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ORIGINAL ARTICLE

Homozygous Missense Variants in *FOXI1* and *TMPRSS3* Genes Associated with Non-syndromic Deafness in Moroccan Families

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Abstract

One of the most prevalent sensorineural disorders, autosomal recessive non-syndromic hearing loss (ARNSHL) which can affect all age groups, from the newborn (congenital) to the elderly (presbycusis). Important etiologic, phenotypic, and genotypic factors can cause deafness. So far, the high genetic variability that explains deafness makes molecular diagnosis challenging. In Morocco, the *GJB2* gene is the primary cause of non-syndromic hereditary deafness, while the existence of a variant in the *LRTOMT* gene is the second cause of this condition. After excluding these two frequently occurring *GJB2* and *LRTOMT* variants, whole-exome sequencing was carried out in two Moroccan consanguineous families with hearing loss. As a result, two novel variants in the *TMPRSS3* (c.1078G>A, p. Ala 360Thr) and *FOXI1* (c.6C>G, p. Ser 2Arg) genes have been discovered in deaf patients and the pathogenic effect has been anticipated by several bioinformatics and molecular modeling systems. For the first time, these variants are identified in the Moroccan population, showing the population heterogeneity and demonstrating the value of the WES in hearing loss diagnosis.

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Introduction

In the entire world, hearing loss is one of the most common sensory problems (Bakhchane et al. 2016). The majority of congenital hearing loss cases have a genetic cause, while it can also be caused by environmental factors (Salime et al. 2017). Hearing loss can result from a single gene pathogenic variant or from a combination of variants in various genes (Pan et al. 2022). Hereditary deafness can be syndromic and non-syndromic which 173 loci have been reported in syndromic presentations, and 131 genes have been discovered for non-syndromic deafness (https://hereditaryhearingloss.org/). Hereditary deafness study in the Moroccan population has been undertaken for a long time whose *GJB2* and *LRTOMT* genes are the primary genetic causes of hereditary hearing loss in Morocco (Bakhchane et al. 2015).

FOXI1 (MIM* 601,093) (also known as *FKHL10*) belongs to the forkhead-box (FOX) transcription factors family, which is characterized by the FOX ~ 100 amino acid monomeric DNA-binding region (Moreno-Estrada et al. 2010). It encodes a transcription factor that binds to the SLC26A4 promoter region and controls the upstream regulation of the gene (Lin et al. 2019). Research done in Sweden in 1998 found that *FOXI1* should be included as a candidate gene for deafness in humans since it was thought to be an early regulator required for the development of the cochlea and vestibule (Hulander et al. 1998). Additionally, FOXI1 has been linked to the control of vascular H^+-ATPase proton pumps in the kidney, epididymis, and inner ear (Landa et al. 2013).

Transmembrane protease serine 3 (*TMPRSS3*) (MIM * 605511) an enzyme present in the stria vascularis of the cochlear duct, spiral ganglion neurons, and inner hair cells is involved in the growth and maintenance of the perilymph and endolymph in the inner ear (Fan et al. 2014). Whereas *TMPRSS3* gene's role in the auditory system is unclear, its modification has been associated with non-syndromic hereditary hearing loss (Moon et al. 2021). *TMPRSS3* variants are linked to two types of hereditary non-syndromic recessive sensorineural deafness (DFNB8 and DFNB10) and appear to alter the proteolytic activity of TMPRSS3 (Holder et al. 2021). In several populations from Palestine, Pakistan, Tunisia, Japan, China, Korea, and Turkey, TMPRSS3 pathogenic variants have been identified (Moon et al. 2021). However, less than 1% of White people with non-syndromic genetic deafness have nonetheless been shown to carry this gene (Moon et al. 2021).

In this study, using whole exome sequencing (WES), we present the identification of two homozygous variants in *FOXI1* and *TMPRSS3* of two Moroccan families affected by hearing loss.

Patients and Methods

In this study, we enrolled two families with several deaf patients, family 1 and 2 (Fig. 1). All of the patients had severe non syndromic bilateral congenital hearing loss and came from consanguineous families. The study was approved by the

Pasteur Institute of Morocco's committee on research ethics and was conducted in line with the Helsinki Declaration's procedure.

