

## The first case of NSHL by direct impression on *EYA1* gene and identification of one novel mutation in *MYO7A* in the Iranian families

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### ABSTRACT

**Objective(s):** Targeted next-generation sequencing (NGS) provides a consequential opportunity to elucidate genetic factors in known diseases, particularly in profoundly heterogeneous disorders such as non-syndromic hearing loss (NSHL). Hearing impairments could be classified into syndromic and non-syndromic types. This study intended to assess the significance of mutations in these genes to the autosomal recessive/dominant non-syndromic genetic load among Iranian families.

**Materials and Methods:** Two families were involved in this research and two patients were examined by targeted next-generation sequencing. Here we report two novel mutations in the *MYO7A* and *EYA1* genes in two patients detected by targeted NGS. They were confirmed by Sanger sequencing and quantitative real-time PCR techniques.

**Results:** In this investigation, we identified a novel mutation in *MYO7A*, c.3751G>C, p.A1251P, along with another previously identified mutation (c.1708C>T) in one of the cases. This mutation is located in the MYTH4 protein domain which is a pivotal domain for the myosin function. Another finding in this research was a novel de-novo deletion which deletes the entire *EYA1* coding region (EX1-18 DEL). Mutations in *EYA1* gene have been found in branchiootorenal (BOR) syndrome. Interestingly the patient with *EYA1* deletion did not show any other additional clinical implications apart from HL. This finding might argue for the sole involvement of *EYA1* function in the mechanism of hearing.

**Conclusion:** This investigation exhibited that the novel mutations in *MYO7A*, c.3751G>C, p.A1251P, and *EYA1*, EX1-18 DEL, were associated with NSHL. Our research increased the mutation spectrum of hearing loss in the Iranian population.

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### Introduction

Sensorineural hearing loss (SNHL) is a multifaceted disease with profoundly medical, social, and educational consequences. The term "hearing impaired" interchangeably refers to people with any degree of SNHL and "Deaf" is a conversational term that implies hearing thresholds in the audiometry severe to profound span.

The diagnosis of SNHL relies on the verification of reduced hearing acuity by auditory testing. Hearing is measured in decibels (dB) and a child's hearing acuity is deemed as normal if it is within 20 dB of defined thresholds. The intensity of hearing loss is classified into five categories: mild (20–40 dB), moderate (41–55 dB), moderately severe (56–70 dB), severe (71–90 dB), or profound (90 dB)(1). The severity of hearing loss is graded as low (500 Hz), middle (501–2000 Hz) and high (2000 Hz) (2). Moreover, hearing loss is categorized by type, onset, severity, and frequency.

In most cases, type of loss is classified as sensori-neural, conductive, mixture of both, and either pro-gressive or stable types. Time of onset is arranged as either hereditary or late-onset (acquired). Causality is categorized as either hereditary (genetic) or environmental (non-genetic) (3). All types of the Mendelian inheritances in hearing loss such as autosomal recessive or dominant and X-linked and mitochondrial manner have been identified (4).

Hearing impairments could be categorized into syndromic and non-syndromic types. Nonsyndromic HL can further be classified by its mode of inheritance. Approximately one-fifth of nonsyndromic sensorineural hearing loss (NSSHL) is inherited as autosomal dominant which is implied to as DFNA. Autosomal recessive (DFNB), non-syndromic hearing loss (ARNSHL) causes around 80% of all cases of hearing loss. The inheritance of X-linked and mitochondrial modes are less than 1 percent. To date, 142 loci have been mapped for NSHL (5).

