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Mutation Screening of Exons 7 and 13 of the *TMC1* Gene in Autosomal Recessive Non-syndromic Hearing Loss (ARNSHL) in Iran

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Abstract

Background: Non-syndromic hearing loss (NSHL) is the most common birth defect and occurs in approximately 1/1,000 newborns. NSHL is a heterogeneous trait and can arise due to both genetic and environmental factors. Mutations of the *transmembrane channel-like 1 (TMC1)* gene cause non-syndromic deafness in humans and mice.
Objectives: The aim of the present study was to investigate the association of *TMC1* gene mutations of the locus DFNB7/11 in exons 7 and 13 in a cohort of 100 patients with hearing loss in Iran using polymerase chain reaction–single-stranded conformation polymorphism (PCR-SSCP), heteroduplex analysis (HA), and DNA sequencing.
Patients and Methods: In this experimental study, the blood samples of 100 NSHL patients were collected from 10 provinces in Iran. These patients had a mean age of 16.5 ± 2.01 years and 74.15% of their parents had consanguinity. DNA was extracted from specimens and mutations of exons 7 and 13 of the *TMC1* gene were investigated using PCR-SSCP. All samples were checked via HA reaction and suspected specimens with shift bands were subjected to DNA sequencing for investigation of any gene variation.
Results: In this study, no mutation was found in the two exons of *TMC1* gene. It was concluded from these results that mutations of the *TMC1* gene's special exons 7 and 13 have a low contribution in patients and are not great of clinical importance in these Iranian provinces.
Conclusions: More studies are needed to investigate the relationship between other parts of this gene with hearing loss in different populations through the country. More research could clarify the role of this gene and its relation with deafness and provide essential information for the prevention and management of auditory disorders caused by genetic factors in the Iranian population.

Keywords: Hearing loss, Heteroduplex Analysis, Iran, PCR-SSCP, *TMC1* Gene

1. Background

One disability that can sufficient to interfere with activities of daily living is non-syndromic hearing loss (NSHL) (1, 2). People with even mild NSHL have problems hearing speech when there is background noise and identifying the sounds sources (3, 4). NSHL is the most common sensory deficit in humans, with an incidence of about 1 in 1,000 newborns. The prevalence increases during childhood, reaching a rate of 2.7 per 1,000 children before the age of 5 years and 3.5 per 1,000 adolescents. NSHL is a major public health concern in developing countries. Two thirds of people with NSHL worldwide live in developing countries (5, 6).

The *transmembrane channel-like 1 (TMC1)* gene is considered a member of a gene family predicted to encode transmembrane proteins (7, 8). Mutations in the *TMC1* gene have been associated with profound prelingual deafness (DFNB7/B11) and progressive postlingual hearing loss (DFNA36); thy have been reported in different populations (9). The DFNA36 and DFNB7/B11 loci are located on chromosome 9q13 - q21 (7). *TMC1* and *TMC2* are members of a gene family predicted to encode transmembrane proteins and are located on p13 of chromosome 20. The *TMC1* gene encodes a sodium sensor and may function as ion transport channel or pump. *TMC1* mRNA is specifically expressed in neurosensory hair cells of the inner ear, and it is required for normal function of cochlear hair cells, although the molecular and cellular functions of the *TMC1* protein are unknown (8).

2. Objectives

Since no report has yet determined the frequency of *TMC1* gene mutations in the Iranian population, the present study was performed to screen and identify the mutations of this gene associated with NSHL using polymerase chain reaction–single-stranded conformation polymorphism (PCR-SSCP) and heteroduplex analysis (HA).

3. Patients and Methods

3.1. Sampling

This experimental study was conducted at the cellular and molecular research center of Shahrekord University of Medical Sciences from February 2011 to January 2012. In the present study, we investigated the mutations of the *TMC1* gene, locus DFNB7/11, in a cohort of 100 patients with NSHL in Iran. The 890 blood samples of families with Iranian origin was obtained in ethylene diamine tetra-acetic acid (EDTA)-containing tubes (Sarstedt) from 10 provinces of Iran, namely Semnan, Sistan & Baloochestan, Fars, Khozestan, Kohgilooye Va Boyer Ahmad, Kordestan, Chaharmahal & Bakhtiari, Booshehr, Golestan, and Gilan. Finally, 100 patients (one proband from each family) were selected (Table 1). NSHL informational questionnaires were filled out for all families. In previous work, these patients had no mutations in the *cx26* gene (10). The blood samples were stored at -20°C until further processing. Known environmental risk factors such as head trauma and use of ototoxic drugs could affect the study, so families with the possibility of exposure to these factors were excluded from the research.

