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A transversion mutation in non-coding exon 3 of the TMC1 gene in two ethnically related Iranian deaf families from different geographical regions; evidence for founder effect

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ABSTRACT

Objectives: Transmembrane channel-like 1 (TMC1) gene is a member of the transmembrane channel-like (TMC) gene family that encodes an integral membrane protein of the inner ear. It is suggested that mutation in this gene is one of the main causes of autosomal recessive non-syndromic hearing loss (ARNSHL) in different populations. The aim of this study was to determine the contribution of the *TMC1* gene mutations in causing hearing loss in Iran.
Methods: In total 54 unrelated Iranian families containing 159 affected individuals with ARNSHL detected by audiometric and otologic examinations were analyzed. Haplotype analysis of all members of 45 GJB2- & GJB6-negative families, using four microsatellite markers linked to DFNB7/11 was performed.
Results: Co-segregation of hearing loss with all investigated markers for the DFNB7/11 locus was found in one family. DNA sequencing of all coding and non-coding exons and intron boundaries of the *TMC1* gene identified c.-258A>C mutation in non-coding exon 3 only in individuals with hearing loss. This mutation has been previously reported in another Iranian family (G9) that share similar ethnicity. This variant was not detected in 300 ethnically matched healthy controls.
Conclusions: These results increase the probability that this nucleotide variation may be a pathogenic mutation. This study showed that the ethnicity may be more useful than geographical location to design research strategy for determining which genes should be considered when a heterogeneous disorder is under investigation.

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1. Introduction

Hearing loss is the most prevalent sensorineural disorder worldwide. It is estimated that genetic causes are responsible for hearing impairment in at least half of the cases with congenital hearing loss. This trait can be inherited as autosomal recessive, dominant, mitochondrial maternally, X- and Y-linked [1,2]. So far over 50 autosomal dominant (DFNA), 70 autosomal recessive (DFNB), 5 X-linked (DFN) and 1 Y-linked deafness loci have been mapped showing extensive genetic heterogeneity for this trait [3]. Non-syndromic inherited congenital hearing loss, with autosomal recessive inheritance, is the most common type of hearing loss and is more prevalent in consanguineous marriage [4].

The vast genetic heterogeneity of inherited non-syndromic hearing loss and limited clinical variation of this defect among deaf are known as the most common pitfalls in genetic diagnosis of hearing loss [5]. Autozygosity mapping is a powerful method to locate genes causing autosomal recessive disorders in large consanguineous families [6–8]. Previous studies have shown that suitable families for these studies can be found in regions such as the Middle East where consanguinity is highly prevalent [5,9].

Transmembrane channel-like gene 1 (*TMC1*) is a member of the subfamily A of transmembrane channel-like (TMC) gene family. It has been shown that the *TMC1* mutations can cause pre-lingual

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profound autosomal recessive (DFNB7/11) and post-lingual progressive autosomal dominant (DFNA36) non-syndromic hearing impairment [10]. This gene has 24 exons and encodes a 760 amino acids protein. TMC1 protein is an integral element of the mechanotransduction complex that is predicted to have intracellular amino and carboxyl termini and contains one conserved TMC and multiple transmembrane domains [11,12]. The *TMC1* is expressed in the mouse inner ear and has been suggested that this protein have a role in the maturation and survival of cochlear hair cells [11,13].

To date, a variety of nonsense, missense, deletion and splice site mutations in the *TMC1* gene have been reported in families from different populations [14–18]. These reports suggest that mutations in the *TMC1* gene are a common cause of autosomal recessive non-syndromic hearing loss (ARNSHL).

In the present study, co-transmission of hearing loss with four DFNB7/11-linked (*TMC1*) microsatellite markers was analyzed in a total of 45 Iranian families. Mutation analysis of the *TMC1* gene in a DFNB7/11 family, led to the identification of a previously reported mutation in this gene [16]. Ethnicity study of these two families (our new case with the one which had already been characterized by Hilgert, Alasti et al.) showed that although these families are not geographically close to one another, both of them belong to related ethnic populations.