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**Table 1.** Distribution of causes for hearing loss in infancy

Environmental-50%	TORCH infection, ototoxicity, drug consumption, prematurity, other infections	
	Syndromic 30%	Alport, Pendred, Usher, Wardenberg, KSS <sup>1</sup> , MERRF <sup>2</sup> , CHARGE, Norrie, Stickler, Treacher Collins, Perrault syndrome, BORS <sup>3</sup> , Jervell & Lange-Nielsen and so on
Genetic-50%	Non-syndromic 70%	Autosomal recessive (70-80%) Autosomal dominant (20-25%) X chromosomal/mitochondrial (1.5%)

TORCH=Toxoplasmosis, other (syphilis, varicella-zoster, parvovirus B19), rubella, cytomegalovirus (CMV), and herpes infections  
Kearns-Sayre syndrome <sup>1</sup>, Myoclonic epilepsy with ragged-red fibers <sup>2</sup>, branchio-oto-renal syndrome <sup>3</sup>  
The etiology of minor degrees of HL in the newborn period is not well perceived

Autosomal dominant non-syndromic hearing loss (NSHL) is usually first detected in school-aged children during common audio-logical screening (6). Some types of syndromic SNHL are also first recognized at this time for the diagnosis of associated comorbidity that was not beforehand noted. Most known examples include Pendred and Usher syndromes; they are both inherited in an autosomal recessive manner. So a family history is contributory for diagnosis and risk assessment (1).

Hearing impairment results from etiologically heterogeneous genetic and environmental factors such as prenatal TORCH infections and postnatal secondary to bacterial meningitis by *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* (2, 7). Approximately, 50% of hearing loss has genetic causes (Table 1).

In this report, we identified 2 novel mutations in two Iranian families. In the AT1220 case, we identified a novel variant c.3751G>C, p.A1251P along with a previously identified mutation (c.1708C>T; p.Arg570 Ter) as compound heterozygote mutations in *MYO7A* (NM\_000260). The p.A1251P mutation has a deleterious impression on Myosin protein by interference in protein folding. Besides, this mutation is located at a highly conserved region of *ELEA*. The pathogenicity of the mutation has been confirmed by *in-silico* predictions. In the second family, a *de novo* and novel deletion (EX1\_18DEL) in the *EYA1* gene was found in the YK1132 patient. Haploinsufficiency of the transcription co-activator *EYA1* causes the branchio-oto-renal syndrome (BOR) that is related to profound hearing loss (8); however, in this family, we could not find any symptoms of the BOR syndrome and it is the first case of NSHL influenced by *EYA1* deletion.

The aim of the present investigation was to determine the spectrum of the deleterious mutations related to NSHL in the Iranian population. Our results increased the mutational spectrum of *MYO7A* and *EYA1* genes. The proteins encoded by these genes are involved in embryonic development, gene regulation, ionic and osmotic homeostasis and are required for normal function of cochlear hair cells.

## Materials and Methods

### Patients

The study was approved by the local ethics committee of Tarbiat Modares University. Informed consents were obtained from all patients and their

healthy relatives. The patients in the YK1132 and AT12120 families were 33 and 30 year old males, respectively. All of the patient's clinical information was collected at DeNA Laboratory, Tehran, Iran and the medical histories were obtained using a special questionnaire that included following issues: type of HL (syndromic or non-syndromic), age of onset, bilateral or unilateral HL, presence of thyroid problems, extraordinary skin pigmentation, tinnitus and vertigo, exposure to teratogenic infections like TORCH in infancy or consuming special drugs during pregnancy, pathological changes in the ear and other related manifestations (9). Audiological evaluations of the patients exhibited symmetrical and bilateral sensorineural hearing loss. According to audiological assessments, the severity of hearing loss varied among affected individuals, ranging from mild to profound. None of the patients displayed any additional symptoms apart from hearing loss, so they were deemed as NSHL patients and the NSHL panel was utilized to survey the pathogenic mutation(s). (The patient's clinical features are described in Table 2).

