Arch Iran Med. April 2019;22(4):189-197

http www.aimjournal.ir

ARCHIVES OF IRANIAN MEDICINE

Original Article

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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_V7.4) and the heterozygous variant identified (c.1647C>G, p.F549L) resulted in the substitution of Phe549 residing in the K_V7.4 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

Cite this article as: Mehregan H, Mohseni M, Akbari M, Jalalvand K, Arzhangi S, Nikzat N, et al. Novel mutations in *KCNQ4*, *LHFPL5* and *COCH* genes in Iranian families with hearing impairment. Arch Iran Med. 2019;22(4):189–197.

Received: November 26, 2018, Accepted: February 13, 2019, ePublished: April 1, 2019

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 flawlessly[1] 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% consanguinity[3] is no exception. HL ranks as the second most prevalent disability in Iran[4] 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*

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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.[16] 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 (K_v7.4) that belongs to the family of voltage-gated K^+ channels (K_v7.1–K_v7.5).[17] 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.[17] The C-terminal domain is composed of 4 segments denoted as A to D and plays a vital role in channel assembly and gating.[18] The variant identified in this family led to a substitution of

Table 1. Pathogenic Variants Identified in This Study

Family Code	Origin	Onset	Severity	Gene	N. Change	AA Change	Zygosity	In Silico Prediction								MAF in ExAC
								S	PP2	MT	MA	F	P	CA	G	Si	
9400034	Ahvaz (South Iran)	Post	Mild to severe[a]	KCNQ4	c.1647C> G	p.F549L	Hetero	D	D	D	M	D	D	32	5.12	9.976	.
9600226	Torbat-jam (West Iran)	Pre.	Profound	LHFPL5	c.34A>T	p.K12X	Homo	.	.	A	.	.	.	36	4.14	11.967	.
9600236	Najafabad (Central Iran)	Post.	Moderate to profound[b]	COCH	c.116T>A	p.L39X	Homo	.	.	A	.	.	.	36	5.67	15.581	.

Pre, Prelingual; Post, Postlingual; N, Nucleotide; AA, Amino acid; Homo, Homozygous; Hetero, Heterozygous; S, Sift [D, Deleterious;]; PP2, PolyPhen2 HDIV [D, Damaging]; MT, Mutation Taster [D, Disease causing; A, Disease_causing_automatic]; MA, Mutation Assessor [M, medium]; F, FATHMM [D, Deleterious]; P, PROVEAN_pred [D, Deleterious]; CA, CADD_phred; G, GERP++_RS; Si, SiPhy_29way_logOdd. MAF, minor allele frequency.
[a] Severe hearing loss at high frequencies.
[b] Profound hearing loss at high frequencies.

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 $K_v7.4$ channel across species (Figure 2b) but also among all 5 known members of the K_v7 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.[19] 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

