Received: 28 July 2020 | Revised: 22 August 2020 | Accepted: 29 October 2020

DOI: 10.1002/mgg3.1550

Molecular Genetics & Genomic Medicine Open Access WILEY

ORIGINAL ARTICLE

Novel homozygous variants in the *TMC1* and *CDH23* genes cause autosomal recessive nonsyndromic hearing loss

Safoura Zardadi[1] | **Ehsan Razmara**[2] | **Golareh Asgaritarghi**[3] | **Ehsan Jafarinia**[2] | **Fatemeh Bitarafan**[4] | **Sima Rayat**[1] | **Navid Almadani**[5] | **Saeid Morovvati**[6] | **Masoud Garshasbi**[2]

[1]Department of Biology, School of Basic Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran

[2]Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

[3]Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

[4]Department of Cellular and Molecular Biology, North Tehran Branch, Islamic Azad University, Tehran, Iran

[5]Department of Genetics, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

[6]Department of Genetics, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

Correspondence
Saeid Morovvati, Department of Genetics, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.
Email: morovvati@iautmu.ac.ir

Masoud Garshasbi, Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
Email: masoud.garshasbi@modares.ac.ir

Abstract

Background: Hereditary hearing loss (HL) is a heterogeneous and most common sensory neural disorder. At least, 76 genes have been reported in association with autosomal recessive nonsyndromic HL (ARNSHL). Herein, we subjected two patients with bilateral sensorineural HL in two distinct consanguineous Iranian families to figure out the underlying genetic factors.

Methods: Physical and sensorineural examinations were performed on the patients. Imaging also was applied to unveil any abnormalities in anatomical structures of the middle and inner ear. In order to decipher the possible genetic causes of the verified *GJB2*-negative samples, the probands were subjected to whole-exome sequencing and, subsequently, Sanger sequencing was applied for variant confirmation.

Results: Clinical examinations showed ARNSHL in the patients. After doing whole exome sequencing, two novel variants were identified that were co-segregating with HL that were absent in 100 ethnically matched controls. In the first family, a novel homozygous variant, NM_138691.2: c.530T>C; p.(lle177Thr), in *TMC1* gene co-segregated with prelingual ARNSHL. In the second family, NM_022124.6: c.2334G>A; p.(Trp778*) was reported as a nonsense variant causing prelingual ARNSHL.

Conclusion: These findings can, in turn, endorse how *TMC1* and *CDH23* screening is critical to detecting HL in Iranian patients. Identifying *TMC1* and *CDH23* pathogenic variants doubtlessly help in the detailed genotypic characterization of HL.

KEYWORDS

cadherin 23, deafness, transmembrane channel-like 1, whole-exome sequencing

TABLE 1 This table summarizes all reported homozygous mutations in *TMC1* with origin information as well as clinical details and impaired domain positions of TMC1

Nucleotide position (cDNA)	Predicted effect	Type of variant	Exon (E) / Intron (I)	Onset of HL	Severity of HL	Domain	Origin and Ref.
-258A>C	–	Regulatory	E3	–	Severe to profound	–	Iran (Davoudi-Dehaghani et al., 2013)
-259C>T	–	Regulatory	E3	–	Severe to profound	–	Iran (Hilgert et al., 2009)
16+1G>T	Splice disruption	Splicing	I5	Prelingual	Severe to profound	–	Pakistan (Kitajiri et al., 2007)
64+2T>A	Splice disruption	Splicing	I6	Congenital/Prelingual	Profound	–	Turkey (Nakanishi et al., 2014)
100C>T	p.R34X	Nonsense	E7	Prelingual	Severe to profound	N-terminus	Pakistan (Kitajiri et al., 2007; Kurima et al., 2002)
150delT	p.N50KfsX26	Frameshift	E7	Congenital	Profound	N-terminus	Iran (Yang et al., 2010)
-195_16del	27 Kb deletion	Deletion	E5	Prelingual	Severe to profound	–	Pakistan (Kurima et al., 2002)
236+1G>A	p.E83X	Nonsense	I7	Congenital	Severe to profound	–	Iran (Hilgert et al., 2008)
237-6T>G	Splice disruption	Splicing	I7	Prelingual	Severe to profound	–	India (El Maghraoui, 2011)
256G>T	p.Glu86X	Nonsense	E8	Prelingual	Profound	N-terminus	Iran (Sadeghian et al., 2019)
295delA	p.K99KfsX4	Frameshift	E8	Prelingual	Severe to profound	N-terminus	North America (Indian) (Kurima et al., 2002)
362+18A>G	p.Glu122Tyrfs*10	Frameshift	I8	Congenital	Severe to profound	–	Pakistan (Shafique et al., 2014)
362+3A>G	Splice disruption	Splicing	E8	Prelingual	Severe to profound	–	Saudi (Ramzan et al., 2020)
453+2T>C	Splice disruption	Splicing	-	Prelingual	Severe to profound	–	India (Ganapathy et al., 2014)
530T>C	p.(lle177Thr)	Missense	E10	Prelingual	Profound	–	Iran (present study)
536-8T>A	Splice disruption	Splicing	I10	Prelingual	Severe to profound	–	Pakistan (Kurima et al., 2002)
536-8T>A	Splice disruption	Splicing	I10	Prelingual	Severe to profound	–	Pakistan (Santos et al., 2005)
c.758C>T	p.Ser253Phe	Missense	E8	Prelingual	Severe	–	Saudi (Ramzan et al., 2020)
767delT	p.F255FfsX14	Frameshift	E13	Congenital	Severe to profound	T1-T2	Turkey (Hilgert et al., 2008)
776A>G	p.T259C	Missense	E13	Prelingual	Profound	T1-T2	Turkey (Kalay et al., 2005)
776+1G>A	Splice disruption	Splicing	E13	Prelingual	Profound	–	Iran (Hildebrand et al., 2010)
797T>C	p.I266T	Missense	E13	Prelingual	Severe to profound	–	China (Wang et al., 2018)
821C>T	p.P274L	Missense	E13	Prelingual	Profound	T2	Turkey (Kalay et al., 2005)
830A>G	p.Y277C	Missense	E13	Prelingual	Severe to profound	T2	Pakistan (Santos et al., 2005)
884+1G>A	Splice disruption	Splicing	E13	Prelingual	Severe to profound	–	Pakistan (Kurima et al., 2002)
1083_1087del	p.R362PfsX6	Frameshift	E15	Prelingual	Profound	T2-T3	Turkey (Kalay et al., 2005)
1114G>A	p.V372M	Missense	E15	Prelingual	Severe to profound	T3	Pakistan (Santos et al., 2005)

(Continues)

TABLE 1 (Continued)