### DNA extraction and Sequencing

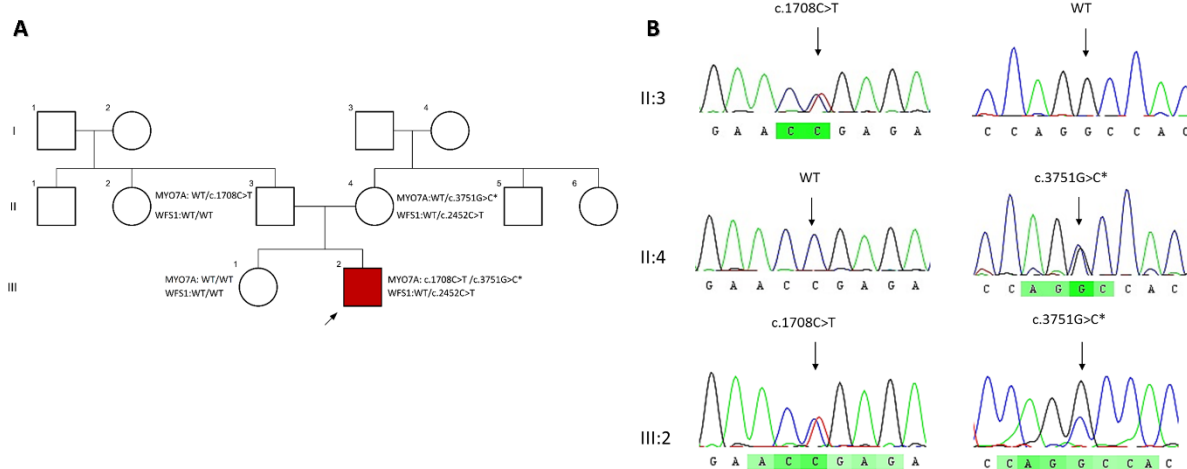
Genomic DNA was extracted from peripheral blood samples using the Roche DNA Extraction Kit (Product No. 11814770001). There are about 127 genes involved in HL based on the reports of deafness variation database (DVD) (<http://deafness-variationdatabase.org/letter>). About 50% of the autosomal recessive types of HL are due to mutations in *GJB2/6* genes. *GJB2* is also a major cause of hearing loss in Iranian patients (10-12). Therefore, at the first imperative step, these two cases were screened for connexin 26 mutations by Sanger sequencing. After ruling out *GJB2* mutations all the contributed genes in HL were studied by utilizing a commercially available targeted NGS panel for hereditary NSHL from BGI, HongKong. In general, point mutations, micro-insertions, deletions and duplications (<20 bp) can be simultaneously detected by this targeted NGS panel with over 90% sensitivity (13). List of genes included in this panel is provided in appendix Table 1.

A filtering pipeline was established to recognize supposedly disease-causing variants. Due to the rarity of HL causing mutations, only variants with a frequency below 0.01 were selected (14). Frequencies of identified variants were also checked in ExAC (*Exome Aggregation Consortium*) (15), DVD (*deafness variants database*), and

**Table 2.** Clinical features of patients and genes/variants that have been found in our study

Patient ID	Phenotype	Gene Exon/CDS	Protein	N.A/pro. alteration	Zygoty	SIFT	Polyphen	Mutation taster	phastCons	EXAC/1K /10K genome frequency
AT1220	Mild hearing loss	MYO7A EX30/CDS29	Myosin 7a	c.3751G>C* p.Ala1251Pro	Het	Damaging	Probably damaging (0.998)	Disease causing	1	Not reported
		MYO7A EX15/CDS14	Myosin 7a	c.1708C>T p.Arg570Ter	Het	Damaging	Damaging	Disease causing	1	Not reported
		WFS1 EX8/CDS7	Wolfamin	c.2452C>T p.Arg818Cys	Het	Damaging	Damaging	Disease causing	1	Reported
YK1132	Severe hearing loss	EYA1 EX1-18	EYES ABSENT 1	C.Ex1_18DEL*	Het	-	-	Disease causing	1	-
		WFS1 EX5/CD4	Wolfamin	c.577A>C p.Lys193Gln	Het	Benign	Possibly damaging	Disease causing	1	Reported