From the patient's peripheral blood, genomic DNA was isolated by QIAamp DNA Blood Mini Kit Qiagen. WES was done at IntegraGen (Evry, France) on a single index case of each family (SF14 and SF144). IntegraGen configures a library, capture, sequence and perform a sequence variant detection and annotation. The libraries preparation is achieved by the Agilent Human exome V5 (50 Mb) capture kit, accompanied by paired end sequencing on an Illumina Hiseq2000. The capturing of the sequence was carried out in accordance with the manufacturer's instructions. An image analysis and base calling may be produced

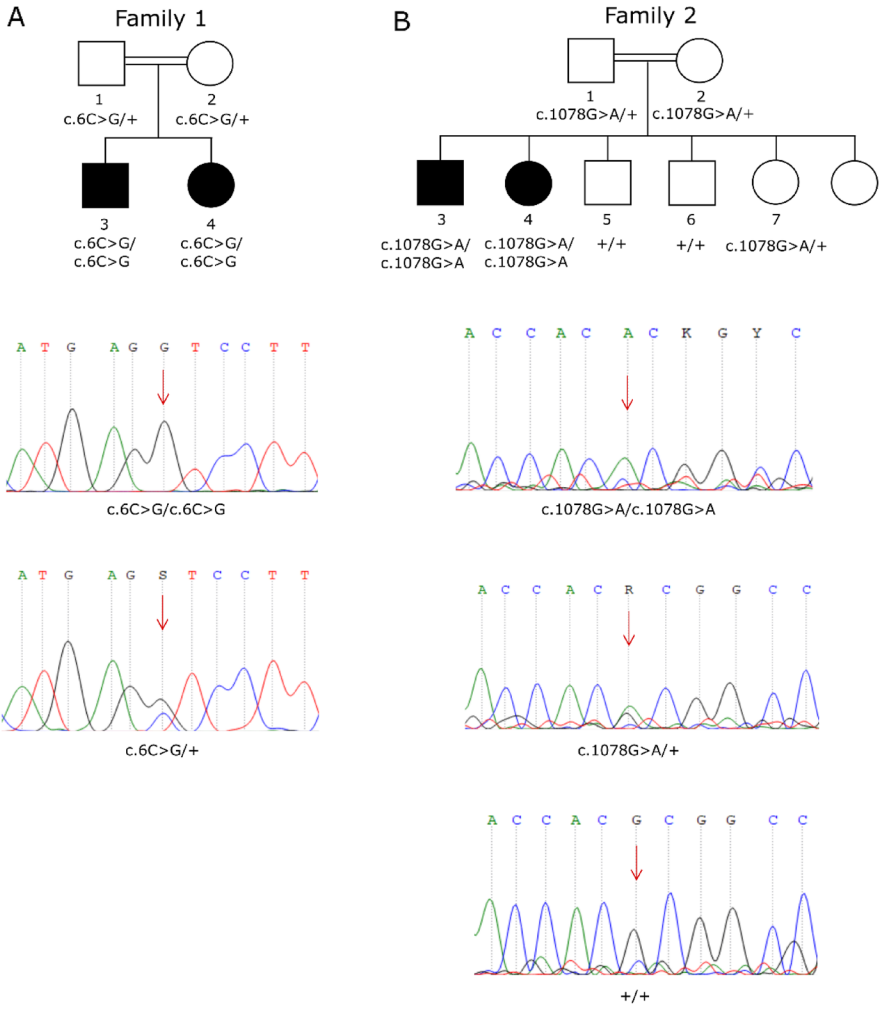

Fig. 1 **A** Pedigree and electropherograms of the family 1 presenting the variant in *FOXI1*. **B** Pedigree and electropherograms of the family 2 harboring *TMPRSS3* variant

using the Illumina Real-Time Analysis Pipeline version 1.14 with the default settings.

