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A Novel Pathologic Variant in *OTOF* in an Iranian Family Segregating Hereditary Hearing Loss

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Abstract

Objective. Hearing loss (HL) is the most common sensory-neural defect and the most heterogeneous trait in humans, with the involvement of >100 genes, which make a molecular diagnosis problematic. Next-generation sequencing (NGS) is a new strategy that can overcome this problem.

Study Design. Descriptive experimental study.

Setting. Diagnostic laboratory.

Subjects and Methods. A comprehensive family history was obtained, and clinical evaluations and pedigree analysis were performed in a family with multiple individuals with HL. As the first tier, *GJB2* was sequenced, and genetic linkage analysis of DFNB1A/B was performed to rule out the most common cause of the disease. Targeted NGS was used to unravel the molecular etiology of the disease in the HL-associated genes in the proband. Two homozygous variants remained in *OTOF* after proper filtration. Cosegregation and in silico analysis were done. Preimplantation genetic diagnosis (PGD) was accomplished via linkage analysis and direct sequencing of the pathogenic variant.

Results. Clinical evaluations suggested autosomal recessive nonsyndromic HL. Two homozygous variants, c.367G>A (p.Gly123Ser) and c.1392+1G>A, were identified in *cis* status. c.1392+1G>A met the criteria for being pathogenic according to the variant interpretation guideline of the American College of Medical Genetics and Genomics. PGD was successfully performed to prevent the recurrence of the disease in the related family.

Conclusion. A novel *OTOF* mutation causing HL was identified. Here, we report the effectiveness of the combined application of targeted NGS and PGD in diagnosis and prevention of hereditary HL.

Keywords

hearing loss, Iran, next-generation sequencing, *OTOF*, pathogenic variant, preimplantation genetic diagnosis

Hearing loss (HL) is the most common human sensory-neural disorder worldwide and the most heterogeneous human trait (http://hereditaryhearingloss.org/). Per every 1000 infants, 1 or 2 are born with prelingual HL.[1] The prevalence rises to 2.7 and 3.5 per 1000 during childhood and adolescence, respectively.[2,3] Severe HL in childhood prevents proper linguistic communication and social engagement, which affects life quality negatively.[4] This trait can be divided into 2 categories: syndromic and nonsyndromic forms. Syndromic HL (SHL) is associated with signs and symptoms affecting other parts of the body, while nonsyndromic HL (NSHL) has no associated clinical symptoms. In addition, 80% of the latter cases show autosomal recessive NSHL (ARNSHL).[5] So far, mutations in >60 genes with different frequencies have been reported to cause ARNSHL in various parts of the world (http://hereditaryhearingloss.org/). *GJB2* mutations at the DFNB1A locus represent about half of the cases in some populations. The contribution of the DFNB1A locus to the molecular etiology of the disease is variable, from 38.3% in

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the north of Iran to near zero in the south. Mutations in the noncoding region of the *GJB2* gene and large deletions of the *GJB6* gene at DFNB1B account for important causes of the disease in some populations. However, there is no report of DFNB1B mutations among Iranian patients with ARNSHL.[6,7] Iran, a country with various ethnic groups and a high prevalence of consanguineous marriage,[8] is a rich source for studying recessive inherited disorders. In Iran, HL is the second-most common disability, after intellectual disability.[9]

Next-generation sequencing (NGS), as a high-throughput technique, has the ability to uncover single-nucleotide variants, small indels, and, to some extent, copy number variants in genetically heterogeneous disorders.[10] Involvement of numerous genes, excessively large genes, and a lack of specific phenotypic features in the majority of cases necessitate the application of this technology for routine molecular diagnosis of ARNSHL.[11,12] Preimplantation genetic diagnosis (PGD) is an efficient method that helps prevent recurrence of single-gene disorders with known molecular etiology.[13,14]

Here, we used a targeted NGS panel for molecular diagnosis of ARNSHL in an Iranian pedigree comprising multiple individuals with HL, and 2 simultaneous *OTOF* variants were identified, 1 of which was a novel pathogenic splice site variant. The results were utilized in a successful practice of PGD in a couple with 1 affected child in this kindred.