3.2. DNA Extraction

Total genomic DNA was extracted from peripheral blood samples of patients using the phenol and chloroform standard procedure (11). The quality of extracted genomic DNA was quantified by Nano-Drop 1000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA) at a wavelength of 260/280 nm according to the method described by Sambrook and Russell (12)

3.3. Gene Amplification

For gene amplification of exons 7 and 13 of the *TMC1* gene by PCR, two sets of overlapping primers were designed due to their length using the Gene Runner software version 3.0 (Hastings Software, Inc., Hastings, NY), and primer sequences were blasted in the National Center for Biotechnology Information's (NCBI's) GenBank. The details of the designed primers are shown in Table 2.

Table 1. Information on Deaf Patients and Their Families in Each Province

Province	Sample Number	Age[a]	Male[b]	Female[b]	Marriage[b]	Number of Families With More Than one Deaf Patient
Sistan & Baloochestan	8	16 ± 2.5	50	50	62.5	5
Kordestan	6	13 ± 1.6	17	83	83	4
Golestan	10	16 ± 2.2	60	40	80	7
Gilan	6	12 ± 1.8	66	34	66	4
Chaharmahal & Bakhtiari	35	21 ± 2.6	48	52	82	19
Semnan	11	19 ± 2.3	37	63	54	7
Booshehr	6	14 ± 1.5	66	34	66	3
Fars	7	19 ± 1.7	71	29	85	4
Kohgilooye & Boyer Ahmad	6	15 ± 1.8	17	83	83	4
Khozestan	5	20 ± 2.1	60	40	80	4
Total	100	16.5 ± 2.01	49.2	50.8	74.15	61

[a]Values are expressed as mean ± SD.
[b]Values are expressed as percentages.

Table 2. The Details of the PCR Primers Used for Gene Amplification

Exon Number/Primer Names	Primer Sequence 5′ → 3′	Product Size, bp
7		187
TMC1-F7	AGGTGAAGAGGAAGAGGAG	
TMC1-R7	ACTTACGCTCCTCTCTTTAG	
13		250
TMC1-F13	GCTCTTCACGACAACTGCTAA	
TMC1-R13	TCCCTCCATTTGATTCCAG	
7		187
TMC1-MF7	AGGTGAAGAGGAAGA*GAG	
TMC1-R7	ACTTACGCTCCTCTCTTTAG	
13		250
TMC1-MF13	GCTCTTCACGACAACTG*TAA	
TMC1-R13	TCCCTCCATTTGATTCCAG	

*Mutant primers created by site-directed mutagenesis (SDM) as positive control, TMC1-M (TMC1 mutant primer).

Site-directed mutagenesis (SDM) after gene amplification using the designed specific primers with changes in one nucleotide was used to generate positive control samples. Standard PCR optimization was carried out in a total volume of 50 μL reaction in 0.5 mL tubes for each amplicon in a gradient palm cycler (Corbett Research, Australia). The PCR reaction consisted of 0.2 pM of each primer, 2.5 mM $MgCl_2$, 200 μM dNTPs mix, 5 μM of 10 X PCR buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 1 unit of Taq DNA polymerase (all Fermentas, Germany), and 100 ng of template DNA. PCR temperature programs involved an initial denaturation at 94°C for 5 minutes followed by 32 cycles consisting of 50 seconds of denaturation at 94°C, 50 seconds of annealing at 57.5°C (exon 7) or 60°C (exon 13), 40 seconds of extension at 72°C, and a final extension at 72°C for 5 minutes. The PCR amplification products (2 μL/lane) were loaded on 8% polyacrylamide gel (29:1 acrylamide: bis-acrylamide) electrophoresis (PAGE) in 1 X TBE buffer (10.8 g of Tris-base 89 mM, 5.5 g of boric acid 2 mM, EDTA (pH = 8.0) 4 mL of 0.5 M EDTA (pH = 8.0), combined all components in sufficient H_2O and were stirred to dissolve) at 85 V for 30 minutes, and the gels were stained using the silver nitrate staining method.

3.4. SSCP Analysis

For SSCP, microtubes containing PCR products were mixed with an equal volume of formamide loading dye, heated to 96°C for 15 minutes, and chilled on ice for 5 minutes before loading on the polyacrylamide gel. The electrophoresis tank was filled by TBE buffer 0.6 X, and TBE 1 X was used in the gel; electrophoresis was performed with 2.5 - 5% glycerol at 20 W at 4°C for 6 - 8 hours. The bands were then visualized using standard silver nitrate staining.