Nucleotide position (cDNA)	Predicted effect	Type of variant	Exon (E) / Intron (I)	Onset of HL	Severity of HL	Domain	Origin and Ref.
1165C>T	p.R389X	Nonsense	E15	Congenital	Profound	T3-T4	Tunisia (Tlili et al., 2008), Jordan (Hilgert et al., 2008)
1166G>A	p.R389Q	Missense	E15	Congenital	Severe to profound	T3-T4	Turkey (Hilgert et al., 2008)
1209G>C	p.W403C	Missense	E15	Prelingual	Severe to profound	T3-T4	(Yang et al., 2013)
1253T>A	p.M418K	Missense	E16	Prelingual	Severe to profound	T4	China (Wang et al., 2018)
1283C>A	p.Ala428Asp	Missense	E16	Prelingual	Severe to profound	T4	India (Singh et al., 2017)
1330G>A	p.G444R	Missense	E16	Congenital/Prelingual	Profound	T4	Turkey (Sirmaci et al., 2009)
1333C>T	p.R445C	Missense	E16	Congenital/Prelingual	Severe to profound	T4	Turkey (Sirmaci et al., 2009)
1334G>A	p.R445H	Missense	E16	Prelingual	Profound	T4	Turkey (Kalay et al., 2005)
c.1404+1G > T	Splice disruption	Splicing	E16	Prelingual	Moderate to severe	-	Pakestan (Imtiaz et al., 2016)
1534C>T	p.R512X	Nonsense	E17	Prelingual	Severe to profound	T4-T5	Pakistan (Kurima et al., 2002)
1541C>T	p.P514L	Missense	E17	Prelingual	Severe to profound	T4-T5	Pakistan (Kitajiri et al., 2007)
1543T>C	p.C515R	Missense	E17	Prelingual	Severe to profound	T4-T5	Pakistan (Kitajiri et al., 2007)
c.1566+1G>A	Splice disruption	Splicing	–	Prelingual	Severe to profound	–	India (Ganapathy et al., 2014)
1586_1587del	-	Frameshift	E18	–	Severe to profound	–	Iran (Sadeghian et al., 2019)
1589_1590del	p.S530X	Nonsense	E18	–	Profound	–	Iran (Bademci et al., 2016)
1703A>G	p.Y568C	Missense	E19	–	Profound	–	Iran (Sloan-Heggen et al., 2015)
1714G>A	p.D572N	Missense	E19	Prelingual	Severe to profound	–	Chine (Wang et al., 2018)
1763+3A>G	p.W588WfsX81	Frameshift	I19	Post-lingual	Profound	–	Netherlands (de Heer et al., 2011)
1764G>A	p.W588X	Nonsense	E20	Congenital	Profound	T4-T5	Tunisia (Tlili et al., 2008)
1810C>T	p.R604X	Nonsense	E20	Congenital	Severe to profound	T4-T5	Greece (Hilgert et al., 2008)
1810C>G	p.R604G	Missense	E20	Prelingual	Severe	T4	Morocco (Bakhchane et al., 2015)
1960A>G	p.M654V	Missense	E20	Prelingual	Severe to profound	T5	India (Kurima et al., 2002)
1979C>T	p.P660L	Missense	E20	Congenital	Profound	T5-T6	China (Sadeghian et al., 2019)
2004T>G	p.S668R	Missense	E21	Prelingual	Severe to profound	T5-T6	Pakistan (Kitajiri et al., 2007; Santos et al., 2005)
2030T>C	p.I677T	Missense	E21	Congenital/Prelingual	Profound	T5-T6	Turkey (Sirmaci et al., 2009)
2035G>A	p.E679K	Missense	E21	Prelingual	Severe to profound	T5-T6	Pakistan (Santos et al., 2005)
2260+2T>A	Splice disruption	Splicing	I23	Prelingual	Severe to profound	–	Tunisia (Riahi et al., 2014)
1696_2283del	Genomic deletion	Deletion	–	Congenital/Prelingual	Profound	–	Turkey (Sirmaci et al., 2009)

1 | INTRODUCTION

According to the World Health Organization, around 466 million individuals throughout the world have been calculated to suffering from hearing loss (HL) while around 35 million are children (Neumann et al., 2019). As the usual sensorineural disorder in human beings, HL has an incidence of approximately 1 in 1000 newborns (Morton & Nance, 2006). It is known as the second most common disability in Iran after different forms of intellectual disability (Najmabadi et al., 2007). Hereditary HL is a heterogeneous disorder and, so far, over 6000 causative variants in approximately 150 independent genes have been identified (Carpena & Lee, 2018).

In most congenital cases of HL, genetic causes take a center stage, and nonsyndromic HL (NSHL) is responsible for almost 80% of inherited deafness (genetic-based HL) (Nakanishi et al., 2014). Considering the Hereditary Hearing Loss Database (http://hereditaryhearingloss.org) (Yan & Liu, 2008), 119 genes have been identified in association with NSHL. Eight of those, including *COL11A2* (OMIM:120290), *GJB2* (OMIM: 121011), *GJB6* (OMIM: 604418), *MYO6* (OMIM: 600970), *MYO7A* (OMIM: 276903), *TBC1D24* (OMIM: 613577), *TECTA* (OMIM: 602574), and *TMC1* (OMIM: 606706), are implicated in both autosomal recessive (ARNSHL) and autosomal dominant nonsyndromic HL (ADNSHL) (Wang et al., 2018). Congenital or prelingual severe-to-profound HL is evident in ARNSHL (Kawashima et al., 2015).

According to worldwide case-studies, impairment of the *TMC1* gene is considered as one of the main causes of ARNSHL (Ballesteros & Swartz, 2020). Furthermore, mutations in *TMC1* make individuals susceptible to autosomal dominant (DFNA36) and recessive (DFNB7/B11) NSHL (Lin et al., 2014). Recently, eight mutations in *TMC1* have been detected in Iranian patients (affected with ARNSHL) (Sadeghian et al., 2019) (Table 1). Though many of the identified mutations are rare in the Iranian population, estimations suggest that 3%–8% of inherited HL can be imputed to *TMC1* mutations (Sloan-Heggen et al., 2016).

The *CDH23* gene encodes a protein of 3354 amino acids with a single transmembrane domain and 27 cadherin repeats. During late embryonic or early postnatal development, the CDH23 protein is imperative for establishing and maintaining the proper organization of the stereocilia bundle of hair cells in the cochlea and the vestibule (Zhang et al., 2020). Not surprisingly, mutations in *CDH23* are responsible for Usher syndrome 1D (OMIM: 601067) and also ARNSHL (Mizutari et al., 2015). It seems that *CDH23* mutations are highly prevalent in patients with congenital high-frequency sporadic or recessively inherited HL, so the patients merit genetic analysis (Mizutari et al., 2015).

