of the study was designed, led and supervised by HN who provided critical feedback as well as the financial support for the project. All authors verified the results and commented on the final version of the manuscript.

**Conflict of Interest Disclosures**

The authors have no conflicts of interest.

**Ethical Statement**

All procedures in this study were approved by the Ethics Committee of the University of Social Welfare and Rehabilitation Sciences in Tehran, Iran. Prior to study enrollment written informed consent was obtained from all of the participants.

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