The human genome reference sequence hg19 is used for the alignment of paired-end short reads (GRCh37). Based on the Illumina pipeline (CASAVA 1.8), bioinformatic analysis of sequencing data was performed. The selection of pathogenic candidate variants was conducted using succession of filters as known genes causing hereditary deafness were prioritized first. As the pedigree of the families suggested an autosomal recessive inheritance pathway, we screened homozygous and heterozygous compound missence, nonsense, frameshift and splice-site variants with allele frequencies < 0.01 using gnomAD, the 1000 genome project and dbSNP (build 132) databases. The pathogenicity of the remaining variants was examined using PolyPhen-2, Mutation Taster, and SIFT. The conservation analysis was done by Consurf (http://consurf.tau.ac.il/), a web server that analyzes the level of conservation to determine the functional sections of a protein. Sanger sequencing was used to confirm the variation of the candidate gene to validate the familial segregation with the disease phenotype.

To determine amino acid change effect on protein structure, a molecular modeling strategy was used. First, the UniProt database was used to download the FOXI1 (Q12951) and TMPRSS3 (P57727) amino acid sequences in FASTA format. Next, we needed the three-dimensional (3D) structure of the native protein. This was done by homology modeling using the ITasser server and Chimera software to obtain the two proteins mutated structure (Yang et al. 2015; Rodríguez-Guerra Pedregal and Maréchal 2018). After obtaining the PDB files, the energy minimization for all these 3D structures was achieved with the YASARA Energy Minimization Server (Krieger and Vriend 2014).

Results

The whole exome sequencing of the family 1 patient's DNA was obtained with an average depth on target of 164.25 and a coverage of 99.4%.

WES results revealed a homozygous missense variant in *FOXI1* (NM 012188.5: c.6C>G; p. Ser 2Arg), present in both the patients of family 1. Sanger sequencing confirmed that this variant segregated with the disease and the healthy parents were heterozygous (Fig. 1A, B). The variation was absent in all data base and it's predicted to be disease causing by Mutation Taster (0.999), damaging by SIFT (0.002) and possibly damaging by Polyphen2 (0.909). The American College of Medical Genetics and Genomics (ACMG) has classified this variation as pathogen by the criteria PM1, PM2, PP2, PP3, and PP1 added after confirming segregation. On the report of the conservation scale findings, we can see that the p. Ser 2Arg substitution is located in a conserved location (8 on the conservation scale), and according to the neural-network algorithm, represents an exposed residue (Fig. 2A).

For the family 2, the WES of its proband's DNA was performed with an average depth on target of 197.42 and a coverage of 99.2%. After the analysis of the WES results, a homozygous variant in the *TMPRSS3* gene (NM_024022.3: c.1078 G>A) leading to a substitution of alanine in position 360 for threonine (p.

