Identification of a Novel Stop Loss Mutation in *P2RX2* **Gene in an Iranian Family with Autosomal Nonsyndromic Hearing Loss**

Reza Azizi Malamiri¹, Javad Mohammadi Asl² and Farideh Ghanbari^{3*}

¹Department of Pediatrics, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; ²NoorGene Genetics Lab, Ahvaz, Iran; ³Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

Received 12 May 2020; accepted 2 June 2020; published online 13 July 2021

ABSTRACT

Background: Hearing loss, a congenital genetic disorder in human, is difficult to diagnose. WES is a powerful approach for ethiological disgnosis of such disorders. **Methods:** One Iranian family with two patients were attented in the study. Sequencing of known NSHL genes was carried out to recognize the genetic causes of HL. **Results:** Molecular analyses identified a novel stop loss mutation, c.1048T>G (p.Term350Glu), whitin the *P2RX2* gene, causing a termination-site modification. This event would lead to continued translation into the 3' UTR of the gene, which in turn may result in a longer protein product. The mutation was segregating with the disease phenotype and predicted to be pathogenic by bioinformatic tools. **Conclusion:** This study is the first Iranian case report of a diagnosis of ADNSHL caused by *P2RX2* mutation. The recognition of other causative mutations in *P2RX2* gene more supports the probable function of this gene in causing ADNSHL. **DOI: 10.52547/ibj.25.5.368**

Keywords: Autosomal dominant 41, Deafness, Mutation, P2RX2, Whole exome sequencing

Corresponding Author: Farideh Ghanbari

Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran; Tel.: (+98-612) 6233884; E-mail: Ghanbari246@gmail.com

INTRODUCTION

earing loss is socially and economically important cause of human morbidity and is the most common sensorineural deficiency in humans. HL affects more than 300 million people worldwide^[1,2]. Genetic factors account for more than 50% of all prelingual HL cases. Hereditary HL is mainly classified into two forms: syndromic HL (approximately 30%), in which HL is accompanied by other clinical manifestations, and NSHL (approximately 70%), in which there are no further abnormal features^[3]. About 20–25% of NSHL forms are ADNSHL and 75–80% are ARNSHL, while only 1–1.5% is X-linked^[3]. To date, 80 genes and up to 140

genetic loci have been identified to be associated with NSHL (http://hereditaryhearingloss.org/). Molecular diagnosis plays a key role in clinical management, prognosis evaluation and pre-implantation genetic diagnosis for NSHL families^[4].

Until now, the extensive genetic heterogeneity of hearing impairment has restricted genetic diagnosis in most patients. Advances in DNA sequencing technologies such as NGS have facilitated the DNA testing and complete genetic analysis ofheterogeneous diseases. Targeted NGS provides a significant opportunity to detect variants in recognized disease genes, mainly in extremely heterogeneous diseases such as deafness^[5].

On the basis of these data, we aimed to recognize

List of Abbreviations:

3'UTR, 3' untranslated region; **ADNSHL**, autosomal dominant nonsyndromic hearing loss; **ARNSHL**, autosomal recessive nonsyndromic hearing loss; **HL**, hearing loss; **NGS**, next-generation sequencing; **NSHL**, non-syndromic HL; **WES**, whole exome sequencing

gene defects in an Iranian family with NSHL. This study reports a pathogenic mutation of *P2RX2* gene, which produces an extended protein-coding transcript that may explain the NSHL phenotype in the family.

MATERIALS AND METHODS

Clinical evaluations

Two patients in a family with a bilateral, sensorineural and progressive form of ADNSHL were enrolled in the study. The index patient was an 11-year-old female, the first child of a couple with consanguineous marriage orginated from the southwest of Iran (Fig. 1A). She was diagnosed with congenital deafness and had no dysmorphic features. The age of onset in the family was in the first decade of life. There was no history of systemic disease in the proband. A

full clinical explanation was obtained to exclude environmental exposures and features indicative of syndromic anomalies in the family. Also, pedigree examination, physical assessments, audiological tests (Fig. 2A), opthalmological evaluation, and electrocardiograms in the proband were carried out.