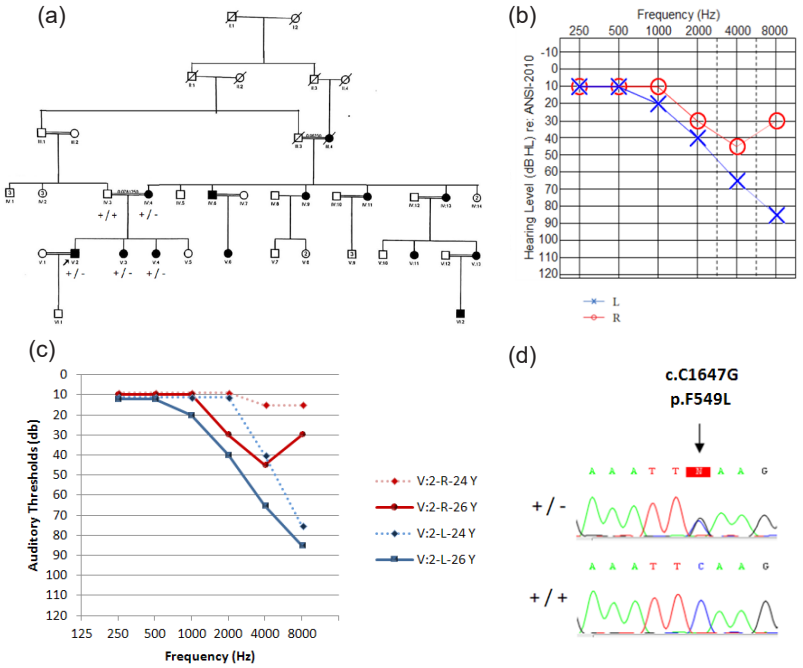

Figure 1. Clinical Features and Segregation Analysis of a Family with Mutation in the *KCNQ4* Gene. **(a)** Pedigree of family 9400034 is consistent with an autosomal dominant mode of inheritance; Open circle, female; filled circle, affected female; open square, male; filled square, affected male; strikethrough, 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.

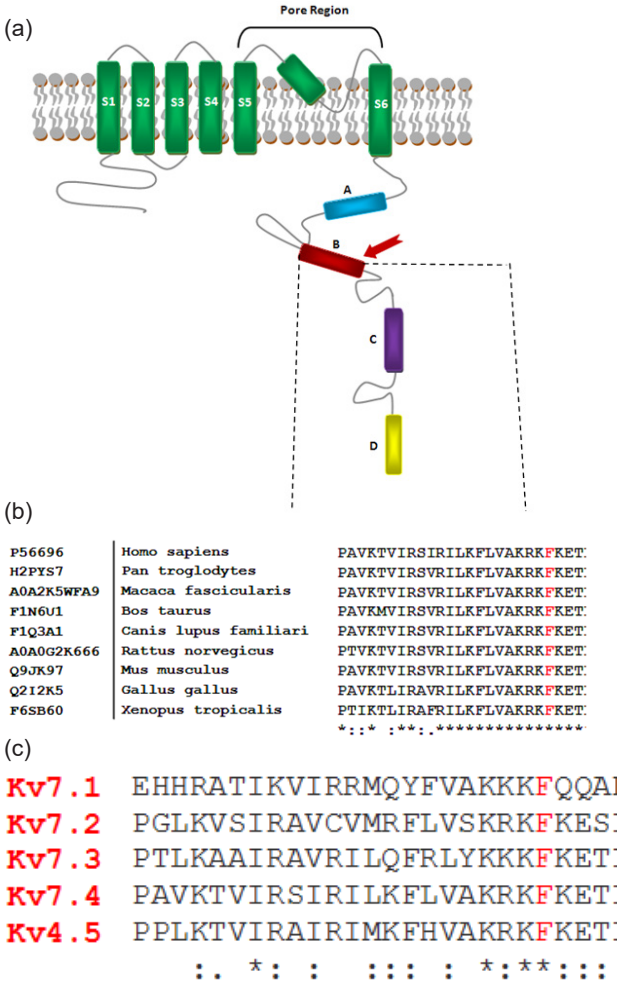

Figure 2. Schematic Drawing of $K_v7.4$ Domain Organization and Phe549 Conservation. (a) The drawing depicts one of the $K_v7.4$ subunits, composed of four helices (S1-S4) which form the voltage sensing domain, followed by the pore region formed by S5 and S6 helices; the subsequent domain is the cytoplasmic C-terminal tail which is composed of four segments denoted as A to D, and the position of Phe549 in the B helix is indicated by an arrow. Multiple sequence alignment of the B helix indicates a high degree of Phe549 conservation **(b)** in $K_v7.4$ across species and **(c)** among all K_v7 family members. Orthologous sequences were obtained from UniProt (https://www.uniprot.org/): [Human (P56696), Chimpanzee (H2PYS7), Cynomolgus monkey (A0A2K5WFA9), Bovine (F1N6U1), Dog(F1Q3A1), Rat(A0A0G2K666), Mouse(Q9JK97), Chicken(Q2I2K5) and Frog(F6SB60)].