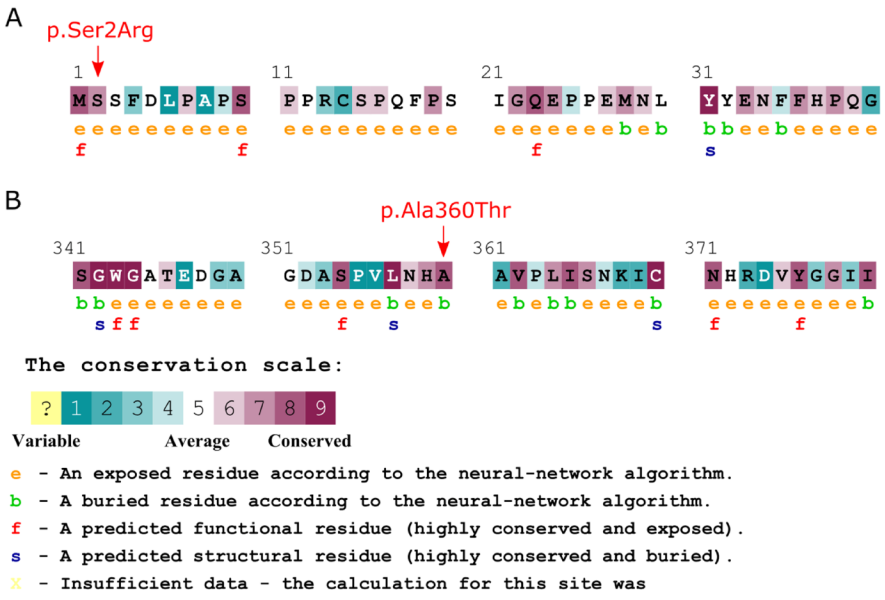

Fig. 2 Conservation results of FOXI1 and TMPRSS3 proteins variant

Ala 360Thr) and referenced in gnomAD Exomes with a frequency of $3.98 \times e{-}6$ was selected as candidate variant. This variant was confirmed in homozygous state in the index patient and his sick brother, in heterozygous state in both unaffected parents and one brother and in wild type sate in two other brothers (Fig. 1B). Prediction tools distributed the variant as disease causing by Mutation Taster (0.999), damaging by SIFT (0.003) and probably damaging by Polyphen2 (0.979). This variant was classified as likely pathogenic according to the ACMG criteria PM1, PM2, PP3 and PP1 added after confirming the segregation. The color-coded conservation scale indicates that the site of the TMPRSS3 protein is in a conserved region (8 on the conservation scale) (Fig. 2B).

Using YASARA, we visualized the structures of wild-type and mutant proteins and discovered differences in the hydrophobic interactions and hydrogen bonds between amino acids. For the p. Ser2Arg substitution, serine had with the amino acid Glu 24, one hydrogen bond and one hydrophobic interaction, the change from serine to arginine lost the previous two bonds and built two hydrogen bonds with Ile 21 and hydrophobic interaction with Glu 24 and Phe4. The RMSD value between mutated and wild-type structures was 1.0259 A (Fig. 3).

Interactions between amino acids showed that p. Ala360Thr amino acid change affected neither the hydrogen bonds nor the hydrophobic bonds, but the RMSD value between mutated and wild type structures was 0.8296 A (Fig. 4).

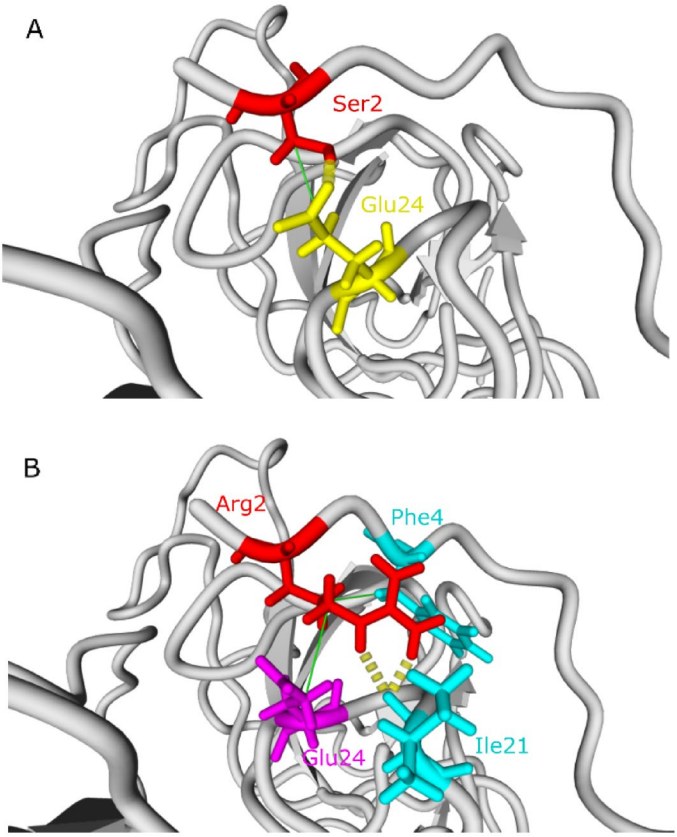

Fig. 3 Potential structural impact of the p.Ser2Arg FOXI1 amino acid change by molecular modeling. **A** Wild-type (Ser2). **B** Muted form (Arg2)

Discussion

Whole exome sequencing is the most effective method for detecting causing disease variant. Two variations of the *FOXI1* and *TMPRSS3* genes were identified in this study in two consanguineous Moroccan families with non-syndromic hearing loss.

