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Case Report

Next-generation sequencing reveals a novel pathological mutation in the *TMC1* gene causing autosomal recessive non-syndromic hearing loss in an Iranian kindred

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ABSTRACT

Objectives: Hearing loss (HL) is the most common sensory-neural disorder with excessive clinical and genetic heterogeneity, which negatively affects life quality. Autosomal recessive non-syndromic hearing loss (ARNSHL) is the most common form of the disease with no specific genotype-phenotype correlation in most of the cases. Whole exome sequencing (WES) is a powerful tool to overcome the problem of finding mutations in heterogeneous disorders.

Methods: A comprehensive clinical and pedigree examination was performed on a multiplex family from Khuzestan province suffering from hereditary HL. Direct sequencing of *GJB2* and genetic linkage analysis of DFNB1A/B was accomplished. WES was utilized to find possible genetic etiology of the disease. Co-segregation analysis of the candidate variant was done. High resolution melting analysis was applied to detect variant status in 50 healthy matched controls.

Results: Clinical investigations suggested ARNSHL in the pedigree. The family was negative for DFNB1A/B. WES revealed a novel nonsense mutation, c.256G > T (p.Glu86*), in *TMC1* segregating with the phenotype in the pedigree. The variant was absent in the controls.

Conclusion: Here, we report successful application of WES to identify the molecular pathogenesis of ARNSHL in a large family. The novel nonsense *TMC1* variant meets the criteria of being pathogenic according to the ACMG-AMP variant interpretation guideline.

1. Introduction

Hearing loss (HL) is the most common sensory-neural defect in human, with incidence of 1–2 new cases in 1000 newborns [1]. This rate rises to 2.83 per 1000 in childhood and is further increased to 3.5 per 1000 in adolescents [2,3]. According to the World Health Organization (WHO) reports, about 360 million people are suffered from HL throughout the world (http://www.who.int/mediacentre/factsheets/fs300/en/). It accounts for the second most common disability in Iran after mental retardation [4]. This trait can negatively affect behavioral, cognitive, physical, social activities and life quality [5].

HL can be pre-lingual or post-lingual according to age of onset. Based on the lesion site, it is categorizing to conductive, if the defect is in the outer and middle ear, sensory-neural, if cochlear dysfunction occurs, and mixed, which is a combination of both forms. The severity of the phenotype is characterized by hearing threshold ranging from mild to profound. Syndromic forms are manifested with involvement of other body organs while HL is the sole clinically relevant feature in non-

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syndromic types [6]. The latter form comprise about 70% of the HL cases and autosomal recessive mode of inheritance is observed in 75–85% of the situations, while autosomal dominant pattern comprises 15–24% of the cases. X-linked recessive and mitochondrial inheritance account for rare causes of the disease, which involve 1–2% of the patients [7].

Autosomal recessive non-syndromic HL (ARNSHL) is a genetically heterogeneous disorder. So far, more than 90 distinct loci and 70 different genes with various functions have been discovered related to this trait (http://hereditaryhearingloss.org/).

Several studies have focused on the importance of consanguineous marriage in clinical genetics [8,9]. Autosomal recessive disorders are more common in populations with high rate of consanguinity, therefore Iran with an average rate of 38.6% consanguinity can be appropriate for studies on such diseases, including hearing loss [10].

DFNB1A/B encompasses Connexin26 (*GJB2*) and Connexin30 (*GJB6*), respectively [11,12]. These two genes account for the most common causes of HL in many countries. Homozygous or compound heterozygous variants in the *GJB2* gene leads to recessive form of non-syndromic HL [11]. Also, large deletions in the *GJB6* gene extend distally to *GJB2,* resulting in ARNSHL [13,14].

The prevalence of the *GJB2* mutations is variable in different populations of Iran, depending on ethnicity and geographical location [15–17]. *GJB2* mutations have an average range of 18.7% [18], with a frequency spectrum of 33% in the north of Iran to less than 2% in southern regions of Iran. DFNB1A/B mutation screening is crucial prior to large-scale analysis [19].