The great genotypic and phenotypic heterogeneity of HL make it too challenging to genuinely identify the underlying genetic factor and also do the clinical diagnosis of the affected individuals. However, the whole-exome sequencing (WES) technique, is often performed as a robust cutting edge technique to detect the underlying mutations in ARNSHL as a heterogeneous disease. Using this technique, performed on two patients affected by prelingual ARNSHL in two distinct consanguineous Iranian families, we identified two novel variants: a novel homozygous variant, NM_138691.2: c.530T>C; p.(lle177Thr), in exon 10 of the *TMC1* gene which may alter the function of TMC1 protein, and also NM_022124.6: c.2334G>A; p.(Trp778*) in *CDH23* as a novel nonsense variant in the second family. According to the report of the American College of Medical Genetics and Genomics (ACMG)-AMP variant interpretation guideline (Green et al., 2013), c.530T>C; p.(lle177Thr) was determined "likely pathogenic," while c.2334G>A; p.(Trp778*) considered as the "Pathogenic variant." We also put forth enough *in silico* evidence endorsing their contribution to the pathogenesis of NSHL. Nonetheless, before applying any genetic consultation, we strongly suggest doing functional analyses.

2 | METHODS

2.1 | Editorial policies and ethical considerations

The study protocol was approved by the local medical ethics committee of Tarbiat Modares University, Tehran, Iran. All participants/legal guardians provided the written, informed consent before enrollment. They also were informed that all derived data would be used only for scientific not for commercial purposes. All clinical information and the medical histories were collected at the Department of Medical Genetics, DeNA laboratory and Rasad Pathobiology & Genetics laboratory, Tehran, Iran.

2.2 | Patients and clinical evaluations

Two inbred four-generation families were ascertained from the Tehran province of Iran. Regarding the first family (Figure 1a), the proband (III.1) was a 7-year-old female, a congenital deaf-mute, while her consanguineous parents/grandparents were normal in auditory and verbal functions. She was suffering a profound HL without any syndromic manifestations. In the second family (Figure 1b), the proband was a 9-year-old male with prelingual HL. Similarly, his parents were also normal.

2.3 | Whole-exome and sanger sequencing

The standard phenol-chloroform method (Chomczynski & Sacchi, 1987) was used to isolate genomic DNA from

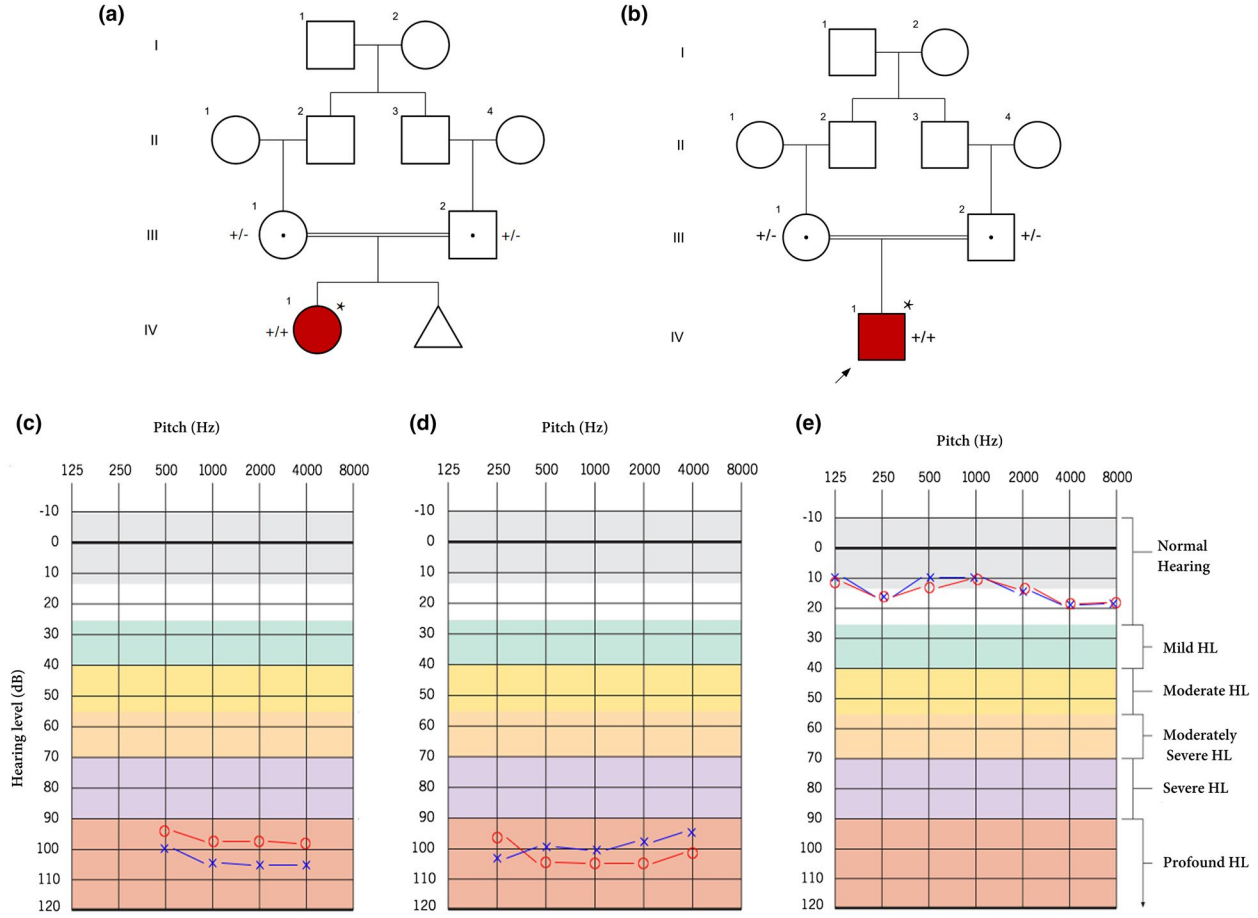

FIGURE 1 (a) pedigree information showing variation spectrum of*TMC1*in the family 1. (+): c.530T>C; (−): wild-type allele. (b) pedigree of family 2 indicates a patient with the*CDH23*variant. In this figure, (+): c.2334G>A. The asterisk (*) shows the samples that were selected for performing whole-exome sequencing. In these figures, white symbols: unaffected; red symbols: affected; squares: men; circles: females; parallel lines: consanguineous marriage. (c) The audiogram showed bilateral profound sensorineural hearing loss of the female affected subject in family 1 (IV.1). (d) The audiogram revealed the profound sensorineural hearing loss of the male proband in family 2 (IV.1). (e) The audiogram of the control individual (III.1), who was selected from family 1. Approximately, the same results were obtained for other next of kin in both families. The graphs are depicted using audiogram-creator (https://www.hearingaidknow.com) according to the original graphs provided by otorinologists. Blue crosses and red circles represent the air conduction hearing threshold levels of the left and right ear, respectively

blood samples. At the first step, samples were screened for *GJB2* mutations using Sanger sequencing (Parzefall et al., 2017), all were *GJB2*-negative. Then, the verified *GJB2*-negative samples (probands) were subjected to WES at Centogene AG (Rostock, Germany) using the Illumina HiSeq4000 platform (Illumina, Inc., San Diego, CA, USA) to achieve an average coverage depth of ~100×. The list of tested genes is accessible in (DiStefano et al., 2019). All information about WES is put forward in Supporting Information S1.