FOXI1, a member of the Forkhead family of transcription factors, is able to interact with the *ATP6V0A4* promoter and directly regulate the expression of the *ATP6V0A4* gene in the kidneys, inner ear, and epididymis (Klarov et al. 2022). Additionally, FOXI1 has been linked to the structuring of the distal nephron epithelium and adequate acid–base homeostasis in the kidney, which leads to distal renal tubular acidosis (dRTA). *FOXI1*'s variants have been associated to sensorineural deafness and dRTA (Table 1) (Enerbäck et al. 2018). In this study, we identified for the first time a homozygous *FOXI1* variant (c.6C>G; p.Ser2Arg) in two patients with isolated hearing loss at the time of enrollment but no follow-up was possible to assess any evolution of a dRTA. This variant is located in a conserved residue of

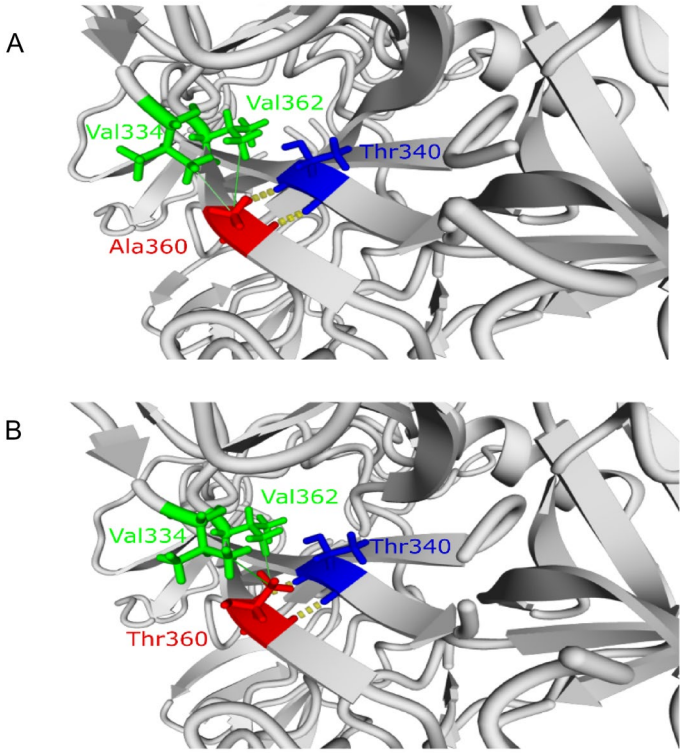

Fig. 4 Potential structural impact of the p.Ala360Thr *TMPRSS3* amino acid change by molecular modeling. **A** Wild-type (Ala360). **B** Muted form (Thr360)

Table 1 All found variants in *FOXI1*

Variants	Amino acid change	Genotype	Source
c.6C>G	p.Ser2Arg	Homozygous	This study
c.214C>A	p.Pro72Thr	Heterozygous	Liu et al. (2020)
c.367C>T	p.Arg123Trp	Heterozygous	Landa et al. (2013)
c.436C>T	p.Leu146Phe	Homozygous	Enerbäck et al. (2018)
c.519C>A	p.His173Gln	Heterozygous	Liu et al. (2016)
c.565G>A	p.Asp189Asn	Heterozygous	Lin et al. (2019)
c.638G>C	p.Arg213Pro	Homozygous	Enerbäck et al. (2018)
c.677C>T	p.Thr226Ile	Heterozygous	Pique et al. (2014)
c.716C>T	p.Pro239Leu	Heterozygous	Cirello et al. (2012)
c.772G>C	p.Gly258Arg	Heterozygous	Yang et al. (2007)
c.773G>A	p.Gly258Glu	Heterozygous	Yang et al. (2007)
c.800G>A	p.Arg267Gln	Heterozygous	Yang et al. (2007)
c.1004G>T	p.Gly335Val	Heterozygous	Yang et al. (2007)

