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

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ABSTRACT

Background: Hearing loss, a congenital genetic disorder in human, is difficult to diagnose. WES is a powerful approach for ethiological diagnosis of such disorders. Methods: One Iranian family with two patients were attended 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), within 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 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.

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

INTRODUCTION

Hearing 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. 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. About 20–25% of NSHL forms are ADNSHL and 75–80% are ARNSHL, while only 1–1.5% is X-linked. 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.

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 of heterogeneous diseases. Targeted NGS provides a significant opportunity to detect variants in recognized disease genes, mainly in extremely heterogeneous diseases such as deafness.

On the basis of these data, we aimed to recognize...
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 originated 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 µg of genomic DNA was fragmented to 200–300 bp. Terminal A residues were added following the incubation with the Klenow Fragment exo-(3’→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.](image-url)
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, followed by NGS approach (BGI-Shenzhen, 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 non-syndromic 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 otomittancence 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[5,6,10]. 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\textsuperscript{18}. These variants mutations are reported in some cases involving different diseases\textsuperscript{19,20}.

The p.Ter350Glu alters the amino acid sequence of P2RX2 protein and typically opens the 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.Ter350Glu; 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 carboxy-terminal 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 ‘non-stop decay’ pathway), proposing haploinsufficiency of P2RX2\textsuperscript{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 possibility phenotypic results in either expression levels of protein or mRNA\textsuperscript{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.

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CONFLICT OF INTEREST. None declared.

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