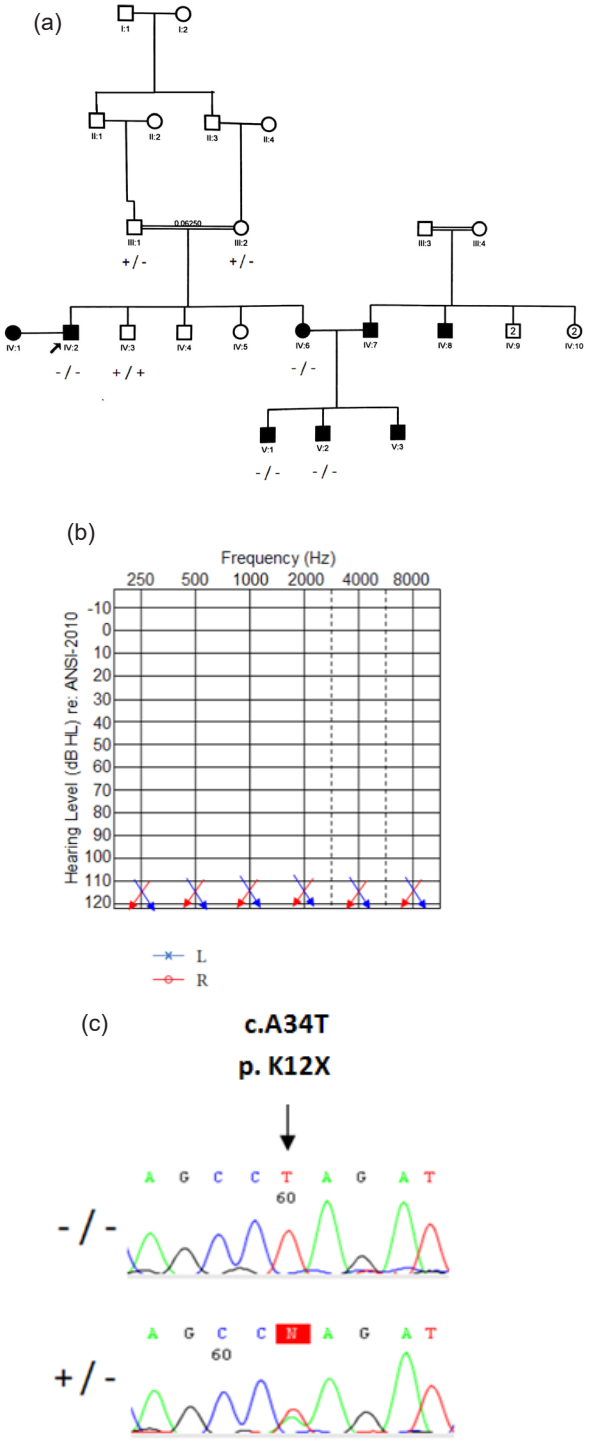

Figure 3. Clinical Features and Segregation Analysis of a Family with Mutation in the *LHFPL5* Gene. (a) Pedigree of family 9600226 is indicative of an autosomal recessive mode of inheritance; **(b)** Pure-tone audiogram for the proband demonstrates profound hearing loss. **(c)** Sanger sequencing chromatogram shows segregation of the identified variant (c.34A>T, p.K12X); -/- and +/- represent the homozygous (mutant) and heterozygous (carrier) genotypes for the wild-type (WT) allele, respectively. For symbols and graph description, see Figure 1.

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,[20] 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.[21] 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.[22] 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.[23,24] The variant identified in this family resulted in the substitution of Leu 39 by a