Methods

Participants

A multiplex family with HL was referred to the Ahvaz Noor Medical Genetics Laboratory. A detailed family history was obtained, and clinical evaluations were performed. Air and bone conduction pure tone audiometry from 250 to 8000 Hz was obtained from the proband (individual VI:1). Written informed consent was taken from available family members, including healthy parents, an affected daughter, and an affected grandfather, before venous blood samples were collected in 0.5M EDTA–containing tubes. The study was approved by the review boards and ethics committees of Ahvaz Jundishapur University of Medical Sciences and Isfahan University of Medical Sciences.

GJB2 Mutation Screening and DFNB1 Linkage Analysis

DNA was isolated from the blood samples with a standard phenol/chloroform method. Purity and concentration of DNA samples were determined with 1.2% agarose gel and a Nanospec cube biophotometer (Nanolytik, Dusseldorf, Germany), respectively. First, the DNA sample of the proband was subjected to polymerase chain reaction (PCR) sequencing of the *GJB2* gene. For this purpose, the forward and reverse primers were used, as described previously,[15] to amplify the 809-bp region containing the coding region of the *GJB2* gene. Afterward, DNA sequencing of the PCR product was performed bidirectionally on an ABI 3130 XL automated sequencer (Applied Biosystems, Foster City, California). The sequencing result was compared with the genomic reference sequence, NG_008358.1, with SeqMan 5.00 software (DNASTAR, Madison, Wisconsin). Genotyping of 3 short tandem repeat polymorphic (STRP) markers (D13S1236, D13S1275, D13S175) was performed through resolving PCR products on 12% nondenaturing polyacrylamide gel, silver-nitrate staining, and visual inspection for genetic linkage analysis of DFNB1A/B loci.

Targeted NGS, Bioinformatics Analysis, and Validation

About 300 ng of genomic DNA from the affected individual (VI:1) was sent to the University of Iowa (Molecular Otolaryngology and Renal Research Laboratories) to carry out targeted NGS. A panel of 89 genes that are known to cause NSHL and some forms of SHL with various modes of inheritance was checked with the OtoSCOPE V.5 platform (designed by the University of Iowa). The examined genes are listed in **Table 1**.

In summary, solution-phase targeted genomic enrichment was utilized to prepare libraries and capture 89 deafness-causing genes. Sequencing was performed on an Illumina Hiseq 2000 (Illumina, San Diego, California) with 100-bp paired-end reads. Bioinformatics analysis included BWA for read mapping to the reference genome (hg19, NCBI Build 37), Picard for removal of duplicate reads, and GATK for variant calling. Variants were annotated with the custom MORL software. Copy number variants were identified with a read depth–based approach. Homozygous missense, start codon change, splice site, nonsense, stop loss, and indel variants with minor allele frequency $<1\%$ were filtered in dbSNP (version 137), 1000 Genomes Project, NHLBI GO Exome Sequencing Project, and Exome Aggregation Consortium. Several computational prediction tools were utilized, and an overall estimate of results was applied to evaluate the pathogenic effect of the variants. We used 10 online software tools—MutationTaster2, FATHMM, PANTHER, SIFT, PROVEAN, MutationAssessor, I-Mutant2.0, PHD-SNP, PolyPhen-2, and ConSurf—for the investigation of the missense variant, and we used BDGP, NetGene2 2.3, Spliceview, and MutationTaster2 for the splice site variant. Next, the variants were investigated in the Human Gene Mutation Database and the literature to seek the novelty of the variant or its association with a phenotype.

Candidate variants were validated by Sanger sequencing in the proband, her parents, and her affected grandfather. SeqMan 5.00 software (DNASTAR) was used to analyze the sequencing results.

PGD Experiment

To perform PGD, a multiplex PCR was carried out with 3 informative and linked STRP markers for the *OTOF* gene (D2S2144, D2S2223, D2S2247) and gene-specific primers for sequencing and mutation detection in exon 13 of the *OTOF* gene, encompassing the splice site variant, on 2 embryonic blastomeres (primer sequences and PCR condition are available upon request). The PCR products of the first round were employed as the template for the second

Table 1. List of Genes Covered by OtoSCOPE V.5 Deafness Panel.