Molecular analysis

Peripheral blood samples were collected from the patients and other member of the family, and the genomic DNA was extracted from blood samples (family members) by using standard protocols^[6]. Libraries were prepared following standard Illumina, sample protocol. In precis, 3 μ g of genomic DNA was fragmented to 200–300 bp. Terminal A residues were added following the incubation with the Klenow Fragment exo-(3' \rightarrow 5' exo-) and dATP. Thereafter,

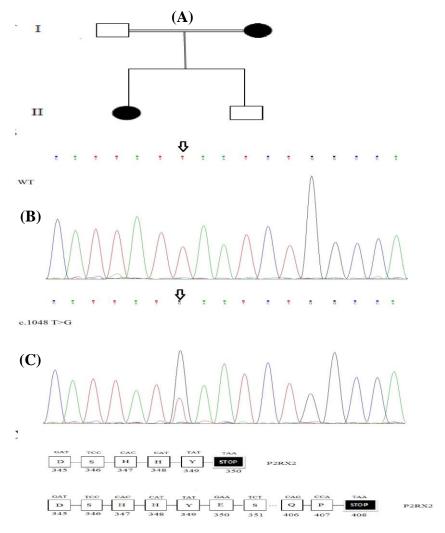


Fig. 1. (A) Pedigree of family with ARNSHL. The c.1048T>G variant is inherited from mother to the affected proband (denoted in black). (B) Electropherograms analysis of P2RX2 in ADNSHL affected probanda c.1048T>G variant (shown with arrow). (C) Zoomed-in view of region containing the variant, including the amino acid sequences of protein-coding isoform and the mutated sequences.

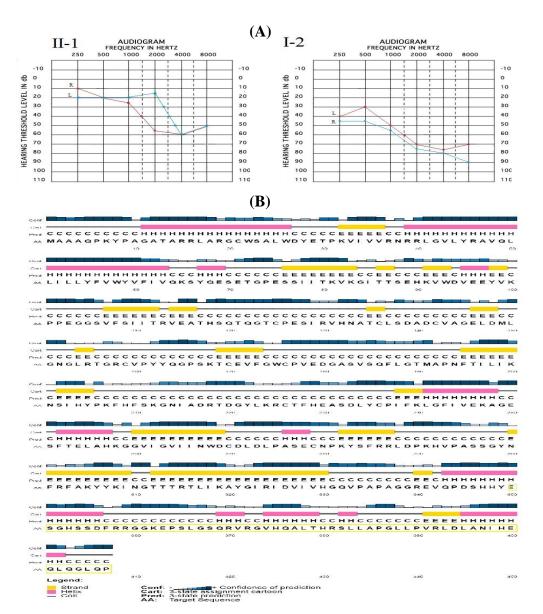


Fig. 2. (A) Audiograms of the affected family members (L and R show left and ears, respectively); ; (B) the predicted secondary structure of elongated mutant P2RX2 protein. The mutant P2RX2 protein is composed of new α -helices (pink) and strands (yellow), compared with the wild-type P2RX2 protein composed of 6 α -helices (data not shown). Coils are presented as straight lines, and 58 additional amino acids residues in the C-terminal highlighted in yellow generate new α -helices and strands.

adapters were ligated to 3' and 5' ends of the fragments. Then, the 200-300 bp product was chosen for further PCR amplification. A Human capture array (Roche NimbleGen, USA) was designed to capture all coding regions, and the intron/exon boundaries of the genes, which are involved in the pathogenesis of HL, by NGS approach (BGI-Shenzhen, followed Guangdong, China). After NGS sequencing, the sequence reads were mapped onto the reference human genomic DNA (UCSC/hg19). For the subsequent variant analysis, GATK software version 4.1 was used to assemble the consensus sequence and detect single nucleotide variants and indels in target regions. Moreover, the search for rare variants (minor allele frequency (< 1%), which were particularly found in the affected girl, was carried out by using single nucleotide polymorphism and 1000 Genomes databases. The effect of these candidate variants on protein structure and phylogenetic conservation was estimated by bioinformatic tools such as SIFT^[7], PolyPhen-2^[8], and Mutation Taster^[9], to predict the pathogenicity of variants. Potential candidate variants observed in each gene using NGS were confirmed by direct sanger sequencing with ABI3500 sequencer (Applied Biosystems, USA). PCR primer sequences and conditions are available on request. Segregation

analysis was carried out for other relatives members. Also, PSIPRED 4 (http://bioinf.cs.ucl.ac.uk/psipred)^[10] was applied to predict the secondary structure of the wild-type P2RX2 and elongated mutant P2RX2 (p.*350Gluext*58).