N.A: Nucleic acid, Pro.: Protein, phastCons: is a program for identifying evolutionarily conserved elements in a multiple alignment, for highly conserved sequences it is 1. \* Novel mutations; EX: Exon; Het: Heterozygote

**Figure 1.** Identification of a novel missense mutation in the *MYO7A* gene

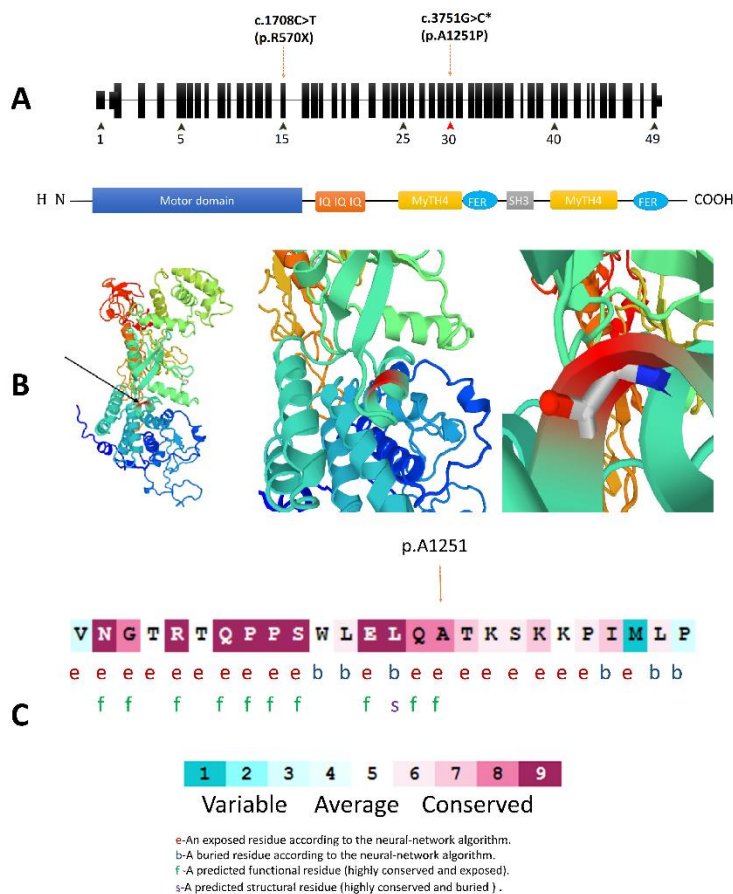
(A) Pedigree of the AT1220 family is comprised of three generations, squares and circles indicate females and males, respectively and the arrow appoints the proband of the family. The *MYO7A* mutations are co-segregating with the disease in this family as compound heterozygote. The star exhibits the novel mutation (B) Chromatograms showing nucleotide sequences of *MYO7A* in the regions of c.3751G>C and c.1708C>T mutations found in AT1220 family. Black arrows are indicating the heterozygous nucleotide substitution

ESP (*Exon Sequencing Projects*) publicly available databases. Mutations that have been described in HGMD (*Human Gene Mutation Database*) (16) were given the highest supremacy. For variants leading to missense mutations, pathogenicity predictions from at least 3 online databases namely SIFT, Polyphen2, and Mutation Taster (17) were compared. To predict the effect of c.3751G>C mutation on splicing, the HSF (*Human Splicing Finder V 3.0*) software was utilized. Also, ConSurf (<http://www.consurf.tau.ac.il>) was applied to provide evolutionary conservation profile for the myosin VII protein (Figure 2).