2. Methods

2.1. Subjects

Blood samples were collected from 54 unrelated Iranian families having 159 affected individuals. Disease segregation followed autosomal recessive pattern for all these non-syndromic pre-lingual hearing loss families. The majority of samples were obtained from deaf families referred to Kawsar Human Genetics Research Center, Genetic Counseling Centers of the Welfare Organization of Iran and Iranian Society for Deaf Families (Tehran, Iran). Bilateral severe to profound sensorineural hearing loss was determined by pure-tone audiometry in all patients. The average number of affected persons in each family was 3, born to consanguineous parents or parents, both from a small village, with high probability of having common ancestor. Adult participants and parents of investigated children completed a questionnaire and signed an informed consent form. Pedigree was drawn for each family based on medical and developmental history. Syndromic form of hearing loss and defects caused by environmental factors such as infections and otoxic drugs were tried to be excluded by physical examination, investigation of medical history of the patients or interviewing parents. The family with affected individuals having the *TMC1* mutation had come from a small village in Lorestan province in the west of Iran. Since it is a small village, its name has not been mentioned due to the ethical considerations related to the privacy of the patients.

This study was approved by the Ethic Committee of Kawsar Human Genetics Research Center.

2.2. Linkage analysis and genotyping

Genomic DNA was extracted from peripheral blood samples of all participants using salting out method [19]. Sequencing of *GJB2* and all 24 coding and non-coding exons of the *TMC1* gene including the exon-intron boundaries were done using Big dye v3.1 terminator kit (Life Technologies, USA, LT). Sequences of primers are not shown but are available upon request. Short tandem repeat (STR) markers were selected from Mapviewer (http://www.ncbi.nlm.nih.gov/projects/mapview) or by using Tandem Repeat Finder (TRF) and Sequence-based Estimation of Repeat Variability (SERV) flanking the DFNB7/11 locus [20,21]. Two-point and multi-point parametric LOD scores were calculated, respectively, by Superlink version 1.6 and Simwalk version 2.91 options of Easylinkage plus version 5.05 software [22]. Since except one STR marker (D9S1876), the other three markers (TMC-SU1, TMC-S13 and TMC-S14) had not been used in the genetic maps, to calculate LOD scores, we manually inserted the related information of the markers, estimated based on the physical map, in the corresponding map file.

Polymerase chain reaction (PCR) amplification of microsatellite markers was performed using fluorescently labeled primers and fragment analysis was done on an ABI 3130 Genetic Analyzer (LT). Genotypes were ascertained using Gene Mapper ID-X V.1 software (LT).

Human Splicing Finder (HSF) software was used to predict the effect of variations on splicing signals [23]. Amplification refractory mutation system (ARMS) was performed for normal and mutant alleles on genomic DNA samples from 300 normal individuals mainly from the same ethnicity or village [24].

2.3. In silico analysis of RNA folding

RNA secondary structure prediction was performed by mfold web server (version 3.5, online: http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) [25].

3. Results

All members of 45 GJB2 & GJB6-negative families were genotyped using four microsatellite markers linked to DFNB7/11 locus. With the aim of finding new markers for this locus and estimating their heterozygosity in the studied population, three markers were selected by Tandem Repeat Finder (TRF) and Sequence-based Estimation of Repeat Variability (SERV) [20,21]. Markers used in this study were named TMC-SU1 at the upstream of the *TMC1* gene and the other three were intragenic markers D9S1876, TMC-SI3 and TMC-SI4. The heterozygosity of these markers and their positions relative to the gene are shown in Table 1.

From 45 families, one (coded Irn-Deaf-119) showed homozygosity for all four STR markers in all 4 affected members of the family. This indicated high probability of disease segregation with DFNB7/11 locus (Fig. 1). Two-point and multi-point maximum LOD scores were obtained to be 2.9 and 3.1, respectively, confirming

Table 1
Heterozygosity and position of selected markers for the DFNB7/11 locus.