Ethical statement

The above-mentioned protocols were approved by the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (ethical code: U-91074). Written informed consents were provided by the patients and their parents.

RESULTS

To identify whether the aggregate contribution inheritance leads to NSHL, we carried out comprehensive clinical genetic testing by sequencing all known and candidate NSHL genes with nonsyndromic mimic genes. We detected a stop loss mutation in the P2RX2 gene, which corresponded to c.1048T>G (p.Ter350Glu). DNA sequence analysis of the other genes failed to identify any disease causing sequence variants in the family. We used in silico pathogenicity prediction algorithms (Mutation Taster, SIFT, and PolyPhen), and all scores showed "damaging" or "disease causing". Also, we performed sanger direct sequencing for a family segregation analysis and for the verification of the detected variant in the proband. As shown in Figure 1B, the Sanger direct sequencing results demonstrated that the proband (II-1) and her mother had the mutation. The c.1048T>G was not found in her father and none of 50 healthy controls investigated in this study. The proband and her mother were tested otoscopically, and otoimmittance measurements were obtained to examine middle ear status, which was normal. Clinical examinations revealed no other abnormalities. Pure tone air and bone thresholds were distinguished at the frequencies of 250, 500, 1000, 2000, 4000, and 8000 Hz, with noise intensities from 1 to 120 dB (Fig. 2B).

DISCUSSION

In present study, we recognized a novel mutation in the *P2RX2* gene in an Iranian pedigree with NSHL. The *P2RX2* gene is one of the most recent genes known as a cause of ADNSHL (OMIM; 608224). The *P2RX2* mutations seem to be very rare for the reason that there have been only two described mutations: c.1057G>C (p.Gly353Arg) and c.178G>T (p.Val60Leu) from Italy and China, respectively^[11,12].

The P2X2 receptor is a protein encoded by the *P2RX2* gene and plays an important function in the cochlea as a ligand-gated ion channel receptor by ATP-mediated regulation^[13]. This ATP-activated ion channel binding to ATP intercedes synaptic transmission between some neurons and from neurons to smooth muscle cells. P2X2 receptors are predominantly expressed in the epithelial cells near the cochlear duct of the endolymphatic compartment in the inner ear, which consists of the organ of Corti^[14]. The P2X2 protein is upregulated due to exposure to noise. ATP is regarded to have a neurotransmission effect at the hair cells synapse and chip in the regulation of the endocochlear potential^[15,16].

Each receptor is composed of three polypeptide subunits (P2X1-7), which all share the general basic structure of intracellular C- and N-terminal, a large extracellular loop and two transmembrane domains. Functional receptors can be organized from triplets of identical subunits or homomeric and can also exist as heteromers complexes^[17]. Yan *et al.*^[11] have previously reported two unrelated Chinese pedigrees with autosomal dominant deafness-41, which carry a missense mutation (p.Val60Leu) in the P2RX2 gene, causing a substitution between two hydrophobic amino acids and removing the P2X2 response to ATP. This missense mutation in the first Chinese pedigree was recognized by WES; the second unrelated Chinese pedigree was 1 of 65 pedigrees in whom the P2RX2 gene was sequenced. Moreover, a missense mutation in the P2RX2 gene (p.Gly353Arg) in an Italian pedigree with dominant deafness-41 was described by Faletra et al. [12], confirming the finding of Yan et al. [11] who implied that mutations in the P2RX2 gene can cause progressive deafness.