### Segregation study for variants: validation by Sanger sequencing

Appropriate primers were designed using primer3 plus a web-based tool (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>) for the regions

of identified variants (Table 3). Primer specificity was checked by *in-silico*-PCR and blat tools of UCSC genome browser and lack of SNPs in the genomic region corresponding to the 3' ends of primers were checked by looking through the dbSNP database. PCR reactions were done according to the standard protocols and followed by Sanger sequencing. Sequence chromatograms were analyzed by *Codon code aligner V.6.0.2* and *DNA Baser Assembler V.4.36* to identify the variants. After confirming the mutation by Sanger sequencing in the studied sample by NGS, the co-segregation in the parents and healthy available members of the family were checked by Sanger sequencing. Additionally, the frequency of the variants was confirmed by utilizing at least 100 unrelated normal controls in the Iranian genome project database (<https://www.irangenomes.com/>).



**Figure 2.** (A) Schematic structure of *MYO7A* and its encoded protein domains. The c.3735C>T located in the myosin tail homology 4 (MYTH4) domain that is coded by Exon 30. (B) A prediction of structural alternation resulted from *MYO7A* c. 3751G>C mutation designed by SWISS-MODEL and PMP online tools. This substitution is not conserved because the substituted amino acid, Proline, has poor helix-forming propensity and it can stir the secondary structure in this protein. (C) Multiple alignments of the *MYO7A* c.3751G homologous sequences of eight different vertebrates based on UCSC Multiz alignments tool. The amino acid substituted by the missense mutation p.A1251P (the red types) is highly conserved among the different vertebrate species. (D) The amino acid sequence MYO7A (p.Ala1251) colored based on conservation scores by ConSurf database. ConSurf demonstrates evolutionary conservation profiles for proteins of known structure in the PDB according to the phylogenetic relations between homologous sequences as well as amino acid's structural and functional importance

### Structural-based analysis and modeling

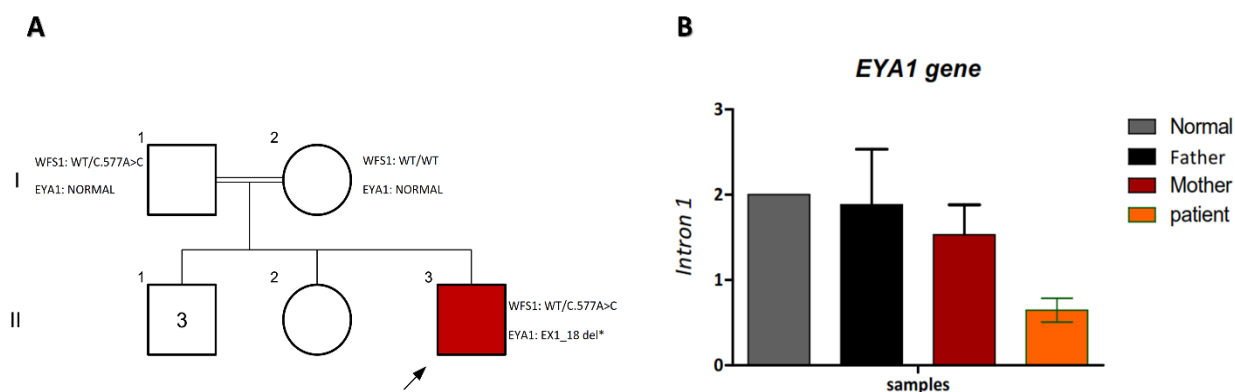
Wild-type and mutant protein comparison based on 3D (three dimensional) for a p.A1251P mutation in *MYO7A* was performed using SWISS-MODEL (18) and protein model portal (PMP) (19), automated homology, comparative modeling programs, and automatic modeling approaches were used to apply the complete protein sequence of human *MYO7A*. The protein structure was fetched from the Protein Data Bank with the accession code 3PVL (20). The target sequences were uploaded in DeepView (21) and mapped on to wild-type template and then sequence alignment optimization was performed. Afterward, the complete project file was submitted to SWISS-MODEL. The effects of amino acid substitution on *MYO7A* were analyzed and predicted by *in-silico* tools such as I-mutant 2.0 (22) and site-directed mutator instruments (SDM) (23). The whole protein aggregation propensities in its folded states were predicted

by Aggrescan3D (24). In order to verify the obtained 3D structure, ProSA (<https://prosa.services.came.sbg.ac.at/prosa.php>) was used, which analyzes the protein structure and matches with X-ray and NMR calculated structures.