Marker name	Heterozygosity (%)	PCR product size (bp)	Chromosome band	Position (Ref: NC_000009.11)	Distance from TMC1 Gene (bp)
TMC-SU1	40	155–181	9q21.13	75101116–75101235	3.5482
TMC-SI2 (D9S1876)	76	257–279	9q21.13	75232710–75232941	Intragenic
TMC-SI3	65.28	229–245	9q21.13	75291971–75292166	Intragenic
TMC-SI4	50	344–364	9q21.13	75315553–75315862	Intragenic

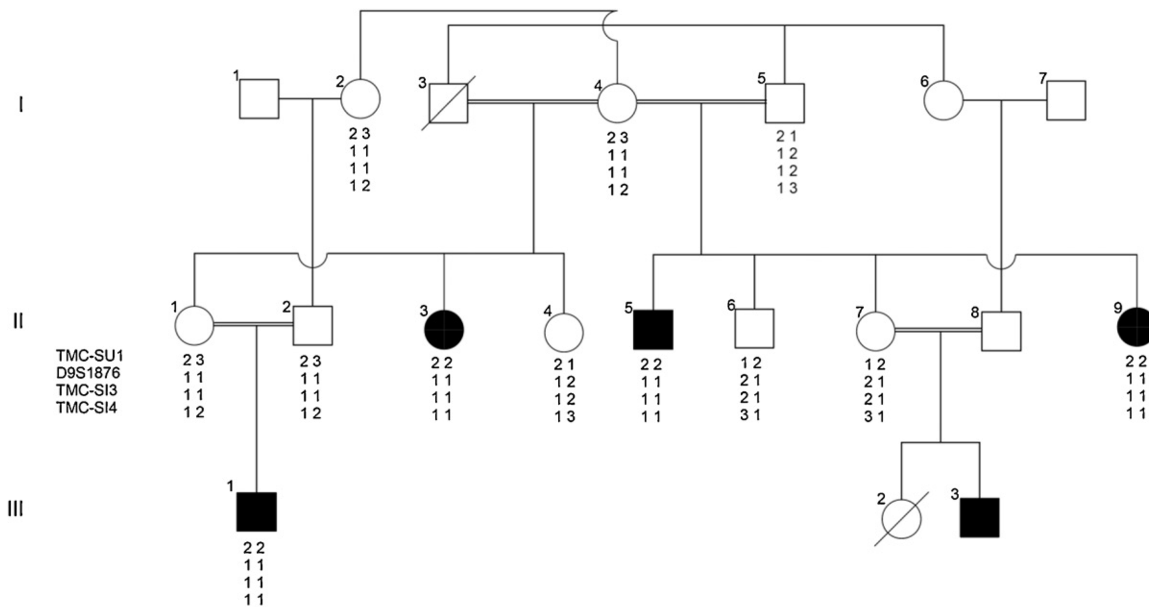

Fig. 1. Irn-Deaf-119 pedigree. Haplotypes for DFNB7/11 microsatellite markers are shown.

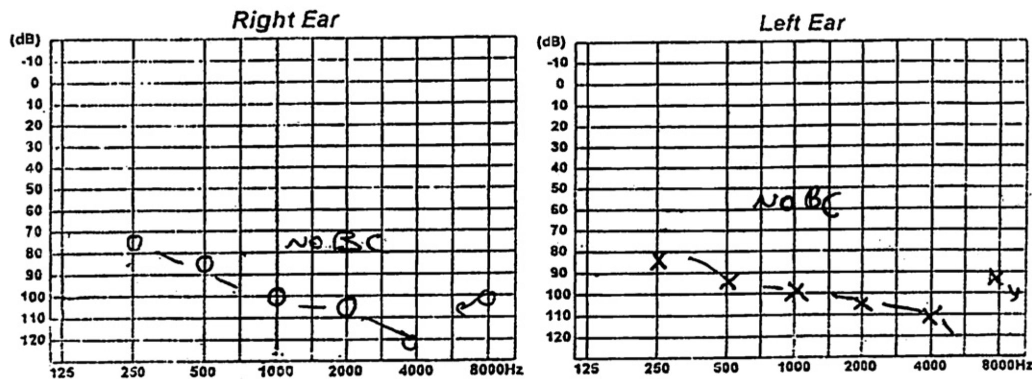

Fig. 2. Audiogram of III-1 (at age 4) in Irn-Deaf-119 pedigree. The *X* and *Y* axes represent frequency (Hz) and hearing threshold (dB), respectively.

genetic linkage. All affected subjects of this family have congenital profound non-progressive sensorineural hearing loss. An audiogram for one of them is shown in Fig. 2.

In order to confirm the result of STR linkage with the disease gene we sequenced all the 24 exons and their boundaries of the *TMC1* gene. This led to finding of several variations in different exons (Table 2).