The substitution of a stop codon with a charged amino acid such as glutamic acid could threaten the fold of the protein and interaction with the membrane. These results strongly propose that the p.Ter350Glu mutation should have a related impact on both the structure of the protein and function. Secondary structural study revealed that the wild-type P2RX2 protein is consisted of six α-helices. Though the extended mutant P2RX2 is consisted of six α -helices, the elongated 58 additional residues in C-terminal sequence produce new α -helix and β strand, finally causing the change of the structure of *P2RX2* (Fig. 2B). Also, multiple sequence alignments of human P2RX2 protein by ConSeq web server (PolyPhen, SIFT, and Mutation Taster) proved high conservation of this amino acid among various types of species; hence, this mutation can affect the ATP-mediated regulation activity of P2RX2 and can cause developmental abnormalities, leading to autosomal dominant deafness. Generally, it seems that this substitution could have a key function in the P2RX2 protein, and mutations at this site give rise to pathogenicity and deafness. In the present study, the modification in *P2RX2* gene was found to interfere with the normal stop codon located at position 350 in exon 10, causing a termination-site change and continuation of translation into the 3' UTR, recognized (Fig. 1C) from one allele of proband. Stop-loss variants are single base-pair exchanges that happen within translational termination codons, which could result in the continued translation of the messenger RNA into the 3' UTR^[18]. These variants mutations are reported in some cases involving different diseases^[19,20].

The p.Ter350Glu alters the amino acid sequence of P2RX2 protein and typically causes the open reading frame of the protein as the wild-type protein has 349 residues, while the mutant type has 407 amino acids. A stop loss mutation in the *P2RX2* gene (c.1048 T>G: p.Term350Glu; Fig. 1B) destroys the functional ochre termination codon (UAA) at the 3' end of P2RX2. The next termination codon is a 174-bp downstream (in the 3' UTR), expecting to add 58 residues to the carboxyterminal end of P2RX2, though no functional analysis procedures were carried out to verify this. There are three hypotheses. First, the mRNA transcript comprising the stop loss allele is degraded (with 'nonstop decay' pathway), proposing haploinsufficiency of $P2RX2^{[21]}$. The second mechanism makes the protein chains unable to assemble in the correct fashion. In this setting, the chain is not generally very stable. Third, while this process of non-stop decay is fairly effective at removing stop loss mRNAs, any protein products generated by the translation of residual stop loss mRNAs are degraded by the proteasome. Therefore quantitative- and translation-based researches are needed to prove the lack of both the mutant RNA and the extended protein. Even though a number of researchers implicitly suppose that the normal open reading frame will simply be developed until the next in-frame termination codon is encountered. Very few stop loss mutations in human have hitherto been recognized to allow any common conclusions to be drawn as to their possiblity phenotypic results in either expression levels of protein or mRNA^[18].

In conclusion, using WES, we identified one novel stop loss mutation (p.Ter350Glu) in *P2RX2* in Iranian family members with ADNSHL. Our findings expand the *P2RX2* mutation spectrum, and the detection of additional disease-causing mutations in this gene could verify more the crucial role of the *P2RX2* in auditory function. Moreover, further functional studies are required to investigate the role of p.Ter350Glu mutation in the function of auditory.

ACKNOWLEDGEMENTS

The authors thank participants and their family members for their participation in this study. The present work was financially supported by the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

CONFLICT OF INTEREST. None declared.