3.5. Heteroduplex Analysis (HA)

HA was used to increase the sensitivity of SSCP. First, 3 μL of EDTA (0.5 M) was added to 2 μL of PCR products. Then, the PCR products were heated at 95°C for 3 minutes and slowly cooled to 37°C over 40 minutes. After mixing with 6 X triple dye loading buffer at a volume ratio of 1:5, the PCR products were loaded on PAGE with 10% urea and electrophoresis were performed at 320 V at 10 - 12°C for 6 - 8 hours (Table 3). Finally, the heteroduplex fragments were visualized using standard silver nitrate staining.

3.6. Sequencing of Amplified Products

The samples containing shift bands on the SSCP gel and after HA were subjected to direct DNA sequencing of exons 7 and 13 of the *TMC1* gene in an ABI 3730xl automated sequencer (Applied Biosystems) by Macrogen Inc. (Seoul, Korea) using the Sanger sequencing method for the final confirmation.

3.7. Ethical Approval

For this study, the regional research ethical committee of Shahrekord University of Medical Sciences (Grant number 91-3-2, January 2011) approved the protocol and informed consent forms. Informed consent was obtained from all hearing loss patients before enrollment in the study based on the declaration of Helsinki (DoH).

3.8. Statistical Analysis

All data were collected in the statistical program for the social sciences software (SPSS, Inc., Chicago, IL, USA) version 20. The mean difference between groups was calculated using a T test. In this study, a P value of ≤ 0.05 was considered statistically significant.

4. Results

In this study, blood samples of 100 patients with hearing loss (mean age 16.5 ± 2.01 years, 49.2% men and 50.8% women, 74.15% married) were collected from 10 provinces in Iran. The medical history and pedigree information of the patients were obtained via questionnaire.

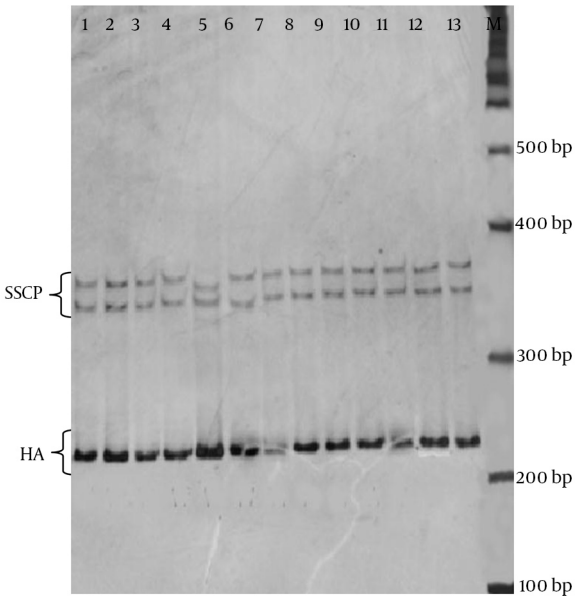
The *Connexin 26* gene is responsible for a large proportion of deafness (about 14.6%), and these patients had no mutations in this gene (10, 13). Extracted DNA with a 260/280 nm absorbance ratio of 1.8 - 2 was subjected to gene amplification. Exons 7 and 13 of the *TMC1* gene were amplified using the PCR technique with specific and mutated oligonucleotide primers. After PAGE, fragments with a length of 187 and 250 bp were revealed for exons 7 and 13, respectively, of the *TMC1* gene.

PCR products were used for SSCP and HA for investigation of mutations in the sequences. The samples with shift bands after HA were used for direct DNA sequencing. Neither PCR-SSCP nor HA showed mutations in exons 7 and 13 of the *TMC1* gene (Figures 1 and 2).