Non-syndromic hearing loss (NSHL) in not associated with specific phenotypic features, except rare conditions with inner ear malformations harboring *SLC26A4* mutations [20] and also those with U-shaped moderate to severe ARNSHL, which might have *TECTA* [21], or *CABP2* [22,23] mutations. Furthermore, in rare circumstances, pathogenic variants in syndromic-related deafness genes can mimic non-syndromic form of HL [24].

Whole exome sequencing (WES) is frequently applied in diagnostic medical genetics laboratories and research in order to unravel molecular etiology of clinically and genetically heterogeneous hereditary disorders [25–27]. This technique has the ability to reveal disease-causing variants in known disease-associated genes [28,29] and also to discover novel genes [22].

In the present study, we applied WES to resolve the genetic diagnosis of ARNSHL in a large inbred Iranian kindred, leading to identification of a novel nonsense mutation in the *TMC1* gene.

2. Materials and methods

2.1. Subjects and clinical investigations

An inbred family (Ahv-18) was ascertained from Khuzestan province of Iran. A comprehensive family history, including age of onset, exposure to ototoxic drugs during pregnancy and childhood infections, was obtained. A five generation pedigree was drawn. Air and bone conduction pure tone audiometry from 250 Hz to 8000 Hz were obtained [30,31]. Further clinical examinations were also carried out to rule out the involvement of other body organs and syndromic HL forms. Informed written consent was taken from participants before obtaining 5–10 ml of venous blood in EDTA-containing tube for DNA testing. The study was approved by Review Boards of the Isfahan University of Medical Sciences and the Shahrekord University of Medical Sciences.

2.2. Mutation screening of DFNB1A/B using both genetic linkage and sequencing

DNA was extracted from peripheral blood lymphocytes according to the standard phenol-chloroform procedure. DNA integrity and concentration were checked by agarose gel and Nanospec cube biophotometer (Nanolytik®, Dusseldorf, Germany). Using forward and reverse primers, the coding exon was PCR-sequenced bi-directionally by an automated sequencer, ABI 3130 XL (Applied Biosystems, Foster City, California, USA), as described before [32]. Also, using three short tandem repeat polymorphic markers (D13S1236, D13S1275 and D13S175) genetic linkage analysis was implemented to find homozygosity-by-descent at DFNB1A/B loci.

2.3. Whole exome sequencing

About 1 μg of genomic DNA sample of the patient (V:3), negative for *GJB2* mutations and without linkage to DFNB1A/B, was subjected to high throughput sequencing. In brief, fragmentation was done by hydrodynamic shearing system (Covaris, Massachusetts, USA) to generate 180–280 bp fragments. Liquid-phase targeted genomic enrichment was utilized to prepare libraries by Agilent SureSelect Human All Exon kit (Agilent Technologies, CA, USA). Sequencing was performed on IlluminaHiseq 2000 to generate 101 bp pair-end reads.

2.4. Bioinformatics analyses

BWA v0.7.8-r455 software was applied to map sequence reads to reference genome (hg19, NCBI Build 37). Samtools v1.0 and Picard v1.111 were used to sort the BAM files and to mark duplicates, respectively. Variant calling was done by GATK v3.1 and variant annotation was accomplished by ANNOVAR.

Variant filtering was performed based on MAF < 1% in dbSNP version 147, 1000 genomes project phase 3 database, NHLBI GO exome sequencing project (ESP), exome aggregation consortium (ExAC) and Iranome database for missense, nonsense, splicesite, stop loss, start codon change, frame-shift and in-frame indels. Human Gene Mutation Database (HGMD) and Clinvar were checked and a comprehensive literature review was performed to evaluate variant novelty and disease association.

2.5. Co-segregation analysis and control screening

Exon 8 of the *TMC1* gene was subjected to direct PCR-sequencing among available affected individuals (7 members) and healthy members of the pedigree to evaluate the co-segregation of the variant with the phenotype. Chromatograms were compared with reference sequence (NM_138691) using SeqMan software version 5.00 © (DNASTAR, Madison, WI, USA). Variant nomenclature was based on HGVS [33].