Another observation was that a 270 bp insertion was seen in the sequenced fragment including exon 5. Further analysis confirmed that this elongation has occurred during DNA sequencing as a result of secondary structure formation and extension which was suggested to be an artifact of sequencing [26].

The result of exon 11–12 sequencing showed an unreported C > T synonymous variation at position c.1167 (NM_138691.2). The mutation analysis by means of the Human Splicing Finder (HSF) software showed that this variation, at 16 bp upstream of the exon 11–12 junction, cannot have a significant effect on splicing signals [23].

Sequencing of exon 3 led to finding a 314-bp insertion c.-196+153_-196+154ins (NM_138691.2) in intron 3 of this gene. This 314-bp inserted segment has already been reported and can be viewed in the NCBI/nucleotide with accession number NW_001839221.1 reference sequence (http://www.ncbi.nlm.nih.gov/nucleotide/157696782). Three affected children (II-3, II-5 & II-9) and their mother (I-4) as well as one of the healthy children (II-1) were homozygous for this insertion (Fig. 1). This indicates that this insertion, most probably, is not a pathogenic mutation.

Further analysis of sequencing result of the non-coding exon 3 in affected individuals led to finding the previously reported c.-258A>C (NM_138691.2) mutation [16] (Fig. 3). Four affected

Table 2
TMC1 gene variations in family Irn-Deaf-119.

Exon/Intron	Nucleotide variation (NM_138691.2)	Reference SNP (rs) number
Exon 1	c.-468G>A	rs7022441 SNP
Intron 2	c.-305-73G>A	rs60049601 SNP
Intron 2	c.-305-67G>A	rs10217204 SNP
Exon 3	c.-258A>C	–
Exon 3	c.-219A>G	rs7026304 SNP
Intron 3	c.-196+153−196+154ins(314bp)	–
Exon 6	c.45C>T	rs2589615 SNP
Exon 11	c.1167C>T	–
Intron 13	c.884+334T>C	rs2501913 SNP
Intron 17	c.1566+169G>C	rs72733079 SNP
Intron 17	c.1567−67T>A	rs7860172 SNP
Exon 19	c.1713C>T	rs34532421 SNP
Intron 19	c.1763+123G>A	rs56349532 SNP

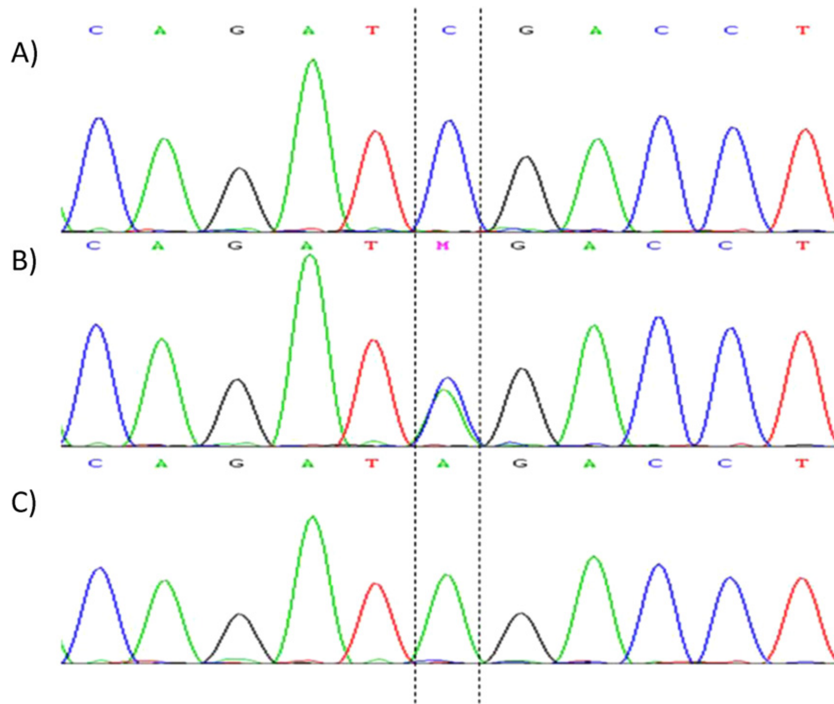

Fig. 3. Sequence chromatograms of the c.-258A>C variant. Partial TMC1 sequences are shown for an affected (II-5) (A), unaffected carrier (II-1) member of the Irn-Deaf-119 family (B) and also for a control person (C). Site of the variation is shown between two dashed lines.