Gene	Locus/Type	Inheritance
ACTG1	DFNA20/26	Autosomal dominant
ADGRV1	USH2C	Autosomal recessive
AIFM1	AUNX1	X-linked recessive
ALMS1	ALMS1	Autosomal recessive
ATP2B2	DFNB12 modifier	Autosomal recessive
CABP2	DFNB93	Autosomal recessive
CACNA1D	SANDD	Autosomal recessive
CATSPER2	DIS	Autosomal recessive
CCDC50	DFNA44	Autosomal recessive
CDH23	DFNB12, USH1D	Autosomal recessive
CEACAM16	DFNA4	Autosomal recessive
CIB2	DFNB48, USH1J	Autosomal recessive
CLDN14	DFNB29	Autosomal recessive
CLRN1	USH3A	Autosomal recessive
COCH	DFNA9	Autosomal dominant
COL11A2	DFNB53/DFNA13/STL3	Autosomal recessive and dominant
CRYL1	DFNB1 marker	Autosomal recessive
CRYM	DFNA40	Autosomal dominant
DFNA5	DFNA5	Autosomal dominant
DFNB31/WHRN	DFNB31/USH2D	Autosomal recessive
DFNB59/PJVK	DFNB59	Autosomal recessive
DIABLO	DFNA64	Autosomal dominant
DIAPH1	DFNA1	Autosomal dominant
DSPP	DFNA39	Autosomal dominant
ESPN	DFNB36	Autosomal recessive
ESRRB	DFNB35	Autosomal recessive
EYA1	BOR1	Autosomal dominant
EYA4	DFNA10	Autosomal dominant
FOXI1	PDS	Autosomal recessive
GIPC3	DFNB15	Autosomal recessive
GJB2	DFNB1/DFNA3	Autosomal recessive and dominant
GJB3	DFNA2	Autosomal recessive
GJB6	DFNB1/DFNA3	Autosomal recessive and dominant
GPSM2	DFNB82/CMC	Autosomal recessive
GRHL2	DFNA28	Autosomal dominant
GRXCR1	DFNB25	Autosomal recessive
HGF	DFNB39	Autosomal recessive
ILDR1	DFNB42	Autosomal recessive
KCNJ10	PDS	Autosomal recessive
KCNQ1	JLNS1	Autosomal recessive
KCNQ4	DFNA2	Autosomal dominant
LHFPL5	DFNB67	Autosomal recessive
LOXHD1	DFNB77	Autosomal recessive
LRTOMT	DFNB63	Autosomal recessive
MARVELD2	DFNB49	Autosomal recessive
miR-96	DFNA50	Autosomal dominant
miR-182	NA	NA
miR-183	NA	NA
MSRB3	DFNB74	Autosomal recessive
MT-RNR1	NA	Maternal
MT-TS1	NA	Maternal
MYH14	DFNA4	Autosomal dominant

(continued)

(continued)

Gene	Locus/Type	Inheritance
MYH9	DFNA17	Autosomal dominant
MYO6	DFNA22	Autosomal dominant
MYO1A	DFNA48	Autosomal dominant
MYO3A	DFNB3	Autosomal recessive
MYO7A	DFNB2/USH1B/DFNA11	Autosomal recessive and dominant
MYO15A	DFNB3	Autosomal recessive
OTOA	DFNB22	Autosomal recessive
OTOF	DFNB9	Autosomal recessive
OTOG	DFNB18	Autosomal recessive
OTOGL	DFNB84	Autosomal recessive
P2RX2	DFNA41	Autosomal dominant
PCDH15	DFNB23/USH1D	Autosomal recessive
PDZD7	USH2C	Digenic
PNPT1	DFNB70	Autosomal recessive
POU3F4	DFNX2	X-linked recessive
POU4F3	DFNA15	Autosomal dominant
PRPS1	DFNX1/CMTX5	X-linked recessive
PTPRQ	DFNB84	Autosomal recessive
RDX	DFNB24	Autosomal recessive
SERPINB6	DFNB91	Autosomal recessive
SIX1	DFNA23/BOR	Autosomal dominant
SLC17A8	DFNA25	Autosomal dominant
SLC26A4	DFNB4/PDS	Autosomal recessive
SLC26A5	DFNB61	Autosomal recessive
SMPX	DFNX4	X-linked recessive
TECTA	DFNB21/DFNA8/12	Autosomal recessive and dominant
TJP2	DFNA51	Autosomal dominant
TMC1	DFNB7/11/DFNA36	Autosomal recessive and dominant
TMIE	DFNB6	Autosomal recessive
TMPRSS3	DFNB8/10	Autosomal recessive
TPRN	DFNB79	Autosomal recessive
TRIOBP	DFNB28	Autosomal recessive
TSPEAR	DFNB98	Autosomal recessive
USH1C	DFNB18/USH1C	Autosomal recessive
USH1G	USH1G	Autosomal recessive
USH2A	USH2A	Autosomal recessive
WFS1	WFS1/DFNA6/14	Autosomal recessive and dominant