High resolution melting technique (HRM) [34] was performed in duplicate for screening the variant in 50 ethnically matched controls. HRM was conducted by a Rotor-Gene 6000 (Corbett Life Science, Qiagen) and using the EvaGreen PCR Master Mix. The reaction mixture (25 μl) contained 50 ng of genomic DNA, 17.5 pM of mixed primers of forward (CCTGCCTTCCTTAAGTTCCAAAGTC) and reverse (CCATGGT TTGCATTTGACAGTAGC) oligonucleotide primers (designed by Primer 3, http://primer3.ut.ee/), 9.25 μl water and 12.5 μl EvaGreen Master Mix. The PCR conditions included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles amplification consisting of denaturation at 95 °C for 10s, annealing at 60 °C for 20s and extension at 72 °C for 30s, followed by melting curve analysis to verify qPCR product identity. Then, the emitted fluorescence in each 0.1 °C temperature, increasing in the range of 65–90 °C was measured. Melting curves were created by the reduction in fluorescence with the increase in the temperature. Data were analyzed using Qiagen HRM software [35]. Totally, 10% (n = 5) of the control samples were randomly subjected to Sanger sequencing to confirm the HRM results.

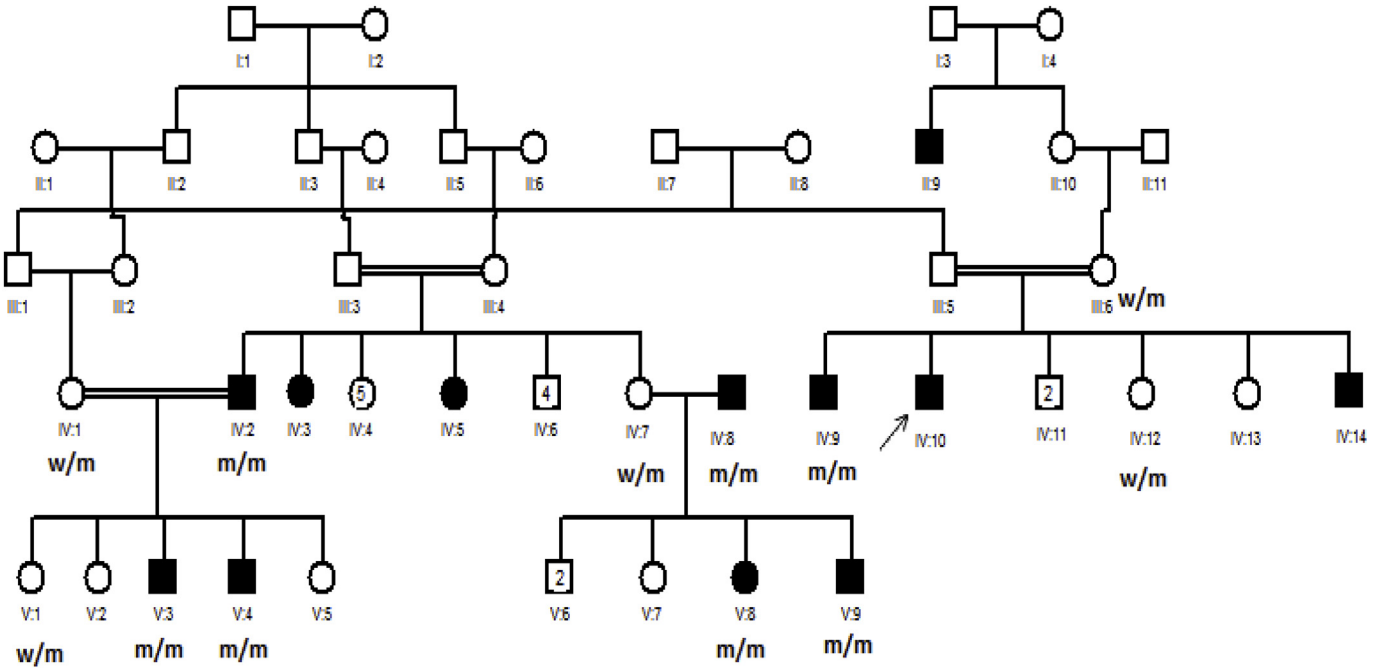

Fig. 1. Pedigree of the family. This figure shows pedigree of family Ahv-18 with ARNSHL. In this pedigree, white symbols: unaffected; black symbols: affected; squares: men; circles: females; parallel lines: consanguineous marriage. Symbols w/m and m/m demonstrate genotype wild type/mutant and mutant/mutant respectively. Selected patient for exome sequencing is indicated as proband.