Samples from all available family members were subjected to Sanger sequencing to show whether the potential homozygous variants in the causative gene, *TMC1* and *CDH23*, co-segregate with HL or not. Primers surrounding the region of the identified variant were designed using Primer3.0 (Untergasser et al., 2007) (Supporting Information S1) and PCR was performed in a standard condition. To detect any alternation in DNA sequences, Sequencher 4.7 (Gene Codes Corporation, MI, USA) was utilized.

2.4 | Three-dimensional structure modeling

To evaluate any possible impacts of p.(Ile177Thr) and p.(Trp778*) on the protein structures (including stability and folding), the protein domains were analyzed employing ScanProsite (Gattiker et al., 2002) and ClustalW (Thompson et al., 2003) was used to recruit sequence alignments of the human TMC1 and CDH23 proteins. We also used a BLAST sequence search to find the closest sequence similarity to the domains of TMC1. Finally, we used the template nhTMEM16 structure (Ballesteros et al., 2018) (Protein Data Bank ID: 4WIS) and Human Cadherin-23 EC6-8 (PDB: 5TFM) to build favorite models. The three-dimensional structure of the

TABLE 2 Characterization of the audiometric data for the three family members including the patient and her parents

Family	Pedigree	Gender	Age at test (years)	Age of onset	Use of aminoglycoside	PTA, dB HL		Type of HL	Other symptoms
						Right ear	Left ear		
Family 1	III.1	Female	7	Congenital	No	>94.75[a]	>100	Profound	Moderate Intellectual Disability
	II.1	Female	28	NA	No	Normal[b]	Normal	NA	Not Observed
	II.2	Male	32	NA	No	Normal	Normal	NA	Not Observed
Family 2	III.1	Female	28	NA	No	Normal	Normal	NA	Not Observed
	III.2	Male	36	NA	No	Normal	Normal	NA	Not Observed
	IV.1	Male	9	Congenital	No	>100	>100	Profound	The patient is asymptomatic

[a]For this proband, 4-PTA (4-frequency pure tone average (0.5, 1, 2, and 4 kHz)) was used.

[b]Normal: <25 dB.

Abbreviation: NA, not appropriate.

proteins and also the probable impacts of the variants were depicted by PyMOL. We also confirmed the structures using the I-TASSER server (Zhang, 2008).

2.5 | Prediction of single point variation on protein stability

We used the I-Mutant2.0 to predict and identify the impact of p.(lle177Thr) on protein stability using the TMC1 protein sequence. I-Mutant2.0 (Capriotti et al., 2005) is used to assess the thermodynamic free energy change upon single-point variations in protein sequences. This tool uses the algorithms of the Support Vector Machine and the ProTherm database (Bava et al., 2004).

2.6 | Prediction of the effects of the variants on protein glycosylation

To predict the possible impacts of p.(lle177Thr) on O-linked glycosylation, GlycoEP (http://crdd.osdd.net/raghava/glycoep) (Chauhan et al., 2013) was applied according to the Average Surface Accessibility and Composition profile of patterns algorithms. We also used GlycoEP to show any abnormality in O-linked or N-linked glycosylation caused by p.(Trp778*). GlycoMine^{struct} (Li et al., 2016) was also utilized to screen and obtain high-confidence predictions for glycosylation sites.

2.7 | Variant pathogenicity

The protein truncation, caused by deletion or indel mutations, are potentially pathogenic mutations since they may lead to loss of several domains and functionally important regions of the protein. This also directly impacts protein functions (Gauthier et al., 2011). The novel variant, p.(Trp778*), leads to the production of a truncated protein. This begs the question whether deleted regions are functionally important, we carried out MetaDome and protein conservation analyses across species using ConSurf (Glaser et al., 2003) and also "2-Way Pseudogene Annotation Set" from UCSC genome browser database. MetaDome predicts the tolerance of the genetic mutations based on the population variation data from ExAC and GnomAD. MetaDome was also applied to visualize the genetically intolerant sites/regions that could have potentially influenced the proteins function (Wiel et al., 2019). Besides, at least four databases were used to evaluate the pathogenicity score of the variants to touch upon MutationTaster (Schwarz et al., 2010), Provean (Choi & Chan, 2015), Polyphen-2 (Adzhubei et al., 2013), and Pmut (Ferrer-Costa et al., 2005).

3 | RESULTS

3.1 | Clinical presentation

To obtain the medical histories, we used a comprehensive questionnaire addressing the following issues: exposure medication, noise, ototoxic, and TORCH (toxoplasma, rubella, cytomegalovirus, herpes simplex), degree of HL, age of onset, the symmetry of HL, utilization of hearing aids, presence of tinnitus and vertigo, pathological changes in the ear, and also other pertinent clinical manifestations (Newton et al., 2001). Further investigations revealed that neither patients nor parents had a positive history of continuous exposure to deleterious noise, serious infection (e.g. TORCH), or even ototoxic drugs. Audiological tests

were executed to categorize HL as mild (20–40 dB HL), moderate (41–70 dB HL), severe (71–95 dB HL), or profound (>95 dB HL) (Shinagawa et al., 2020). These assessments were executed in a standard anechoic chamber with a pure-tone audiometer at frequencies ranging from 250 to 4000 Hz (Bayat et al., 2019). Imaging investigations, for example, computed tomography (CT) scans and magnetic resonance imaging (MRI), did not reveal any abnormalities in anatomical structures of middle and inner parts of the ear in each patient. Some of the important clinical findings are summarized in Table 2.

3.1.1 | Family 1

The proband (a 7-year-old female; III.1) was delivered full-term, although, her mother previously experienced an abortion in 8 weeks. The proband (Figure 1a) was subjected to common audiological assessments including auditory brainstem response (ABR), distortion production otoacoustic emissions (DPOAE), and also multiple auditory steady-state evoked responses (ASSR). Using a 4-pure tone audiometry (4-PTA) test, the patient showed a bilateral profound HL at all frequencies from 500 to 4000 Hz (Figure 1c).

Further clinical assessments did not show any abnormality in the proband's cardiovascular, endocrine, skin, and particularly visual organs. Hence, the syndromic HL was excluded. Other auxiliary symptoms were detected and also observed in the proband (III.1), for example, moderate and intellectual disability. No hearing symptoms (pertinent- or non-pertinent) were identified in each parent (II.1 and II.2). Her parents had a consanguineous marriage, suggesting ARNSHL in the offspring. No HL history was identified in three previous generations of the family or even in their next of kin.

3.1.2 | Family 2

The proband was a 9-year-old Iranian male who had prelingual HL (Figure 1b). As the different mutations of *CDH23* had been reported in association with Usher syndrome, in order to exclude the germane phenotypes, the patient examined meticulously. For example, fundus examinations did not show any macular changes in both eyes. To obtain medical history, the aforementioned questionnaire was also used. Also, no other visual complaints such as night blindness, visual field loss, and decrease in central vision were detected. PTA test subsequently confirmed the presence of sensorineural HL, while his parents tested negatively for HL (Figure 1d,e). Imaging investigations did not show any abnormalities in anatomical structures of each middle and inner ear. Cochlear implantation was performed on the patient at the age of 6 years.