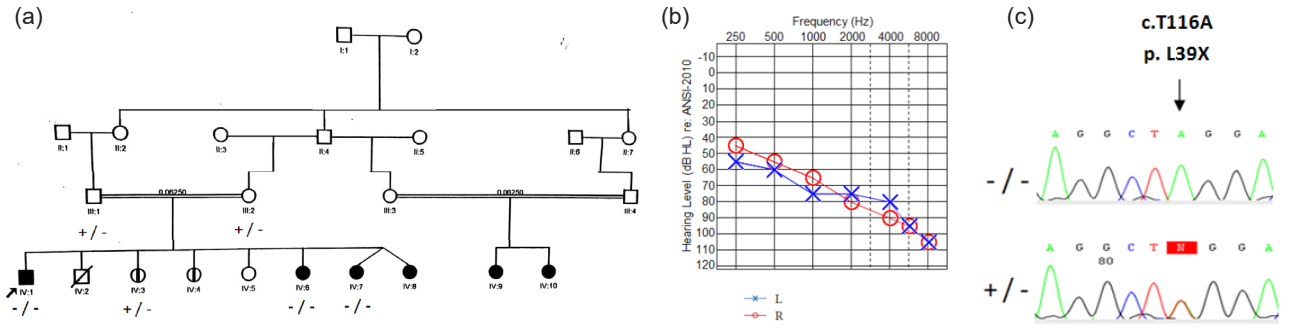

Figure 4. Clinical Features and Segregation Analysis of a Family with Mutation in the *COCH* Gene. (a) Pedigree of family 9600236 displays an autosomal recessive mode of inheritance; **(b)** Pure-tone audiogram for the proband is indicative of moderate hearing loss at low frequencies sloping down to 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.

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.[25] 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[26] where high frequencies are sensed.[27] It is speculated that lack of native K^+ currents due to impaired $K_v7.4$ channels in the sensory outer hair cells (OHCs) of these regions might lead to an overload of K^+ and result in gradual cell degeneration.[28] 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).[29] 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 Ca^{2+}-binding protein calmodulin (CaM) in both Apo-CaM and Ca^{2+}-CaM forms (Figure 5a).[30] CaM is a strong modulator of K_v7 function and studies have shown that disruption of

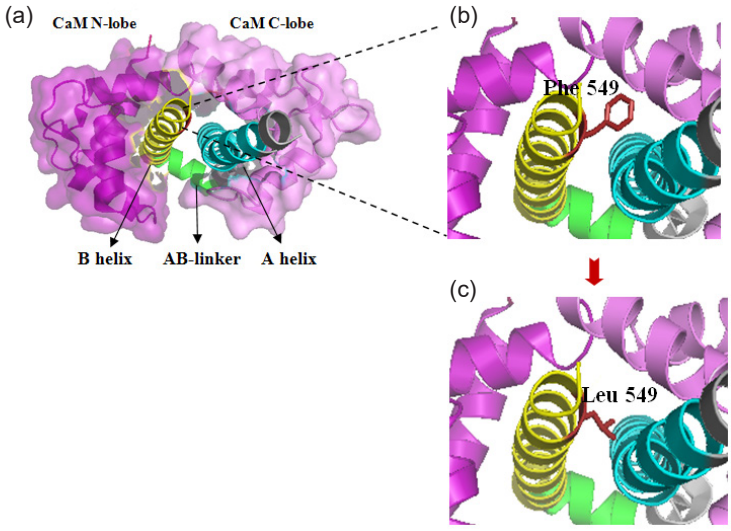

Figure 5. 3D Structure of the AB Domain of $K_v7.4$ Interacting with CaM. (a) The A and B helices of the Kv7.4 cytoplasmic domain form an antiparallel helical pair separated by a short helix (AB linker). X-ray crystallography of Ca^{2+}-CaM (PDB code: 6B8N), visualized by PyMOL, depicts how CaM embraces 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 **(c)** 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_V7 interferes with proper channel assembly and trafficking.[31] Based on structural studies, the A and B helices of $K_V7.4$ are in contact with the CaM C-lobe and N-lobe, respectively and mutations of some residues critical for Apo/CaM:$K_v7.4$ are shown to impair channel trafficking.[30] 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 LHFPL5 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 *LHFPL5*.[34] The encoded protein by this gene, Lhfpl5 (or Tmhs), localizes near the lower point of tip links in hairs cells and along with 3 other transmembrane proteins Tmc1 (transmembrane channel protein 1), Tmc2 (transmembrane channel protein 2)[35] and Tmie (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 *LHFPL5* have been reported in ARNSHL families from the Middle East and northern Africa and unlike *hscy* mice, none of the human *LHFPL5* mutations are accompanied by vestibular symptoms (Table 2). TGE in a cohort of 302 GJB2-negative families led to the first report of *LHFPL5*-related HLin the Iranian population, including 2 previously reported as well as a novel variant in 3 families[15] and recently, in a separate study on 5 Iranian families using NGS, another novel mutation in *LHFPL5* 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)[45] 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[46] 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.[47,49] Reported mutations thus far are missense variants which predominantly involve LCCL and vWFA2 domains of