## Results

In this study, we identified two NSHL affected families and utilized the targeted next-generation sequencing for recognizing the pathogenic variants. After applying different filtering steps in case *AT1220*, one variant in *WFS1* and two variants in *MYO7A* genes, and in case *YK1132* a deletion in *EYA1* gene and another variant in *WFS1* remained as candidates (Table 2). The co-segregation study was performed for all of these variants in each family.

In *AT1220* a novel variant (c.3751G>C, p.A1251P) and a previously identified mutation c.1708C>T; p.R570Ter in *MYO7A* (NM\_000260) were identified as



**Figure 3.** A) mutations identified in family YF-KHZ with hearing loss.

A) Pedigree of the YK1132 family, presenting the segregation of the c.577A>C mutation. B) Validation of EYA1 deletion by Real-time CQ-PCR. The relative quantity of Introns 1 in the Patient was almost half of the patient's father and mother. Values are expressed as the mean  $\pm$  standard error

**Table 3.** Sequences of the primers used to validate the mutations by Sanger sequencing

Patient ID	Gene and Variants	Primers
AT1220	<i>MYO7A</i>	F5'-TGTGGTGGAACTAGGTGGAT-3'
	c.1708C>T	R5'-TTCCCGAACAAACAGAAACCG-3'
	<i>MYO7A</i>	F5'-AGAGAGCCAAAGTCCAGAGG-3'
	c.3751G>C*	F5'-ACAGGGCAATGTAGAGGGAG-3'
YK1132	<i>WFS1</i>	F5'-TTGAGATTACCGTGGGCATG-3'
	c.2452C>T	R5'-CTGGTGGGTGAGAGCTGG-3'
	<i>EYA1</i> * (Real-Time primer)	F 5'-AGTGGTTGCTGAAATGTTGCT-3' (INTRON 3)
	<i>WFS1</i>	R 5'-TGCCTAACCAATTTGAAGACCT-3'
	c.577A>C	F5'-CGAGACCGACCTGGAGAG-3'
		R5'-TGACCTGGCCGACATTCT-3'

compound heterozygotes. The AT1220, was a 33-year old male with mild hearing loss. In addition to this mutation, another known mutation in *WFS1* (NM\_006005, c.2452C>T, p.R818C) was also found in this patient. The novel c.3751G>C variant affects the exon 30 of *MYO7A* coding the myosin tail homology 4 (MyTH4) domain, which is known to play a role in the connection between actin-based motor proteins and the microtubule cytoskeleton (Figure 2) (25). The MyTH4 domain comprises four highly conserved regions designated as *MG* or *MGDhP*, *LRDE*, *RGW*, and *ELEA*. Associations between this gene and *Usher syndrome type 1B* and autosomal dominant deafness type 11 have been previously reported (OMIM: 276903, OMIM: 600060). The novel p.A1251P is located in the conserved region of *ELEA*. Co-segregation study in this family showed that the *MYO7A* variants, c.1708C>T, and c.3751G>C, co-segregated with the disease as a compound heterozygote but the variant found in *WFS1* (c.2452C>T) did not co-segregate with the disease (Figure 1A).

In the YK1132 patient also after applying different filtering steps, only two mutations remained as candidates: a heterozygote c.577A>C, p.K193Q mutation in *WFS1* (NM\_006005) and a heterozygote EX1-18 DEL deletion in the *EYA1* (NM\_000503) gene.