3. Result

3.1. Clinical outcomes

A large highly inbred family with multiple (n = 11) affected individuals was enrolled in the study. The kindred had Arab ethnicity from Khuzestan province, southwest of Iran. The proband, with congenital HL, was born to a second cousin marriage (Fig. 1).

More clinical examinations did not indicate any cardiovascular, skin, ophthalmic abnormality in affected members. Therefore, syndromic form of HL was ruled out. Audiogram profile of the patients displayed profound hearing loss in all frequencies. The pedigree revealed several consanguineous marriages, suggesting ARNSHL in the offspring.

3.2. Molecular findings

Direct sequencing of the coding exon of the *GJB2* gene did not show any mutation. In addition, homozygosity mapping for DFNB1A/B ruled out existence of pathogenic variants in the non-coding region of *GJB2* and large deletions in *GJB6*.

WES was applied to identify the genetic etiology of the disease. The mean depth of coverage was 50X and 92% of targeted regions were covered.

The total number of variants was 363287. By applying the following strategy for variant filtering, the number of variants was limited to eleven in five genes. In the first step of variant filtering, we focused on nonsense, missense and frameshift indels and excluded noncoding or synonymous variants. Deleterious variants with higher impact on mRNA stability and protein structure were retained. According to the pedigree, as autosomal recessive mode of inheritance, homozygous variants were selected. Variants with MAF > 1% based on dbSNP and other population databases were discarded. The *TMC1* gene, known to cause ARNSHL was selected.

A novel homozygous transversion nonsense variant was identified in exon 8 of the *TMC1* gene. *TMC1*: c.256G > T (p.Glu86*) (NM_138691) leads to a truncated protein located in the first intracellular domain. The nonsense variant was absent from dbSNP version 147, 1000 genomes project phase 3, NHLBI GO ESP, ExAC, Iranome, HGMD and clinvar databases. The variant was not found in the literature. Sequencing of exon 8 of the *TMC1* gene among available healthy and affected family members confirmed co-segregation of the variant with ARNSHL in the pedigree (Fig. 2). The variant was observed as homozygote in all affected members. Screening of the variant via HRM in control samples revealed no mutation.

4. Discussion

HL is an extremely heterogeneous trait with over 100 known genes, which are rarely related to a distinctive phenotype. Lack of precise DNA diagnostics is a major obstacle for patient monitoring and genetic counseling in the family [36]. Fortunately, NGS has provided the opportunity for DNA testing in a time-saving manner [37,38]. Moreover, it reduces the expenses to one third in comparison with other routine genetic diagnostics methods [36].

Following the guidelines release by the ACMG for hearing loss [39], in the first tier of diagnosis, direct sequencing of the *GJB2* gene was performed. In addition, we performed the genetic linkage analysis of DFNB1A/B to rule out the most common cause of the disease. In the next step, WES was done, which upon further investigations unraveled a novel pathogenic nonsense variant co-segregating with the disease in the pedigree.

The *TMC1* gene is responsible for both autosomal recessive and dominant HL at DFNB7/11 and DFNA36 loci, respectively. This gene is located on chromosome 9q21.13 containing 24 exons, including 4 noncoding exons upstream of a methionine codon in exon 5. The protein encoded by this gene is predicted to contain 6 transmembrane domains with a cytoplasmic orientation of the N and C termini [40]. Although the amino acid sequence is completely known, the protein function is unclear. It was suggested that Tmc1 might be an ion channel or transporter which mediates K+ homeostasis in the inner ear [41]. Animal model studies have shown an orthologous recessive deafness locus for DFNB7/11 on mouse chromosome 19 causing profound HL [42]. The difference between them is 57 amino acids of Tmc1 (exon 14)