Abbreviation: NA, not available.

round. Fragment analysis of the STRP markers was performed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The PCR product containing the splice site variant was subjected to Sanger sequencing on an ABI 3130 XL automated sequencer (Applied Biosystems).

Results

Clinical Findings

A family with a history of 3-generation consanguineous marriages and multiple members who were deaf underwent clinical evaluations. The proband was a 12-year-old girl who was congenitally deaf and born to first-cousin parents (**Figure 1A**). No additional abnormal phenotypic features were observed in the proband, including visual impairment or any facial and limb malformations. Pedigree data suggested ARNSHL. Audiogram pattern revealed that the patient suffered from profound ARNSHL (**Figure 1B**).

DNA Testing Findings

Molecular investigations showed that the affected individual was negative for the *GJB2* mutations. In addition, haplotype reconstruction ruled out involvement of DFNB1A/B loci. Upon variant prioritization, 2 homozygous variants were

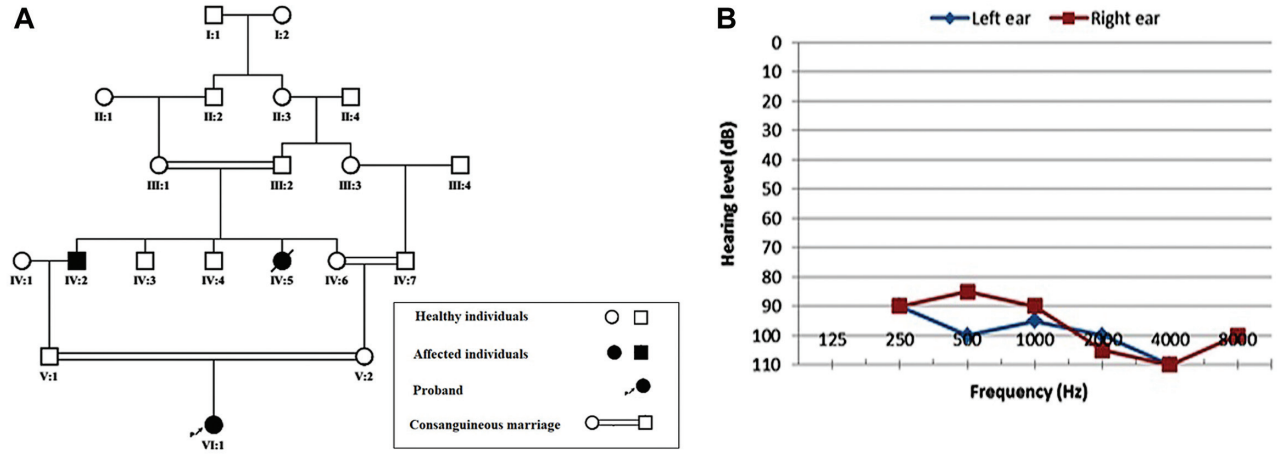

Figure 1. Pedigree and the proband audiogram. (a) The pedigree shows the occurrence of several consanguineous marriages and the presence of multiple individuals with hearing loss. (b) Profound deafness in right (rectangle) and left (diamond) ears.