Segregation study in the family showed that the c.577A>C variant in the *WFS1* gene cannot be the cause of disease in this family as the healthy father was also a carrier for this variant. On the other hand, the EX1\_18 DEL deletion was a de-novo mutation that occurred in the patient only and none of the parents was the carrier for this deletion; therefore, this variant can be considered as the cause of disease in this patient. This is a de-novo interstitial deletion with the size of ~165 Kb on the long arm of chromosome 8 (8q13.3). This region contains the entire *EYA1* gene. It is known that haploinsufficiency of *EYA1* gene is responsible for the clinical features of patients with branchiootorenal (BOR) syndrome. According to the deafness variation database (DVD) to date, this

deletion has not been reported for nonsyndromic hearing loss. Apart from hearing loss, any other obvious clinical features were not observed in this patient so it can be considered as NSHL. Quantitative real-time PCR was applied to confirm the deletion (Figure 3B).

## Discussion

Hereditary hearing loss is strongly heterogeneous in different populations. To date, 142 loci have been mapped and over 127 genes have been found for HL. Despite such heterogeneity, mutations in some of the genes play important roles in the nonsyndromic hearing loss. These genes belong to the wide variety of the protein classes such as Myosins and related cytoskeletal proteins, transcription factors, proteins that belong to channels and gap junction structures, extracellular proteins and other genes with unknown functions (26).

The advent and development of the next-generation sequencing (NGS) technologies have tended to increase the rate of gene detection for genetically heterogeneous diseases such as hearing loss with reducing costs and producing massive sequencing data (27). By high-throughput sequencing technologies, it could be possible to sequence a large number of exons or other genomic sequences in a person to confirm or rule out a suspected genetic condition (28). As for a plurality of genetic factors that account for hearing loss, we utilized the NGS technologies to detect the pathogenic mutations in previously known causative genes.

In this study, we identified two novel variants in *MYO7A* and *EYA1* that were associated with NSHL. Myosin VIIA belongs to the unconventional myosin superfamily of proteins that consists of over 35 classes (29). The unconventional myosin VIIa is required for normal auditory functions and its mutations are associated with NSHL. This gene is expressed in hair cell stereocilia. Several disruptive variants in this gene can result in hearing impairment associated with vestibular dysfunction and retinitis pigmentosa.

The most conserved sequence in these proteins is the N-terminal region with the characteristic head domain followed by a more variable neck region. The N-terminal domain binds to actin and ATP, and regulatory domain or neck domain consists of IQ domains that bind to calmodulin or calmodulin-like light chains. The C-terminal domain corresponds to the tail domain and it is the highly variable region between myosin classes (30). Mutations in *MYO7A* have been previously associated with the *Usher Syndrome*, especially *Usher syndrome type IB*, and autosomal dominant or recessive NSHL (31). *MYO7A* mutations have been shown to be the cause of hearing

loss in a significant percentage of deaf Iranian families (32).

Myosins comprise a superfamily of ATP-dependent motor proteins and they are well-known for their function in muscle contraction, vesicles transportation, cell division, and so on (33). It is shown that myosin VIIa plays a critical role in transporting *harmonin* to the tip region of the stereocilium and has a significant role in shaping the sensory hair cell bundle (34).

Myosin VIIa has two *MyTH4* domains and several mutations in this domain have been described previously to be the common causes of *DFNB3* hearing loss (35). Two domains of MyTH4-FERM (MF; myosin tail homology 4; band 4.1, ezrin, radixin, moesin), which are separated by an SH3 domain, are located in the Myo7 tail region and essential for interaction with PDZ domain and microtubules (36, 37).

In this study, we report a novel mutation in ELEM conserved region of the MyTH4 domain in *MYO7A*. The alanine-to-proline substitution results in obstacles in  $\alpha$ -helix formation due to poor helix-forming propensity of proline. This alteration is predicted to negatively effect the function of the myosin VIIa protein and it could be the cause of hearing loss. Based on I-mutant 2.0, this substitution can change the stability and flexibility of the protein because of proline's unique propensities (38). According to Aggrescan3D, no certain alteration in protein folding is predicted. In a nutshell, additional studies, especially in animal models, must be applied to identify certain pathogenic role(s) of the mutation.