3.2 | Molecular findings

WES was applied according to previous studies (Binaafar et al., 2020). The mean depth of coverage was around 100× and approximately 97% of targeted regions were covered (Supporting Information S1). Among the total number of variants, we focused on non-synonymous, splice variants, and also coding Indels. By assuming autosomal recessive mode of inheritance, heterozygous variants were excluded

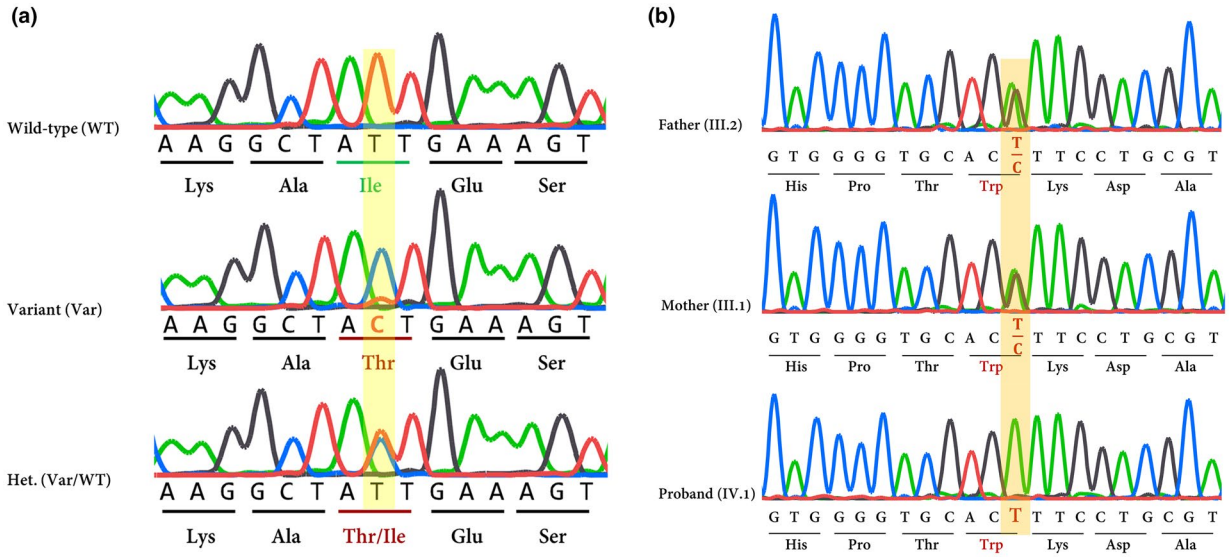

FIGURE 2 (a) Chromatograms show nucleotide sequences of*TMC1*in the regions of c.530T>C which is found in family 1. Het: heterozygote. (b) mutation analysis of*CDH23*gene: the chromatogram shows the nucleotide alternation caused by a novel nonsense variant in exon 22 of*CDH23*(c.2334G>A) in family 2. Affected amino acids are indicated by red color