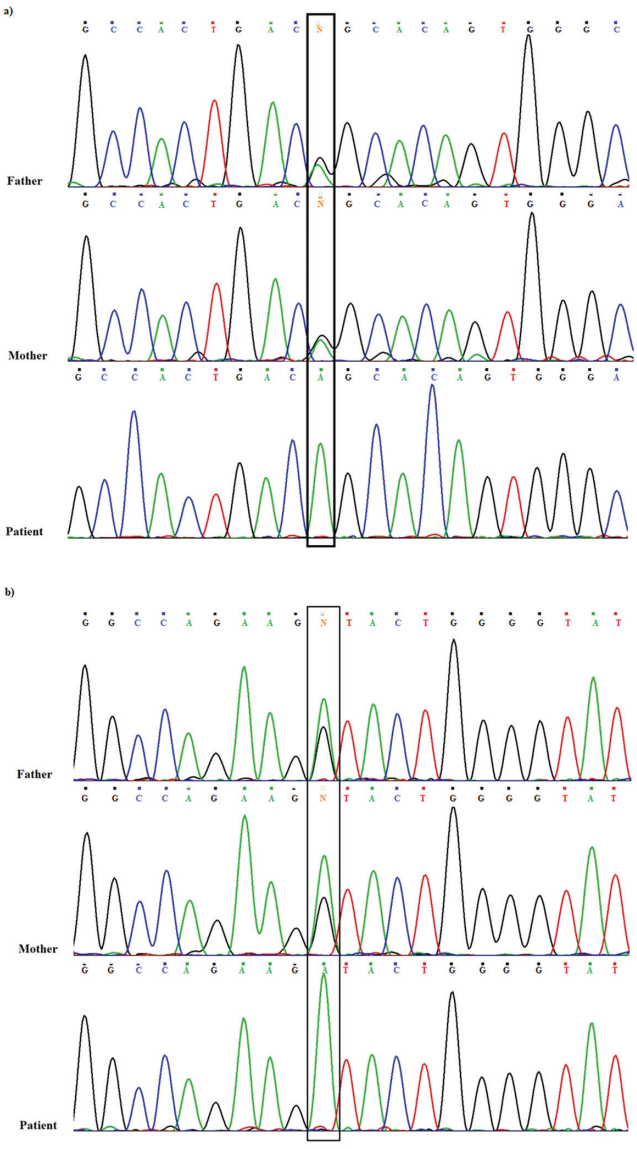

Figure 2. Electropherograms: (a) c.367G>A (p.G123S) and (b) c.1392+1G>A. variants are shown in rectangle. The patient is homozygous for both variants, while her parents are heterozygous.

identified within the *OTOF* gene simultaneously (**Figure 2**): a rare missense variant c.367G>A (p.Gly123Ser) in exon 5 and a novel splice site variant (c.1392+1G>A). The latter substitution is located in the donor splice site of intron 13, in which G nucleotide is replaced by A. Her affected grandfather was homozygous for both variants, while her parents were heterozygous in *cis* status for both variants.

In this case, 6 of 10 software tools predicted p.Gly123Ser as a neutral or benign variant without significant consequence on protein structure or function (**Table 2**). In contrast, all online splice site software tools and MutationTaster2 predicted the pathogenic effect of the splice site variant through losing the donor splice site.

PGD Outcome

Two embryonic blastomeres were examined through 2 methods, including linkage analysis and direct sequencing of exon 13, encompassing the c.1392+1G>A splice site variant. Through haplotype and sequence analysis, 1 of the blastomeres was heterozygous like the parents; the other displayed the homozygous haplotype containing the splice site variant.

Discussion

The genetic diagnosis of ARNSHL is the prenecessary step before its prevention through PGD and prenatal diagnosis. For minor disorders such as HL, PGD appears to be the best option in prevention. PGD has been accomplished for many monogenic diseases.[16] While there are several reports of PGD for DFNB1 and DFNB4 loci as the common causes of ARNSHL,[17,18] this is the first report of an attempt for the DFNB9 locus (*OTOF*).

NGS technology has provided a new way to advance scientific research and genetic diagnosis. This technology provides a powerful method for finding disease-causing variants in known as well as new disease genes.[11,12,19] Due to the ultimate heterogeneous nature of NSHL, NGS will help in DNA testing of the disease. NGS gene panels provide the possibility of screening for known disease-causing

Table 2. In Silico and Bioinformatics Analysis of the Variants.