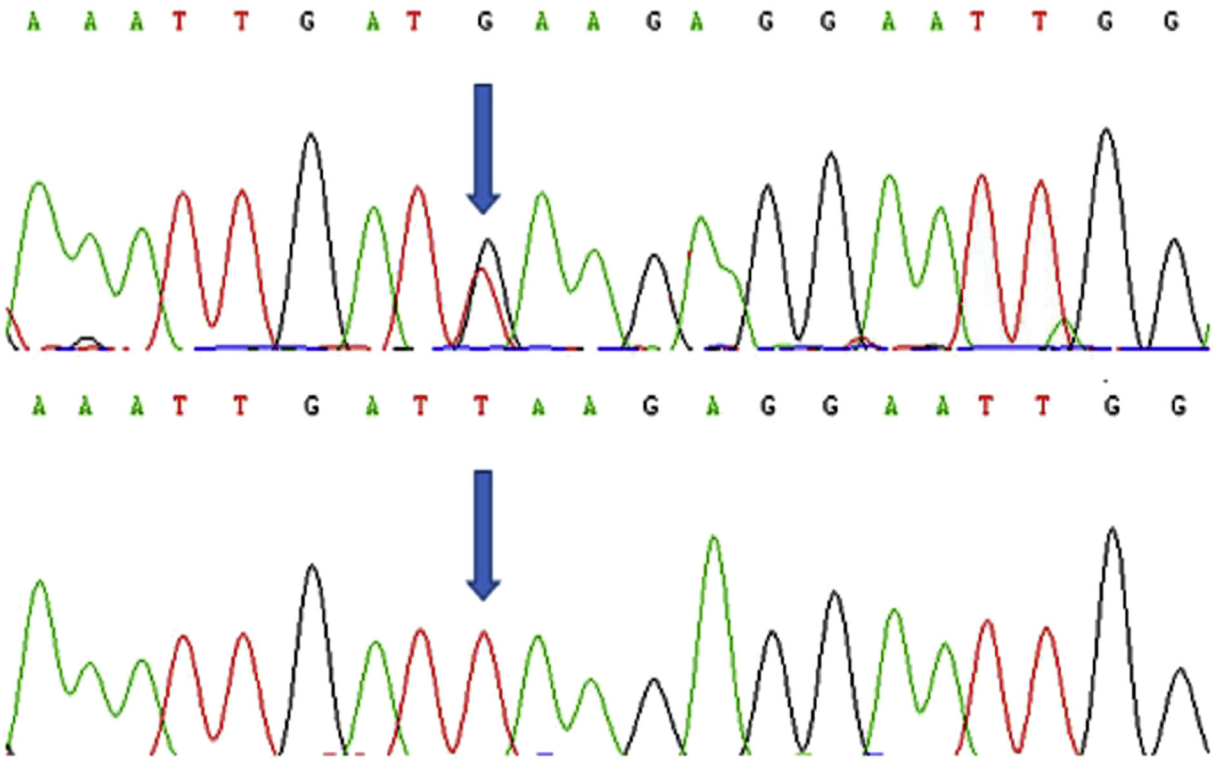

Fig. 2. Electropherogram showing the heterozygous (top) and homozygous (bottom) variant in c.256 position.

deleted in *dn* mice.

The *TMC1* gene is transcribed in human fetal cochlea and inner-ear tissue of postnatal mouse. The N- and C- termini of Tmc1 are unlikely to be tightly folded into a single and stable conformation. The distinct clusters of alternating opposite charge in the N terminus suggest an accordion model in which electrostatic interactions mediate the reversible association of adjacent clusters. Alternatively, these interactions may mediate the association of the entire domain with a similar domain on another polypeptide [40]. The *TMC1*: c.256G > T (p.Glu86*) is located on the first intracellular (IC1) region with high proportion of charged amino-acid residues in a relatively conserved nucleotide sequence across the mammals. This nonsense mutation lead to an early truncated protein; it is expected to cause HL due to nonsense-mediated decay or loss of downstream functional domain. Stop codon mutations in nucleotides closed to the end of sequence might have no major effect on protein level but disturb the transcription rate, the efficiency of mRNA processing or transport to the cytoplasm, or mRNA stability [43]. Furthermore, multiple sequence alignment of human TMC1 protein across other species revealed downstream residues are conserved, too. This fact indicates the importance of these residues in protein function and proper conformation. Loss of these amino acids might adversely influence natural function of the protein.