Table 3. SSCP conditions for Exons 7 and 13 of *TMC1* Gene

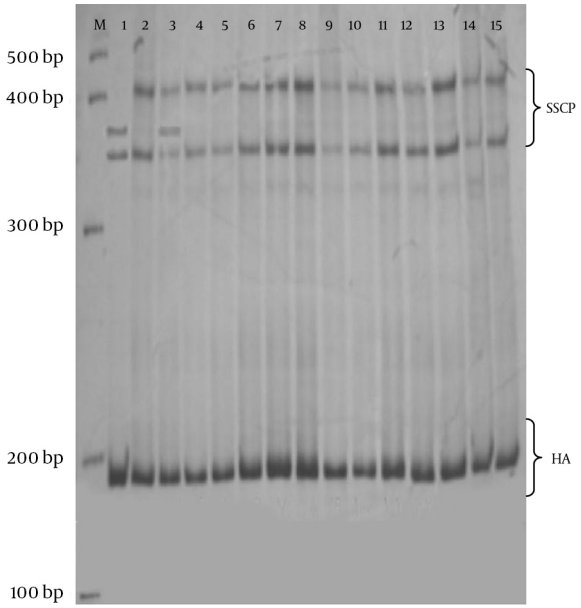
Exon	Gel Density, %	Time, h	Milliampere (MA)	Voltage, V	Temperature, °C
Exon 7	10	6	30	320	12
Exon 13	12	7	32	330	10

Figure 1. SSCP Bands and Heteroduplex Analysis of Exon 13 and Mutant Sample on PAGE



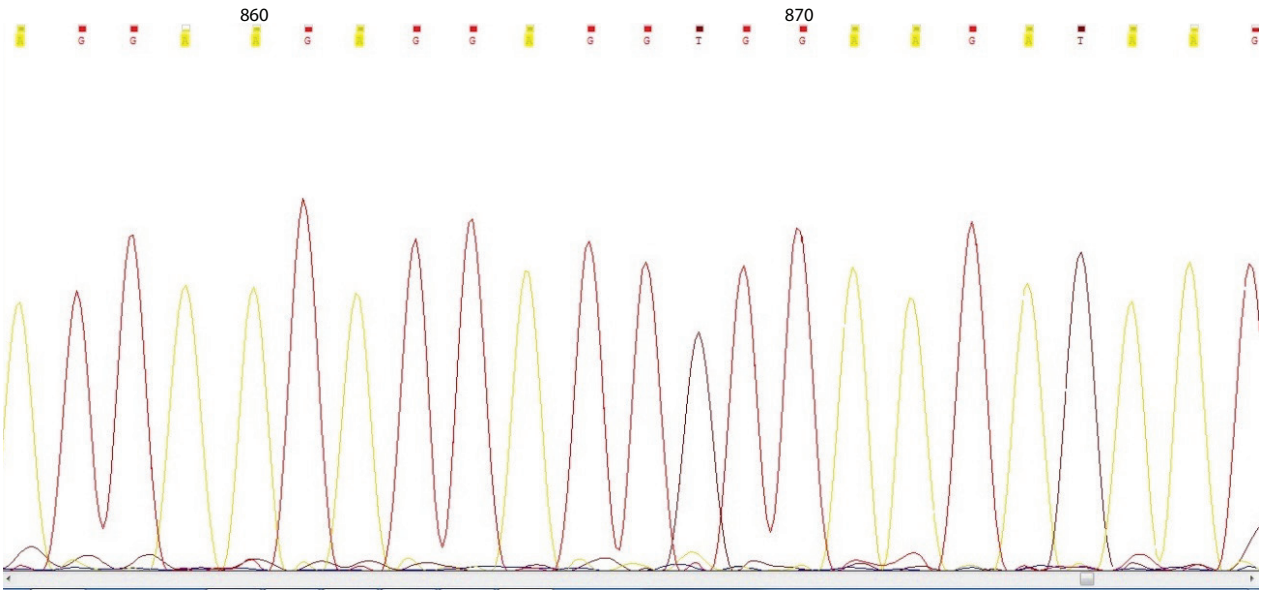
Line M is a 100 bp DNA ladder (Fermentas, Germany), line 5 is a sample amplified by mutant primers with typical shift (positive control), and lines 2 - 4 and 6 - 13 are deafness patients samples. All specimens have the same template bands without shifts in the SSCP bands and after HA.

Figure 2. SSCP and Heteroduplex Analysis of Exon 7 and the Mutant Sample via PAGE



Line M is a 100 bp DNA ladder (Fermentas, Germany), line 1 is positive control, line 3 is a suspected sample containing different bands compared to other samples, and lines 2 and 4 - 15 are samples from deaf patients. In the present study, to increase the accuracy of the SSCP reaction, HA and mutant control (positive control created by SDM) were performed. Only a number of suspected fragments in exon 7 showed a different banding pattern, but after sequencing, the mutations were not confirmed (Figure 3). This different pattern may have been the result of experimental error.

Figure 3. A Diagram of the DNA Sequencing of Exon 7 in the Suspected Sample



Mutations were not confirmed.