Variant	c.367G>A (p.G123S)	c.1392+1G>A
dbSNP rsID	rs116314622	Novel
NHLBI allele frequency	0.0026	0
1000 genomes MAF	0.0026	0
ExAC all MAF	0.0032	0
ConSurf score	6	NA
MutationTaster2	Disease causing	Disease causing
FATHMM	Damaging	NA
PANTHER	Probably damaging	NA
SIFT	Tolerated	NA
PROVEAN	Neutral	NA
MutationAssessor	Neutral	NA
I-Mutant2.0	Decrease stability	NA
PHD-SNP	Neutral	NA
PolyPhen-2	Benign	NA
BDGP	NA	Wild 0.87, mutant NR
NetGene2 2.3	NA	Wild 0.95, mutant NR
Spliceview	NA	Wild 81, mutant NR
Segregates in the family	Yes	Yes
ACMG variant category[35]	Pathogenic	Uncertain significance

Abbreviations: ACMG, American College of Medical Genetics and Genomics; ExAC, exome aggregation consortium; MAF, minor allele frequency; NA, not available; NR, not recognized.

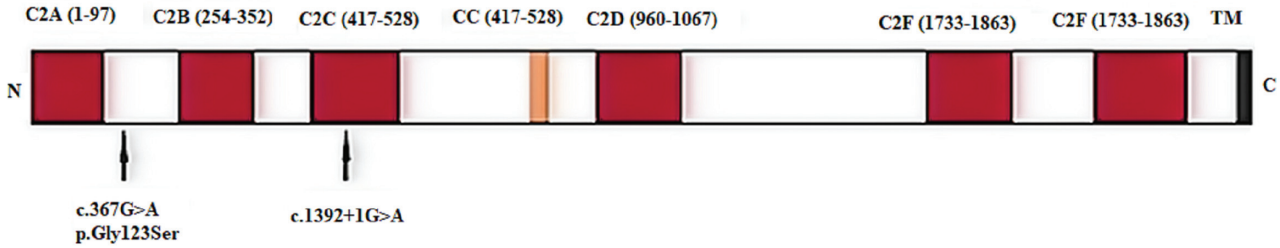

Figure 3. Structure of the otoferlin protein and its domains. The black arrows indicate the positions of the variants.

genes simultaneously with higher vertical and horizontal coverage of the genomic DNA as compared with whole exome sequencing.[20]

In the present study, targeted HL gene sequencing was performed for a panel of 89 genes, and 2 homozygous variants within the *OTOF* gene were found in the patient.

In 1999, Yasunaga and colleagues showed that pathogenic variants in the *OTOF* gene are responsible for ARNSHL at the DFNB9 locus.[21] The *OTOF* gene is located at position 2p23.1 and consists of 47 exons, the first of which is noncoding.[22] This gene is responsible for encoding the otoferlin protein, which is a member of the mammalian ferlin family. Several translation initiation sites and alternative splicing contribute to short and long otoferlin isoforms encoded by *OTOF*. It is believed that variants affecting the long isoform cause ARNSHL. About half the cases with the *OTOF* gene pathogenic variants are associated with auditory neuropathy,[23,24] which is a sensory-neural hearing defect characterized by a lack of or a significantly abnormal auditory brainstem response but normal otoacoustic emission responses.[24] The long isoform contains 6 C2 domains allowing for binding to Ca^{2+} and Ca^{2+}-dependent interactions with SNARE proteins,[25] and its presence is required for the final step in calcium-activated release of neurotransmitter vesicles at inner hair cells.[25-27] High expression of the *OTOF* gene has been shown in cochlear auditory inner hair cells, vestibule, and the mouse brain.[28,29]

In this study, the patient simultaneously had 2 homozygous variants: c.367G>A (p.Gly123Ser) and c.1392+1G>A. The missense variant is located in the region between the C2A and C2B domains in the protein (**Figure 3**). The effect of this variant was investigated with 10 online software tools (**Table 2**), and the results showed that the identified substitution may not be pathogenic despite cosegregating in the family. Notably, no pathogenic missense variant has been identified in this exon. However, the low global minor allele frequency and lack of homozygous reports of the variant are in favor of its pathogenic effect. Thus, functional in vivo and

Table 3. *OTOF* Pathogenic Variants Reported in Middle East Populations.