Based on the ACMG-AMP guideline [44], the variant is categorized as pathogenic: it is a nonsense variant at early positions (PVS1), it is absent in controls (PM2) and is co-segregating with the phenotype in the pedigree (PP1). Due to this guideline and combination of three evidences, 1 very strong (PVS1), 1 moderate (PM2) and 1 supporting criteria (PP1), it can be concluded that this variant is pathogenic.

Based on the known mutations in deafness genes, *TMC1* is the sixth most common cause of recessive HL worldwide and in Indian, Pakistani, Turkish, and Tunisian families, it is a common cause of recessive HL with prevalence of 2%, 3.4%, 6% (in another study 8%) and 4%, respectively [40,45–49]. *TMC1* mutations are considered to be common causes of ARNSHL in Iran with prevalence of 2%–2.2% [50–52]. However, in some ethnicities such as Moroccan Jewish population, higher prevalence of approximately 38% has been observed [41]. The variety of the identified mutations in the *TMC1* gene among Iranian ARNSHL families represent allelic heterogeneity among Iranian DFNB7/11 families [28,32]. All the mutations that have been identified to date are presented in Table 1. According to Table 1, here we report the 12th nonsense variant in the *TMC1* gene causing ARNSHL. Except our variant, two pathogenic stop gain variants have been reported in exon 8 [40,53].

By far, the most common recessive mutation for ARNSHL in the *TMC1* gene is p.R34X (c.100C > T) [54]. This mutation has been reported from several different populations such as Lebanon, Algeria, Iraq, Pakistan, Tunisia, Turkey and Iran [40,45,47,54–57]. Two different haplotypes associated to this variant was identified in these populations [57]. Only one non-coding variant with pathogenic role in the *TMC1* gene has been identified in an Iranian family. Variant g.94615 lies in exon3 of *TMC1* and may be located in the regulatory region or promoter of *TMC1* [54].

In summary, applying WES, we identified a novel pathogenic variant (c.256G > T) in a large Iranian ARNSHL family. The DFNB7/11 HL seems to show a significant allelic heterogeneity among Iranian families studied so far.

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Conflicts of interest

Authors, hereby, declare no conflict of interest.

Table 1
Overview of all *TMC1* mutations so far identified.