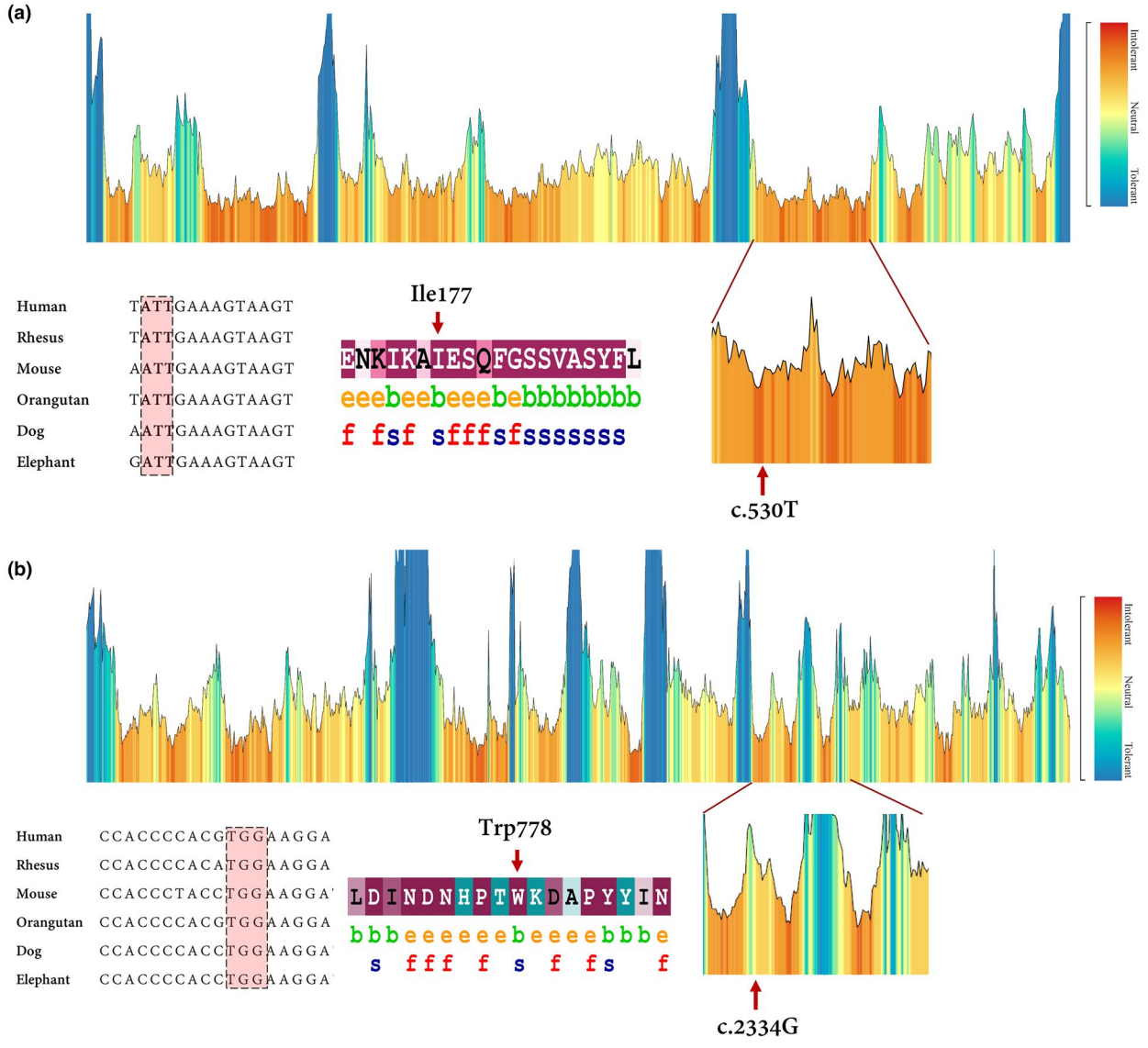

FIGURE 3 (a) the tolerance landscape depicts a missense over the synonymous ratio calculated as a sliding window over the entirety of the protein. The missense variation is annotated from the gnomAD data set and the landscape provides some indication of regions that are intolerant to missense variation. In this TMC1 tolerance landscape, the region harboring the novel missense variant can be seen as intolerant if compared with other parts in this protein. Nucleotide alignment showing high conservation of the codon residue which encodes Ile 177. The ConSurf server was applied to estimate conservation scores for the amino acid residue substituted by the missense variant. Scores ranged from 1 to 9, where a score of 9 represented a highly conserved residue (Glaser et al.,2003). ConSurf demonstrates evolutionary conservation profiles for proteins of unknown/known structure in the PDB according to the phylogenetic relations. (b) MetaDome database was used to identify the intolerant regions (surrounding the c.2334G>A variant). As depicted, the novel variant is located in a highly intolerant region. Data derived from nucleotide alignment and ConSurf show that the c.2334G or Trp778 is highly conserved

and all previously identified SNPs with MAF ≥1% were filtered out using publicly available data of ExAC (Karczewski et al., 2017), Exon Sequencing Projects (ESP), the Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/), Human Gene Mutation Database (HGMD) (Stenson et al., 2003), and Iranome (Fattahi et al., 2019). Consequently, variant functionality was applied using SIFT, Pmut, Provean, MutationsTaster, and Polyphen-2. As an essential filtering step, variants were sorted out according to the identified associated genes with NSHL. Finally, two novel variants including c.530T>C; p.(lle177Thr) in *TMC1* (Family 1) and also c.2334G>A; p.(Trp778*) in the *CDH23* gene (Family 2) were identified as the most possible causative variants (Figure 2a,b).

Evolutionary conservation of the detected region harboring the variants was analyzed by aligning the amino acids and nucleotide sequences from several species using the ConSurf, UCSC database (Karolchik et al., 2003) and MetaDome. It was shown that the affected regions in *TMC1* and *CDH23* were highly intolerable (Figure 3a,b). Eventually, we

TABLE 3 Several online databases that used to predict the pathogenicity of the variants in the *TMC1* and *CDH23* genes. The annotation was applied according to the Homo sapiens genome assembly GRCh37 (hg 19)

Gene	Exon	Variation				PolyPhen-2	MutationTaster	SIFT	Pmut	Provean	ExAC	Iranome	1k Genome
		Nucleotide	Protein	Type	Status								
TMC1	10[a]	c.530T>C	p.(Ile177Thr)	Missense	Hom.	DC	DC	DC	DC	DC	Novel	Novel	Novel
CDH23	22[b]	c.2334G>A	(p.Trp778*)	Nonsense	Hom.	ND	DC	DC	ND	DC	Novel	Novel	Novel

[a]Has been annotated according to NM_138691.2.

[b]Has been annotated based on NM_022124.6.

Abbreviations: Hom, homozygote; ND, not defined.

reclassified the novel variant of *TMC1* using ACMG-AMP guidelines (http://wintervar.wglab.org) (Green et al., 2013) into the "Likely Pathogenic" group, while the nonsense variant in *CDH23* was categorized as "Pathogenic" variant. For detailed filtering steps and the number of variants in each step, refer to Table S1.

In summary, the novel variants were not reported in dbSNP147, 1000 genome project, ESP, ExAC (Karczewski et al., 2017), HGMD®, ClinVar (Landrum et al., 2016), and Deafness Variation Database (Azaiez et al., 2018). Using a local database (i.e. Iranome), the allele frequency of both variants was checked in at least 100 people with the same ethnicity. Sequencing of the surrounding regions of variants in *TMC1* and also *CDH23* genes using available family members verified that the variant co-segregated with ARNSHL phenotype in the families (Figure 2a,b; Table 3).

4 | DISCUSSION

Hearing loss (HL) is a heterogeneous disease with more than 150 known genes, which often show overlapped phenotypes in patients (Razmara et al., 2018). In this study, according to guidelines released by the ACMG for HL (Oza et al., 2018), the screening of *GJB2* mutations was initially performed, but no variant was identified in both families under research. In the next step, WES was performed and this successfully resulted in the identification of two novel variants in *TMC1* (Family 1) and *CDH23* (Family 2) co-segregated with HL.

The *TMC1* has 24 exons (Kawashima et al., 2015) and its encoded protein involves 760 amino acids with 6 transmembrane domains along with an intracellular N-terminal domain, three extracellular loops, two intracellular loops, and a short intracellular C-terminal domain (Jiang et al., 2018) (Figure 4a). The exact structure and function of TMC1 are uncertain but proposed structures show that the protein can potentially function as a transporter or a channel. It also has a similarity to the α-subunit of voltage-dependent K^+ channels and mediates K^+ homeostasis in the inner ear (Santos et al., 2005) (Figure 4a). The mechanotransduction channel in inner ear hair cells of vertebrates converts mechanical stimuli of sound, gravity, and head accelerations into electrical signals (Lin et al., 2014). The auditory or vestibular nerves transmit these signals into the central nervous system for perception of sound, this process is known as mechanoelectrical transduction (MET).

The homozygous c.530T>C substitution was identified in exon 10 of the *TMC1* gene. This variant causes isoleucine (Ile) substitution to threonine (Thr) at codon 177 which is located within the long intracellular N-terminus of TMC1 protein (Figure 4a). The alignment of amino acid and nucleotide sequences of different species indicated that this variant is located in a highly conserved region of TMC1 protein. By