Meanwhile, we described another family with hearing loss phenotype with a mutation in *WFS1* and a *de novo* mutation in *EYA1* gene.

An interesting point is that in both families studied here, after various stringent filtering steps, we still ended up with previously reported mutations in *WFS1*, c.577A>C and c.2452C>T, and surprisingly in both cases, segregation study in the families could rule out these mutations as the pathogen, because they were present in the healthy members as well despite the fact that both of these mutations have been previously reported in several publications as pathogenic (39-41). This finding shows how much a study like this one is important to elucidate the repertoires of mutations in different populations. It is also highly needed to publish and submit these variants and their clinical outcomes in the public databases in order to avoid misinterpretation of the neutral variants.

*EYA1* gene encodes the *EYA1* protein which is identified as a transcription factor or transcription coactivator. This protein interacts with several other proteins, such as *SIX* proteins. In the organogenesis stage, these protein interactions are necessary for the normal formation of many tissues (42). It has been illustrated that *Eya1* knocked out mice show several deficiencies in ears

and kidneys as well as abnormal apoptosis of organ primordia (43). This protein plays important roles in the control of the cell polarity, cell fate, spindle orientation and Notch signaling in distal embryonic lung epithelium (44). It can explain the lung deficiencies in BOR patients (45). At least 160 mutations in the *EYA1* gene have been reported in individuals with BOR syndrome so far (46-50). This syndrome is being identified by the famous manifestation of malformations in ears and kidneys. Several mutations in this gene are related to eye disorders such as clouding of the clear front surface of the eye (the cornea) and clouding of the lens (cataracts) (51). Mutations in *EYA1* gene have been found in 40% of BOR patients (47). In patients with BOR syndrome, the malformations of the middle and inner ear structures have been revealed by analysis of computed tomography (CT) imaging of the temporal bone (52, 53).

Given the large number of *EYA1* mutations identified in various populations, in this study, we implicated pathogenic variants in *EYA1* as a novel cause of NSHL. *EYA1* encodes a member of the eyes absent (EYA) family of proteins. This protein has various important functions in developing several organs such as ears, branchial arches, eyes, and kidneys. Mutations in this gene are associated with the branchiootorenal dysplasia syndrome, branchiootic syndrome, sporadic cases of congenital cataracts, and NSHL (54).

The YK1132 is a sporadic case and parents who were clinically healthy did not carry the *EYA1* mutation, indicating a de-novo mutation in this case. After precise physical examinations we did not find any obvious expected symptoms of the BOR syndrome in this patient. Interestingly our findings suggest that the complete deletion of *EYA1* leads to non-syndrome hearing loss without any other additional clinical implications. This finding might argue for the sole involvement of *EYA1* function in the mechanism of hearing.

## Conclusion

We have utilized the targeted NGS panel for the discovery and diagnosis of the new variants and causative mutations in syndromic hearing loss. In this report, we described two novel mutations in the highly conserved regions of *MYO7A* (c.3751G>C) and *EYA1* (EX1\_18 deletion) genes, suggesting the potentially conserved role of these genes. Although in-silico predictions have shown that the novel mutation in *MYO7A* (c.3751G>C) is a pathogenic variant, more *in vivo* studies or animal models are necessary to conclude the pathogenic impact. Furthermore, this study illustrates for the first time the impression of *EYA1* gene deletion in a patient with NSHL. Besides, these novel mutations expand the mutational spectrum of *MYO7A* and *EYA1* in the Iranian population, which will contribute to the clinical understanding of hearing loss, caused by mutations in these genes and will

help physicians in better understanding the hearing loss etiology.

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## Conflicts of interest

The authors declare that they have no conflicts of interest.

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