members of the Irn-Deaf-119 family showed homozygosity for this mutation. Homozygosity for this mutation was not found in any of the unaffected members of this family. As another peculiar sequencing result, individual I-5 (Fig. 1) showed an A/A genotype while he had two affected children with C/C genotype for the same position. Repeating the sequencing led to the same result but STR analysis confirmed paternity. Heterozygosity of father for the insertion in intron 3 and preferential amplification of smaller segment (allelic drop-out) is the most probable explanation for this observation. The analysis of this mutation by ARMS-PCR method showed heterozygosity for this mutation in I-5 confirming the above hypothesis.

The Mfold web server, which was used to investigate the c.-258A>C mutation effect on secondary structure of the *TMC1* mRNA, showed that this mutation can change the mRNA folding. The TMC1 mRNA from nucleotide 131 to 750 was modeled with the mfold program. With regard to finding another variation in this exon (c.-219A>G, Table 2), we analyzed the results of two situations: the change c.-258A>C, without c.-219A>G variation and the change c.-258A>C in combination with the c.-219A>G variation (the results have not shown).The c.-258A>C variation was predicted to alter mRNA folding from a three-branch, stem–loop structure to a four-branch structure in both situations.

To check the presence of the c.-258A>C mutation in homozygous form in normal controls, a total of 300 ethnically matched controls, including 28 individuals from family Irn-Deaf-119 and 76 other individuals from Lorestan province, where the family was from, were investigated. Genotypes of studied individuals are shown in Fig. 4. The c.-258A>C mutation analysis by ARMS-PCR was shown that homozygous form of this mutation was absent in the 300 control individuals in Lorestan province.

During our investigation on molecular basis of DFNB in Iran, another family (G9) was referred to our center. This family also had the same mutation. Mutation in this family had already been reported by Hilgert et al. [16]. The G9 family was from Looshan in Gilan province in the north of Iran. To determine whether these two families are related or share similar ancestral origin, we interviewed the families and inquired about their ethnicity. Results showed that these two families belong to different branches of Lak people who mostly live in the Lorestan province. Our molecular investigation also confirmed the founder effect. In view of the hypothesis, all of the four linked STR markers in the affected

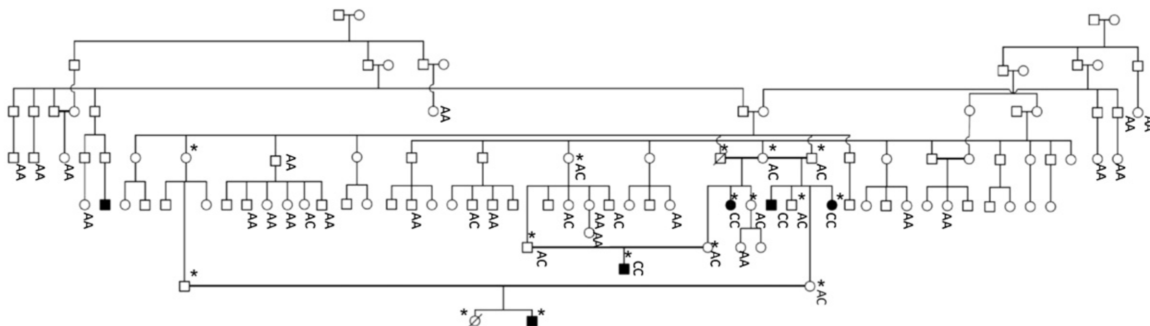

Fig. 4. Enlarged Irn-Deaf-119 pedigree. The genotypes of 38 individuals of this family are shown. The members of the family that are shown in Fig. 1 are marked by star symbol.

individuals from the two apparently distinct families were the same confirming related ethnicity or founder effect.

4. Discussion

Iran has high frequency (%38.6) of consanguineous marriage [9]. The consanguineous marriage has been identified as a risk factor for different autosomal recessive disorders because it increases frequency of homozygosity for the deleterious recessive alleles [27]. Previous studies have shown that populations with high frequency of consanguineous marriage and large families are appropriate for studies on heterogeneous disorders. These kinds of studies can provide useful knowledge about etiology of studied traits which in turn can be helpful for genetic counseling for example in hearing loss in this country.