Nucleotide Change	Amino Acid Change	Population	Reference
c.1469C>A	p.P490Q	Turkey	36
c.1544T>C	p.I515T	Turkey	36
c.1718T>G	p.L573R	Pakistan	32
c.3032T>C	p.L1011P	Turkey	37
c.3269C>A	p.A1090E	Pakistan	32
c.5197G>A	p.E1733K	Pakistan	32
c.5567G>A	p.R1856Q	Pakistan	32
c.5815C>T	p.R1939W	Pakistan	32
c.367G>A	p.G123S	Iran	This study
c.3265C>T	p.R1089W	Iran	38
c.2417T>C	p.I806T	Iran	38
c.1469C>G	P490R	Oman	39
c.709C>T	p.R237X	UAE	40
c.1273C>T	p.R425X	Pakistan	32
c.1607G>A	p.W536X	Pakistan	32
c.4157C>T	p.R577X	Palestine	41
c.4491T>A	p.Y1497X	Lebanon	21
c.4809C>A	p.Y1603X	Pakistan	32
c.2122C.T	p.R708X	Pakistan	32
c.3679C>T	p.R1227X	Turkey	10
c.2866+1G>A	Intronic	Israel	42
c.1392+1G>A	Intronic	Iran	This study
c.1329+2T>C	Intronic	Iran	43
c.1103_1104delinsC	p.G368AfsX2	Pakistan	32
c.1958delC	p.P653LX13	Turkey	33
c.1981dupG	p.D661GfsX2	Iran	31
c.4467dupC	p.I1490HfsX19	Turkey	33
c.3636_3637del	p.Phe1212fs	Iran	11
c.2295_2297del	p.E766del	Pakistan	32

in vitro experiments are required for a precise decision. Available evidence suggest it as a variant of uncertain significance based on the guidelines of the American College of Medical Genetics and Genomics for interpretation of variants. However, the c.1392+1G>A variant in the donor splice site affects splicing and can lead to intron retention. Based on the guidelines, the latter variant is categorized as pathogenic (**Table 2**). Previous studies have shown that a single amino acid change, even in nonconserved residues, in 1 C2 domain severely affects protein stability and localization.[30] Variants that affect 2 domains are likely to have a higher negative effect on the protein structure and function. Pathogenic variants in different domains are associated with profound deafness in most cases.[31] This could explain the profound deafness phenotype cosegregating in the pedigree due to severe effect of the splice site variant on C2C and downstream domains of the protein.

Up to now, >100 *OTOF* mutations have been reported, 29 of which are from the Middle East. They involve a wide spectrum of missense, nonsense, frame shift, splice site, deletion, and duplication variants (**Table 3**). Missense variants represent 12 (41.3%) of the reported pathogenic variants in this part of the world, followed by nonsense variants (n = 8 reports, 27.5%) and frameshift variants (n = 5 reports, 17.2%). There are 3 reports of splice site variants (10.3%) and 1 of a small deletion variant (3.4%). Among the Middle Eastern countries, Pakistan and Turkey have the highest diversity of these mutations. According to previous studies, mutations in the *OTOF* gene are the cause of 5% and 2.3% of ARNSHL in Turkish and Pakistani families, respectively.[32,33] Due to the presence of various ethnic groups living in Iran, a high genetic heterogeneity is expected. Various studies in Iran suggested that the role of the DFNB9 locus in ARNSHL might be low, from 0.7% to 2.6%.[34]

Conclusion

In the present study, we used a combined application of NGS and PGD for diagnosis and prevention of ARNSHL in an Iranian family. Two homozygous variants, a rare missense and a novel pathogenic splice site, were identified in *cis* status in the patient. To the best of our knowledge, PGD

of the *OTOF* gene has not been reported in the literature. The diagnostic routing that we followed in this study could be useful for prevention of HL.

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Author Contributions

Mohammad Amin Tabatabaiefar, study design, validated data, wrote the manuscript, answered reviewers comments, proved manuscript final version; **Mohammad Reza Pourreza**, performed experiments, data analysis, wrote the manuscript, answered reviewers comments, proved manuscript final version; **Parisa Tahmasebi**, performed experiments, data analysis, wrote the manuscript, proved manuscript final version; **Nader Saki**, clinical examinations, technical editing and revising the manuscript, answered reviewers comments, proved manuscript final version; **Morteza Hashemzadeh Chaleshtori**, prepared reagents/tools, technical editing and revising the manuscript, proved manuscript final version; **Rasoul Salehi**, performed preimplantation genetic diagnosis, technical editing and revising the manuscript, proved manuscript final version; **Javad Mohammadi-asl**, study design, validated data, technical editing and revising the manuscript, answered reviewers comments, proved manuscript final version.

Disclosures

Competing interests: None.

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