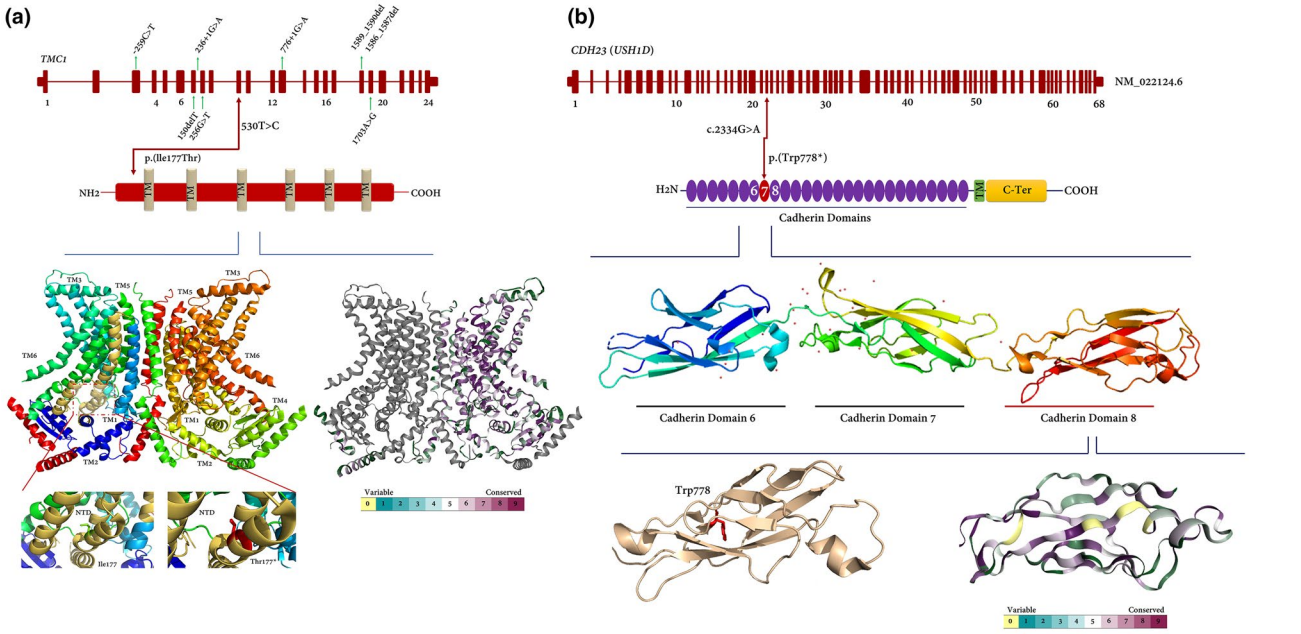

FIGURE 4 (a) Organization of the*TMC1*gene (NM_138691.2) and TMC1 protein showing the position of the c.530T>C and p.(Ile177Thr) variant (red arrow), respectively. Important novel variants/mutations identified in Iranian populations are also shown (green arrows). The cDNA size of*TMC1*is around 3.2 Kb. In the figure, TM: transmembrane domain, (+) variant, (−) wild-type allele. A comparison of normal and mutated TMC1 predicted structure was applied. The normal and the variation site of p.(Ile177Thr) is emphasized by a highlighted zone and locally zoomed. The three-dimensional structure of TMC1 is also colored by the ConSurf evolutionary conservation. (b) genomic and protein structure of cadherin-23. The novel nonsense variant is located in exon 22 encoding cadherin domain 8 (shown as red). The affected amino acid is indicated by red color. CDH23 consists of 27 extracellular cadherin repeats (shown as violet), a transmembrane (TM) domain (green box), and a cytoplasmic domain (C-Ter, yellow box). The three-dimensional structure of CDH23 was also shown and colored according to algorithms of ConSurf to show the entire conservation throughout the protein

circumventing data from predicting tools for glycosylation, we showed that the substitution cannot make a new site of O-linked glycosylation in protein (Score: −1.24; Figure S1), whereas, a prediction based on I-Mutant2.0 showed that this substitution may decrease protein stability (DDG: −2.62). Variant pathogenicity showed that the substitution is a disease-causing alternation (Table 3). The data are consistent with previous investigations showing that double *Tmc1/2* knockout mice suffer from severe auditory and vestibular deficits, and also thoroughly lack normal mechanotransduction currents in auditory and vestibular hair cells (Kawashima et al., 2015). Certainly, mutations in the *TMC1* gene at the DFNB7/11 locus are one of the common causes of ARNSHL. Also, it seems that DFNB7/11 HL shows a significant allelic heterogeneity among Iranian populations that have been studied (Table 1).

Herein, by using WES, Sanger sequencing, and co-segregation analysis, a novel nonsense variant, NM_022124.6: c.2334G>A; p.(Trp778*), was successfully identified in the *CDH23* gene (Figure 4b). Using conservational analysis, we showed that the affected residue is in a highly conserved region. Cadherin 23 plays an important role as a calcium-dependent cell-cell adhesion glycoprotein (Zhang et al., 2017). This novel nonsense variant potentially makes truncated protein. There are two fates for mRNAs containing premature termination codons (PTCs): nonsense-mediated mRNA decay (NMD) (Maquat, 2004) or translation to truncated proteins. The former one is an evolutionarily conserved quality control pathway in eukaryotic cells that is responsible for inspecting mRNA for any possible errors, so eliminating any error-containing transcripts and controlling the amount of nonmutated transcript in the transcriptome. Therefore, NMD results in loss-of-function allele (Khajavi et al., 2006). Second, translation to truncated protein can also put the proteins on the brink of instability or even inactivation, depending on how many residues are deleted. Regardless of two possible mechanisms, we believe that the CDH23 protein containing p.(Trp778*) will be a malfunctioned or an inactive protein.

TMC1 and CDH23 are implicated in mechanotransduction complex in mouse hair cells (Müller, 2008; Pan et al., 2013) (Figure 5a), though how they interact with other components of the complex is shrouded in mystery. The molecular identity of the MET channel remains unknown but there are studies cogently showing that TMC proteins (TMC1 and TMC2) are pore-forming subunits of the hair cells MET channels (Fettiplace, 2016; Kawashima et al., 2015; Kurima et al., 2015). Studies using the Zebrafish model showed that Tmc1 is capable of binding to the