5. Discussion

In various human populations, different dominant (e.g., *COCH*, *DFNA5*, and *POU4F3*) and recessive genes (e.g., *GJB2* and *SLC26A4*) have been reported as the cause of hearing loss worldwide (7, 9, 14-16). The *TMC1* gene is an autosomal recessive gene and a common cause of hearing loss in many countries, such as India, Pakistan, Algeria, Iraq, Lebanon, Sudan, Tunisia, and Turkey (15, 17-19). The prevalence of non-syndromic hearing impairment due to *TMC1* in the Pakistani population is 4.4%, and one study has indicated that the *TMC1* protein might have an important function in the K^+ channels of the inner hair cells (16). Kurima et al. (2002) detected a 1.6 kb genomic deletion encompassing exon 14 of *TMC1* in a recessive deafness mouse mutant, which lacks auditory responses and has hair-cell degeneration (7).

In the present study, the association of exons 7 and 13 mutations of *TMC1* gene locus DFNB7/11 in deaf patients was investigated in 10 Iranian provinces. After gene amplification, neither SSCP nor HA showed mutations in exons 7 and 13 of this gene related to hearing loss in these patients. Only a suspected sample in exon 7 had a different banding pattern, but after sequencing, mutations in this exon were not identified. The strength of this study was the number of samples, as 890 blood samples were provided; however, due to a lack of financial resources, we chose two exons of this gene. Via the molecular analysis of the *TMC1* gene in Korean patients, one study showed that this gene was not the cause of NSHL in the Korean population (20). In our study, as in the research in Korea, a relation between the *TMC1* gene and NSHL in the Iranian population was not observed. Kalay et al. (2005) investigated four novel *TMC1* (DFNB7/DFNB11) mutations in Turkish patients with congenital autosomal recessive NSHL (ARNSHL), and they indicated that *TMC1* mutations account for at least 6% (4/65) of ARNSHL cases in *GJB2*-negative Turkish families from the northeast and east of Turkey; however, in our study, mutation in exons 7 and 13 of *TMC1* gene was not observed (14). In another study in Sudan, it was shown that *TMC1* mutations contribute to deafness; this confirmed and extended previous reports on the role of *TMC1* in recessive non-syndromic deafness (20, 21). Meanwhile, in the present study on the Iranian population, no significant relation between exon 7 and 13 mutations of the *TMC1* gene were observed.

In Pakistan, Kitajiri et al. (2007) identified 10 new families segregating DFNB7/B11 deafness and *TMC1* mutations, including three novel alleles; moreover, they identified a C.100C > T mutation in exon 7 (16). Furthermore, Hilgert et al. (2008) identified four new mutations in the *TMC1* gene and suggested an additional deafness gene at loci DFNA36 and DFNB7/11 in 51 familial of Turkish patients with autosomal recessive hearing loss; these results implied the presence of mutations outside the coding region of this gene, or alternatively, at least one additional deafness-causing gene in this region (9). Another study in Turkey reported five novel mutations in the *TMC1* gene related to ARNSHL (19). In comparison with the other populations discussed, in a review study in Iran by Mahdieh et al. the frequencies and distributions of NSHL included *GJB2*, *GJB6* (large deletion), TECTA, SLC26A4, and *PEJVK* mutations. The researchers indicated that mutations in *GJB2*, *SLC26*, *TECTA*, and *PJVK* genes have an important role in deafness in Iran, and a screening test should be generated for better intervention and diagnostic programs (22). The study of Hildebrand et al. in Iranian families showed that two families are related to locus DFNB7/11, and one of them had a c.776 + 1G > A mutation in exon 7 (23). In 2014, Lin et al. identified novel compound heterozygous mutant alleles of *TMC1* responsible for ARNSHL in a Tibetan Chinese family (24); meanwhile, in the present work, we did not observe a relation with mutations of exons 7 and 13 of the *TMC1* gene in Iranian NSHL.

The findings of the current study indicate that mutations in exons 7 and 13 of *TMC1* gene are not related to hearing loss in the Iranian population. Therefore, the *TMC1* gene may not related to NSHL, but further studies investigating related mutations in other parts of this gene in Iranian population are necessary and could help in the genetic counseling of patients and design of practical strategies for the management of auditory disorder.

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Footnotes

Authors' Contribution: All authors contributed in all parts of the research. Negar Moradipour: samples collection and contribute in molecular tests compeletly; Payam Ghasemi-Dehkordi: data collection and writing the manuscript; Shahrbanuo Parchami-Barjui, Marziyeh Abolhasani, Ahmad Rashki, and Morteza Hashemzadeh-Chaleshtori: contributed to performing of molecular tests and analyzing of data.

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