Table 2. Overview of LHFPL5 Mutations Described in DFNB67

Nucleotide Change	Amino Acid Change	HL Severity	Zygosity	Age of Onset	Origin	Author/Year [Ref]
c.1A>G[a]	p.Met1Val	Severe	Homozygous	Prelingual	Palestinian	Shahin et al, 2010 [40]
c.16+1G>A	-----	Profound	Homozygous	Prelingual	Pakistani	Liaqat et al, 2018 [41]
c. 34A>T	p.Lys12X	Profound	Homozygous	Prelingual	Iranian	This study
c.89dupG	p. Thr31TyrfsX41	Profound	Homozygous	ND	Tunisian	Bensaïd et al, 2011 [42]
c.246delC	P.Leu84X	Profound	Homozygous	Prelingual	Pakistani	Shabbir et al, 2006 [34]
c.258_260delCTC	---	Severe to profound	Homozygous	Postlingual	Iranian	Sloan et al, 2015 [15]
c.269 C>G	p.Pro90Arg	Profound	Homozygous	Prelingual	Iranian	Shang et al, 2018 [39]
c.380A>G[a]	p.Tyr127Cys	Severe	Homozygous	Prelingual	Pakistani	Shabbir et al, 2006 [34]
c.452 G > T	P.Gly151Val	Profound	Homozygous	Prelingual	Pakistani	Liaqat et al, 2018 [41]
c.494C > T	p.Thr165Met	Severe to profound	Homozygous	Prelingual	Turkish	kalay et al, 2006 [43]
c.518T > A	p.Cys173Ser	Profound	Homozygous	Prelingual	Algerian	Ammar-Khodja et al, 2015 [44]
c.649delG	p.Glu216ArgfsX26	Severe to profound	Homozygous	Prelingual	Turkish	kalay et al, 2006 [43]

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 (DNFA9 related mutations in the *COCH* gene with detailed description of clinical features are discussed by Tsukada et al).[49] Recently, a nonsense variant (c.292C>T, p.R98X) in a consanguineous family of Moroccan origin has been reported which recessively leads to vestibular dysfunction with moderate prelingual sensorineural hearing loss.[50] 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.[51,52] These pathological aggregations of impaired proteins seem to build up over time, consistent with the late onset of manifestations in DFNA9 mutations.[51] As opposed to the defining characteristics of DFNA9, the variant identified in this study (p.L39X) 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 and are speculated to trigger a NMD mechanism similar to the 50-bp rule mentioned earlier, thereby leading to the subsequent total lack of a functional protein. $Coch^{-/-}$ mice harboring a genomic deletion downstream the LCCL domain,[53] exhibit hearing impairment by the age of 21 months but $Coch^{+/-}$ mice do not display any auditory deficit.[54] This finding as well as a report of a normal individual carrying a heterozygous frameshift mutation (c.146dupT, p.C50LfsX8)[55] corroborate the hypothesis that truncating variants probably demonstrate a recessive pattern of inheritance due to the protein loss of function.

In conclusion, with regard to the 50% genetic contribution to hearing impairment, identification of the underlying genetic causes is of considerable importance, particularly when this knowledge is beneficial to those who are susceptible to drug or noise-induced hearing loss, or when the impairment runs in the family and a consanguineous marriage is being considered. As more details get revealed about the molecular networks of hearing loss, more progress will be made towards taking preventing measures or developing effective treatments. In a country such as Iran where there is a high rate of consanguinity and *GJB2*-related HL contributes, on average, up to 16% of cases, identification of the remaining causes is crucial to public health. WES has proven to be a powerful technique to reveal causative rare variants and utilizing this technique in the current study led to the identification of 3 novel variants in *KCNQ4*, *LHFPL5* and *COCH* genes and mutations in 2 out of the 3 genes (i.e. *KCNQ4* and *COCH*) are reported for the first time in families of Iranian origin. These findings yet again provide further evidence of the diagnostic potential of this technique to identify variants which would otherwise remain unresolved or hard to be identified.

Authors' Contribution

HM performed WES tertiary analysis, variant interpretation, co-segregation 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.

Acknowledgments

We do appreciate all of the participants for their cooperation, and Dr. Ahmad Daneshi and Dr Zakie Arab for their efforts in recruiting patients and sample collection.

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