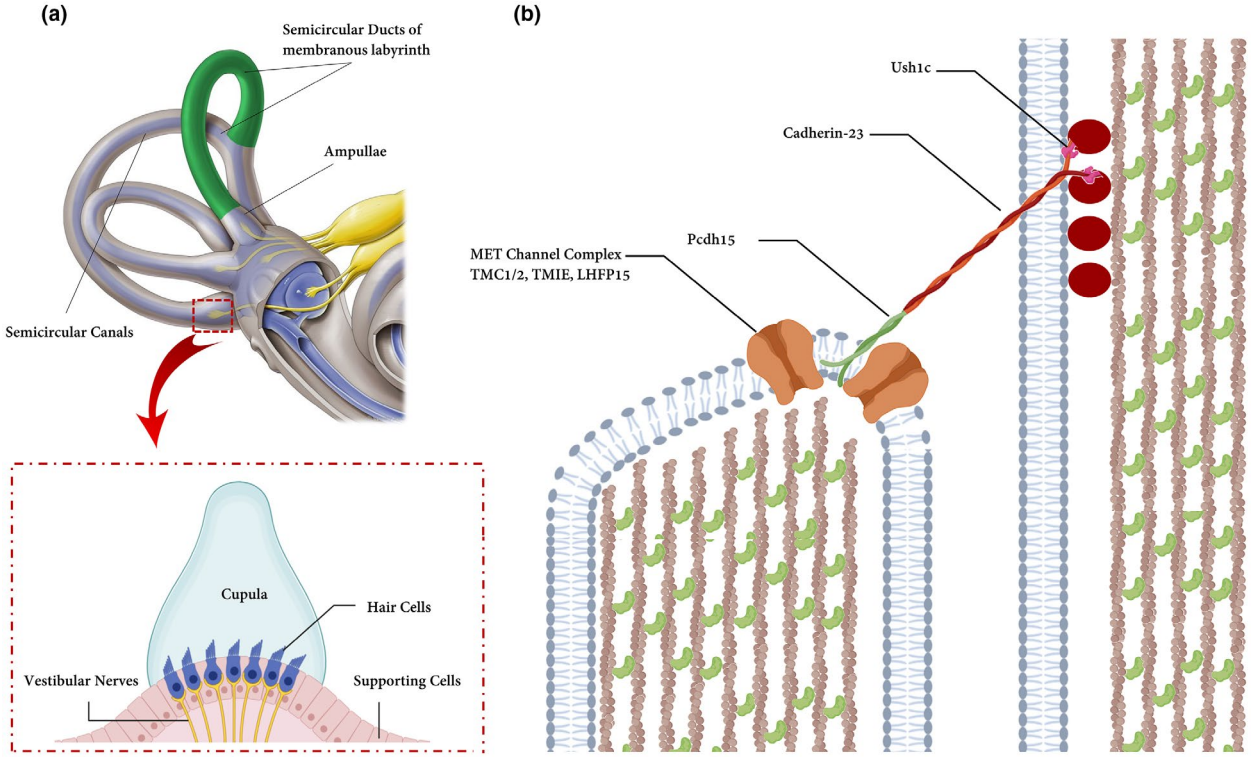

FIGURE 5 (a) Hair bundles and tip links. The diagram of a hair cell is depicted the hair bundle and the tip-link filaments that connect the stereocilia in the direction of their mechanical sensitivity. Cupula is a structure in the vestibular system, providing a sense of spatial orientation. (b) Molecules form tip links and putative components of the mechanotransduction channels in hair cells. Cadherin-23 interacts directly with protocadherin-15 (Pcdh15) to form the upper and lower parts of tip links. Ush1c and Myosin7a (is not shown) play an important role in connecting molecular components of hair cells. LHFPL5, TMIE, and TMC1/2 form MET channel complex and localize at the lower end of tip links near Pcdh15 where transduction channels are located. The figure is redrawn from a published paper (Lukacs,2016)

C-terminus of Pcdh15a, which in turn is a fundamental component of the mechanotransduction complex in auditory and vestibular hair cells (Figure 5b). Corresponding amino acids 1–229 of Tmc1 may contribute to protein–protein interactions (Maeda et al., 2014). Besides, Maeda et al. showed that the N-terminus of TMC1, including 1–179 aa, also could interact with the cytoplasmic tail of each isoform of PCDH15 (Maeda et al., 2014). This interaction is restricted to the MET site at the tips of stereocilia and does not involve kinociliary links (Kurima et al., 2015). In this study, we reported the 9th case of Iranian patients affected by ARNSHL who was homozygote for a novel missense *TMC1* variant. We conjectured that p.(lle177Thr) may disrupt/enervate the interaction between TMC1 with PCDH15. Thus, we can propose two probably pathological mechanisms: impairment of *TMC1* which causes ARNSHL or decreased activity of PCDH15 which can justify the phenotype in the patient. However, these mechanisms should be evaluated meticulously in other complementary studies. Besides, because Cdh23-deficient mice have splayed stereocilia, it was suggested that CDH23 is part of a transmembrane complex that connects stereocilia into a bundle (Siemens et al., 2004) (Figure 5b), as a result, any truncating defects in the formation of this complex may disrupt stereocilia bundles and cause deafness (Okano et al., 2019).

We believe that the findings of this study hopefully broaden the horizons toward better understanding the impact on patient clinical management, genetic counseling, carrier testing, and premarital screening. Further screening is required to finding out the contribution of this missense variant to ARNSHL and also its allele frequency among Iranian HL patients. We also recommend doing functional analysis of the identified variant *in vitro* and *in vivo*.

5 | CONCLUSIONS

Herein, we described c.530T>C or p.(lle177Thr) as a novel variant in the *TMC1* gene and also c.2334G>A; p.(Trp778*) in the *CDH23* gene causing ARNSHL in two distinct Iranian families. Detecting additional *TMC1* and *CDH23* variants provides an additional endorsement that mutations in *TMC1* and *CDH23* play a pivotal role in the etiology of ARNSHL. Our findings indicate that screening for *TMC1* and *CDH23* variants may provide appropriate information for diagnosis

and counseling in Iranian ARNSHL patients. Moreover, we reconfirmed that the solo-WES can properly detect underlying genetic factors contributing to ARNSHL. It can, in turn, provides priceless information on genetic counseling and personalized health maintenance measures to prevent the transmission of HL mutations.

INFORMED CONSENT

All participants provided the written, informed consent before enrollment. They also were informed that all derived data would be used only for scientific not for commercial purposes. All clinical information and the medical histories were collected at the Department of Medical Genetics, DeNA laboratory and Rasad Pathobiology and Genetics laboratory, Tehran, Iran.

CONSENT FOR PUBLICATION

Written consent for publication of clinical data and results of the whole exome analysis was obtained from each participant.

COMPETING INTEREST

None.

ACKNOWLEDGMENTS

We are especially grateful to the staff of the DeNA and Rasad laboratories, Tehran, Iran, for helping us in this research. We also appreciate Mr. Amirreza Bitaraf, Tarbiat Modares University, Tehran, Iran, for his supports.

AUTHOR CONTRIBUTIONS

MG and SM are responsible for the design of this study, acquisition, analysis, and interpretation of data for the work. MG and SZ drafted the work; ER, GA, and SR revised the draft critically for important intellectual content; SZ, MG, and EJ provided approval for publication of the content; SZ, MG, SNA, and ER collect the detailed information and blood samples of pedigrees; MG, GA, and ER analyzed whole-exome sequencing data; ER, SZ, EJ, and MG agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

ETHICAL APPROVAL

The study protocol was approved by the local medical ethics committee of Tarbiat Modares University, Tehran, Iran. The present study had been performed from 2017 to 2019.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher. The variant and pertinent phenotype caused by a mutation in *TMC1* are accessible at ClinVar (accession number: SCV000992684), Leiden Open Variation Database (LOVD; https://databases.lovd.nl/shared/individuals/00265280). The information for the *CDH23* novel variant is also accessible at ClinVar (accession number: SUB7804220) and LOVD (https://databases.lovd.nl/shared/individuals/00306910).

ORCID

Ehsan Razmara https://orcid.org/0000-0001-9926-3975
Ehsan Jafarinia https://orcid.org/0000-0002-1152-2862
Fatemeh Bitarafan https://orcid.org/0000-0002-4489-2923
Masoud Garshasbi https://orcid.org/0000-0002-5508-7903

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Fig S1

Table S1

Supplementary Material

How to cite this article: Zardadi S, Razmara E, Asgaritarghi G, et al. Novel homozygous variants in the *TMC1* and *CDH23* genes cause autosomal recessive nonsyndromic hearing loss. *Mol Genet Genomic Med.* 2020;8:e1550. https://doi.org/10.1002/mgg3.1550