REFERENCES

- Shearer AE, Hildebrand MS, Sloan CM, Smith RJ. Deafness in the genomics era. *Hearing research* 2011; 282(1-2):1-9.
- Talebi F, Mardasi FG, Asl JM, Sayahi M. Nextgeneration sequencing identifies three novel missense variants in ILDR1 and MYO6 genes in an iranian family with hearing loss with review of the literature. *International journal of pediatric otorhinolaryngology* 2017; 103:103-108.
- Kemperman MH, Hoefsloot LH, Cremers CW. Hearing loss and connexin 26. *Journal of the royal society of medicine* 2002; 95(4):171-177.
- Chang MY, Choi BY. Strategy for the customized mass screening of genetic sensorineural hearing loss in koreans. Korean journal of audiology 2014; 18(2): 45-49.
- Rehm HL. Disease-targeted sequencing: a cornerstone in the clinic. *Nature reviews genetics* 2013; 14(4): 295-300.
- 6. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic acids research* 1988; **16**(3): 1215.
- Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nature protocols* 2009; 4(7):1073-1081.
- Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. *Nature methods* 2010; 7(4): 248-249.
- Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deepsequencing age. *Nature methods* 2014; 11(4): 361-362.
- 10. Buchan DW, Ward SM, Lobley AE, Nugent TC, Bryson K, Jones DT. Protein annotation and modelling servers at University College London. *Nucleic acids research* 2010; **38** (2): 563-568.
- 11. Yan D, Zhu Y, Walsh T, Xie D, Yuan H, Sirmaci A, Fujikawa T, Wong AC, Loh TL, Du L, Grati MH, Vlajkovic SM, Blanton S, Ryan AF, Chen ZY, Thorme PR, Kachar B, Tekin M, Zhao HB, Housley GD, King MC Liu |XZ. Mutation of the ATP-gated P2X2 receptor leads to progressive hearing loss and increased susceptibility to noise. *Proceedings of the national academy of sciences* 2013; **110**(6): 2228-2233.
- 12. Faletra F, Girotto G, D'Adamo AP, Vozzi D, Morgan A,

- Gasparini P. A novel P2RX2 mutation in an Italian family affected by autosomal dominant nonsyndromic hearing loss. *Gene* 2014; **534**(2): 236-239.
- 13. Housley GD, Kanjhan R, Raybould NP, Greenwood D, Salih SG, Järlebark L, Burton LD, Setz VC, Cannell MB, Soeller C, Christie D, Usami S, Matsubara A, Yoshie H, Ryan AF, Thorme PR. Expression of the P2X2 receptor subunit of the ATP-gated ion channel in the cochlea: implications for sound transduction and auditory neurotransmission. *Journal of neuroscience* 1999; **19**(19): 8377-8388.
- 14. Housley GD, Bringmann A, Reichenbach A. Purinergic signaling in special senses. *Trends in neurosciences* 2009; **32**(3):128-141.
- Telang RS, Paramananthasivam V, Vlajkovic SM, Munoz DJ, Housley GD, Thorne PR. Reduced P2x 2 receptor-mediated regulation of endocochlear potential in the ageing mouse cochlea. *Purinergic signalling* 2010; 6(2): 263-672.
- 16. Housley GD, Morton-Jones R, Vlajkovic SM, Telang RS, Paramananthasivam V, Tadros SF, Wong AC, Froud KE, Cederholm JM, Sivakumaran Y, Snguanwongchai P, Khakh BS, Cockayne DA, Thorne PR, Ryan AF. ATP-gated ion channels mediate adaptation to elevated sound levels. *Proceedings of the national academy of sciences* 2013; 110(18):7494-7499.
- 17. Mammano F. ATP-dependent intercellular Ca2+

- signaling in the developing cochlea: facts, fantasies and perspectives. *Inseminars in cell and developmental biology* 2013; **24**: 31-39.
- 18. Hamby SE, Thomas NS, Cooper DN, Chuzhanova N. A meta-analysis of single base-pair substitutions in translational termination codons ('nonstop'mutations) that cause human inherited disease. *Human genomics* 2011; **5**(4): 241-264.
- 19. Pang S, Wang W, Rich B, David R, Chang YT, Carbunaru G, Myers SE, Howie AF, Smillie KJ, Mason JI. A novel nonstop mutation in the stop codon and a novel missense mutation in the type II 3β-hydroxysteroid dehydrogenase (3β-HSD) gene causing, respectively, nonclassic and classic 3β-HSD deficiency congenital adrenal hyperplasia. *The journal of clinical endocrinology & metabolism* 2002; **87**(6): 2556-2563.
- 20. Ameri A, Machiah DK, Tran TT, Channell C, Crenshaw V, Fernstrom K, Khachidze M, Duncan A, Fuchs S, Howard TE. A nonstop mutation in the factor (F) X gene of a severely haemorrhagic patient with complete absence of coagulation FX. *Thrombosis and haemostasis* 2007; 98(6):1165-1169.
- Venkataraman K, Garcia-Diaz M, Karzai W. Non-stop mRNA decay: a special attribute of trans-translation mediated ribosome rescue. Frontiers in microbiology 2014; 5: 93.