the FOXI1 protein and exposed according to the neural network algorithm According to the ACMG, this variant's classification is uncertain significance. However, the structural modeling analysis conducted to examine the effects of this causative variant on protein structure has able to demonstrate that the p.Ser2Arg variant affected the protein structure as it gained and lost interactions and bonds with surrounding amino acids. These changes may have affected the protein's integrity and stability evaluated by the high RMSD score between wild type and mutated structure. Most of *FOXI1* variants were heterozygous causing a syndromic hearing loss, and located in a functional region between 121 and 211 named as Fork-head Domain, a conserved DNA-binding domain of about 100 amino acid residues, also known as a (winged helix) (Weigel et al. 1989). Although this domain is present in a variety of transcription factors, they all play a role in the early developmental decisions about cell fate during embryogenesis (Blomqvist et al. 2004). Enerbäck et al. reported two homozygous *FOXI1* missense variants (p.Leu146Phe and p.Arg213Pro) that do not directly affect membrane transport proteins but may affect the highly preserved DNA binding domain. This significantly lowers the activation of several target genes, especially membrane transport proteins, which appropriate expression depends on FOXI1 connections causing a severe acidosis and deafness syndrome two novel loss-of-function variants (Enerbäck et al. 2018). The heterozygous variant c.214C>A (p.Pro72Thr) in exon1, was discovered in the Chinese population, according to (Yalan Liu), who claims that the transcription factor FOXI1 is a key player in deafness associated with EVA (Liu et al. 2020). Another variant, c.565G>A (p.Asp189Asn), was reported in this gene in the Chinese population as well. This pathogenic variant compromised the binding affinity of *FOXI1* with the promoter region of SLC26A4 and was situated in the conserved fork-head DNA-binding domain of *FOXI1* (Lin et al. 2019).

The type II transmembrane serine protease encoded by TMPRSS3 is essential for the morphological and functional development of the inner ear as well as the preservation of the perilymph and endolymph's contents (Lee et al. 2023). TMPRSS3's variants are associated with two different phenotypes which are a hearing loss linked to the prelingual DFNB10 and another linked to the post-lingual DFNB8 (Liang et al. 2022). To our knowledge, this is the first report of a TMPRSS3 missense variant, c.1078G>A; p.Ala360Thr, causing hearing loss in the Moroccan population. Analysis using Polyphen-2 software predicted him to be probably damaging, and it was also identified as damaging by analysis using SIFT and the site of the TMPRSS3 protein is in a conserved region. Moon and al summarized and examined all TMPRSS3 variants related to hearing loss between May 2000 and August 2021 (Moon et al. 2021). The p.Ala360Thr variant found in this study is located in exon 11 which is in a region between two pathogenic variants p.Glu347X and p.Tyr376Tyr in a serine protease domain (Ben-Yosef et al. 2001; Song et al. 2020). According to the data on variants overviews by (Gao et al.), missense mutations especially those that are present in the serine protease domain or adjacent to the active site, have severe effects (Gao et al. 2017). In the Chinese population the c.535G>A missense variant in exon 6 was found (c.535G>A) in a homozygous state, predicted as a disease-causing variant affecting the protein function by prediction algorithms (Fan et al. 2014). Following Next Generation Sequencing, Battelino et al. discovered a

frame shift variation of *TMPRSS3* c.208delC (p.His70Thrfs * 19) linked to hereditary non-syndromic deafness in Slovenia as it was carried in a homozygous state by a patient and his mother and as a digenic compound heterozygote by his father (Battelino et al. 2016). Furthermore, to evaluate the structural impact of variations, a molecular modeling analysis for FOXI1 and TMPRSS3 was carried out. Due to changes in hydrophobic and hydrogen interactions, the FOXI1 protein's 3D structure has changed between its native and mutant states, while the TMPRSS3 protein has not changed between its native and mutated states.

Conclusion

Whole exome sequencing of two deaf Moroccan consanguineous families discovered novel variations on *TMPRSS3* and *FOXI1* in hearing-impaired patients. This study confirmed the population heterogeneity for this disease and demonstrates the value of the WES on molecular analysis.

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Data Availability Data will be provided by the authors upon request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval The genetic study was approved by the ethics committee for the biomedical research of Rabat.

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