Genetic studies on hearing loss can also help to increase our knowledge about molecular mechanism of hearing and hearing loss. In spite of importance of genetic studies on hearing loss in Iran, these studies are limited and knowledge about frequency of different mutations in deafness genes in this country is inadequate [28].

In this study linkage between hearing loss and the DFNB7/11 was investigated in 45 Iranian DFNB1-negative deaf families. Co-segregation was found in one of the investigated families. The initial sequence analysis of all 24 exons of the *TMC1* gene revealed that exons 3, 5 and 11 need more analysis. The existence of pathogenic mutation was rejected in exon 11 because mutation analysis by using the Human Splicing Finder (HSF) showed that synonymous variation C>T at position c.1167 cannot have a significant effect on splicing signals [23].

Our study showed that the c.-258A>C mutation in exon 3 of the *TMC1* gene is most probably the cause of hearing loss in the family Irn-Deaf-119. This mutation can change the *TMC1* mRNA folding which may have an effect on the translation of this gene. The c.-258A>C mutation was not found in 300 ethnically matched controls. The segregation of this mutation with the hearing loss in another Iranian deaf family (G9) and its absence in 100 Iranian and 100 Belgian control samples has also been reported by Hilgert et al. [16]. These findings strongly support the claim that c.-258A>C mutation is the only pathogenic mutation in these two families. Since the mutation resides in the non-coding exon 3, it is possible that it affects regulatory domain. An alternative explanation is that c.-258A>C defines a TMC1 haplotype that harbors a pathogenic mutation. Therefore functional studies will need to be performed to determine whether this mutation is causative. Hilgert et al. (2008) previously reported the G9 family that lived in Looshan, Gilan province in the north of Iran while the investigated family Irn-Deaf-119 in this study lives in a small village in Lorestan province in the western Iran. Although these families are from two geographically distant regions, both of them belong to Lak ethnic population. The Laks are one of the Iranian tribes that speak Laki, a Kurdish dialect of Northwestern Iranian language [29]. Because of linguistic and cultural proximity of the Laks to both Lurs and Kurds, some references classify this tribe as a branch of Lur while others categorize it as a part of Kurd population. The majority of Laks live in the western provinces of Iran such as Ilam and Lorestan. Some have migrated from these provinces to reside elsewhere in the country. Looshan is a city in north of Iran that inhabits some Laks [30–32].

Finding of a very rare mutation in these two Lak families residing in two different places separated by Zagros and Alborz Chain Mountains as a geographical barrier can be explained by a founder effect and separation due to migration [33]. The result of haplotype analysis in these two families confirmed this hypothesis. This study showed that the ethnicity may be more useful than geographical location to design research strategy for determining which genes should be considered when a heterogeneous disorder is under investigation.

Previous studies have shown that the *TMC1* mutations are a prevalent cause of *GJB2* mutation-negative ARNSHL in different populations such as Pakistani, Indian, Tunisian and Turkish populations with the frequency of 3–6% [17,18,34–36]. The studies on the *TMC1* mutations in Iran also have shown that mutations in this gene can be one of the most frequent causes of ARNSHL in the Iranian population [15].

From 45 *GJB2* mutation-negative ARNSHL Iranian families in this study, one family showed co-segregating with the DFNB7/11 markers and a mutation in the *TMC1* gene. Therefore the prevalence of hearing loss due to the *TMC1* mutation in this investigated population was estimated to be about 2.2%. In Tabatabaiefar et al.'s study, one out of 31 Iranian DFNB1-negative families (3.2%) was linked to the DFNB7/11 [37]. Another study performed by Babanejad et al. led to finding of 4 families linked to the DFNB7/11 out of 144 Iranian families (2.8%) negative for *GJB2* & *GJB6* mutations [38]. Finding 6 DFNB7/11 families out of 220 Iranian DFNB1-negative families investigated in these three studies shows that the prevalence of hearing loss due to the *TMC1* mutation among Iranian DFNB1-negative families is about 2.7%.

More studies on genetic causes of hearing loss with larger sample sizes are required to determine types of deafness gene mutations and their frequencies in Iran.

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