Coding region	variation	Protein	Exon (E)/Intron (I)	Type of variant	domain	Phenotype	Origin and ref.
−259	C > T	–	E3	Regulatory	–	severe to profound	Iran [54]
100	C > T	p.R34X	E7	Nonsense	IC1	Profound	Pakistan [40]
236 + 1	G > A	–	I7	Splice site	–	NA	Iran [48]
247[1a]	G > T	p.E83X	E8	Nonsense	IC1	severe to profound	[53]
295	delA	–	E8	Deletion	IC1	severe-to-profound	Pakistan [40]
458[2]	G > A	p.W153X	E9	Nonsense	NA	NA	Belgium [58]
534	A > C	p.E178D	E11	Missense	NA	severe to profound	Turkey [59]
545[3]	G > A	p.Gly182Asp	E11	Missense	NA	severe to profound	[53]
582	G > A	p.W194X	E11	Nonsense	NA	NA	Turkey [48]
589[4]	G > A	p.G197R	E11	Missense	TM1	NA	China [60]
596	A > T	p.N199I	E11	Missense	NA	Moderate to severe	pakistan [61]
767	delT	p.F255FfsX14	E13	Deletion	EC1	severe to profound	Turkey [54]
776	A > G	p.Y259C	E13	Missense	EC1	NA	Turkey [54]
800	G > A	p.G267E	E13	Missense	EC1	severe to profound	india [62]
804[5]	G > A	p.W268X	E13	Nonsense	NA	NA	Turkey [49]
821	C > T	p.P274L	E13	Missense	TM2	Profound	turkey [46]
830	A > G	p.Y277C	E13	Missense	TM2	severe to profound	pakistan [63]
1080_1084	delGATCA	p.R362PfsX6	E15	Deletion	IC2	NA	Turkey [48]
1083_1087	delCAGAT	p.R362PfsX6	E15	Deletion	IC2	severe to profound	turkey [46]
1107	C > A	p.N369K	E15	Missense	NA	severe to profound	China [64]
1114	G > A	p.V372 M	E15	Missense	TM3	severe to profound	pakistan [63]
1165	C > T	p.R389X	E15	Nonsense	EC2	Profound	Sudan [65]
1166	G > A	p.R389Q	E15	Missense	EC2	severe to profound	turkey [54]
1171[4]	C > T	p.Q391X	E15	Nonsense	EC2	profound	China [60]
1209	G > C	p.W403C	E15	Missense	NA	severe to profound	China [64]
1210[6]	T > C	p.W404R	E15	Missense	NA	Profound	Jewish Moroccan [41]
1247	T > G	p.L416R	E16	Missense	NA	severe to profound	[53]
1283	C > A	p.Ala428Asp	E16	Missense	NA	severe-to-profound	India [66]
1330	G > A	p.G444R	E16	Missense	TM4	NA	Turkey [48]
1333	C > T	p.G445R	E16	Missense	TM4	NA	turkey [48]
1334	G > A	p.R455H	E16	Missense	TM4	severe to profound	turkey [63]
1396_1398	delAAC	p.466del	E16	Deletion	TM4	severe to profound	China [67]
1404 + 1	G > T	–	I16	Splice site	–	Moderate to severe/severe to profound	pakistan [61]
1405–13	C > G	–	I16	Splice site	–	severe to profound	india [62]
1534	C > T	p.R512X	E17	Nonsense	NA	severe-to-profound	pakistan [40]
1566 + 1	G > A	–	I17	Splice site	–	severe to profound	india [62]
1566 + 4	delA	–	I17	Splice site	–	severe to profound	india [62]
1586_1587	delTC		E18	Deletion	NA	sever to profound	iran [68]
1589_1590	del CT	p.S530X	E18	Deletion	NA	profound	iran [69]
1685_2280	del	–	E19–24	Deletion	Na	sever to profound	turkey [45]
1703	A > G	p.Y568C	E19	Missense	NA	Profound	iran [68]
1718[7]	T > A	p.I573 N	E19	Missense	NA	NA	Ecuador [49]
1763 + 3[2]	A > G	p.W588WfsX81	I19	splice site	TM6	NA	Netherland [58]
1764	G > A	p.W588X	E20	Nonsense	IC2	profound	[56]
1788	C > A	p.S596R	E20	Missense	IC2	Moderate to severe	Pakistan [61]
1808	T > A	p.L603H	E20	Missense	IC2	profound	Qatar
1810	C > G	p.R604G	E20	Missense	IC2	profound	Morocca [70]
1809	C > T	p.R604G	E20	Nonsense	IC2	NA	Greece [54]
1939[6]	G > T	p.S647P	E20	Missense	TM6	profound	Jewish Morocca [41]
1959	C > G	p.Y653X	E20	Nonsense	TM6	NA	turkey [49]
1960	A > G	p.V654 M	E20	Missense	TM6	severe-to-profound	pakistan [40]
1979	C > T	p.P660L	E20	Missense	TM5-6	severe to profound	china [71]
2027[1]	T > A	p.V676Asp	E21	Missense	NA	severe to profound	[53]
2030	T > C	p.I667T	E21	Missense	EC3	severe to profound	turkey [40]
2050[5]	G > A	p.D684 N	E21	Missense	EC3	severe to profound	turkey [49]
2050	G > C	p.D684H	E21	Missense	EC3	severe to profound	turkey [59]
2130–1[7]	delG	–	I22	Splice site	NA	NA	Ecuador [49]
E14-15[3]	del	–	–	Large deletion	NA	severe to profound	[53]
E19-24	del	–	–	Large deletion	NA	severe to profound	turkey [48]

[a] The Superscript numbers represent the heterozygote variants.

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