Molecular insights into MYO3A kinase domain variants explain variability in both severity and progression of DFNB30 hearing impairment

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ABSTRACT
Hereditary hearing impairment (HI) is a common disease with the highest incidence among sensory defects. Several genes have been identified to affect stereocilia structure causing HI, including the unconventional myosin3A. Interestingly, we noticed that variants in MYO3A gene have been previously found to cause variable HI onset and severity. Using clinical exome sequencing, we identified a novel pathogenic variant p.(Lys50Arg) in the MYO3A kinase domain (MYO3A-KD). Previous in vitro studies supported its damaging effect as a ‘kinase-dead’ mutant. We further analyzed this variation through molecular dynamics which predicts that changes in flexibility of MYO3A structure would influence the protein-ATP binding properties. This Lys50Arg mutation segregated with congenital profound non-syndromic HI. To better investigate this variability, we collected previously identified MYO3A-KDs variants, p.(Tyr129Cys), p.(His142Gln) and p.(Pro189Thr), and built both wild type and mutant 3D MYO3A-KD models to assess their impact on the protein structure and function. Our results suggest that KD mutations could either cause a congenital profound form of HI, when particularly affecting the kinase activity and preventing the auto-phosphorylation of the motor, or a late onset and progressive form, when partially or completely inactivating the MYO3A protein. In conclusion, we report a novel pathogenic variant affecting the ATP-binding site within the MYO3A-KD causing congenital profound HI. Through computational approaches we provide a deeper understanding on the correlation between the effects of MYO3A-KD mutations and the variable hearing phenotypes. To the best of our knowledge this is the first study to correlate mutations’ genotypes with the variable phenotypes of DFNB30.

1. Introduction
Hearing impairment (HI) is the most common sensory human disease affecting almost 466 million people worldwide (World Health Organization (WHO), March 2020, https://www.who.int/en/news-room/fact-sheets/detail/deafness-and-hearing-loss). Over 50% of congenital or early onset non-syndromic HI cases are caused by genetic factors (Morton & Nance, 2006; Shinagawa, 2020). Hereditary non-syndromic HI is characterized by significant genetic and phenotypic heterogeneities. Introduction of Next Generation Sequencing (NGS) represents an effective tool to comprehensively address these heterogeneities (Liker et al., 2018; Neveling et al., 2013; Shearer & Smith, 2015; Yang et al., 2013). Therefore, the advent of NGS has expedited the discovery of several non-syndromic HI genes and mutations (over 123 genes are identified to date according to Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage (https://hereditaryhearingloss.org). Most recent update: 5/02/2021). These genes are implicated in a variety of molecular processes such as motor control, hair-cell structure, neuronal innervation and signal transduction.

Deafness autosomal recessive 30 (DFNB30) is caused by variants affecting MYO3A gene that is expressed in both photoreceptor and auditory hair cells (Berg et al., 2001; Sellers, 2000; Walsh et al., 2002). MYO3A (NM_017433.4) encoding MYO3A protein, a member of the myosin superfamily that contains more than 20 classes. Expression of this gene is highly restricted, with the strongest expression in retina and cochlea. In fact, Myosins are actin-dependent motor proteins categorized into conventional myosins (class II) and unconventional myosins (classes I and III through XV) based on their variable C-terminal cargo-binding domains. Particularly, the class III myosins, expressed in photoreceptors, uniquely have a kinase domain at their N-terminal (from aa 21 to aa 287), followed by the conserved motor domain (From aa 338 to aa 1053), three IQ motifs and a class specific
C-terminal tail region (Dosé et al., 2003; Dosé & Burnside, 2000).

In 2002, Walsh et al. identified three different mutations in MYO3A gene in a three-generation Jewish family suffering from bilateral progressive autosomal recessive non-syndromic HI (ARNSHI). Since then, six additional reports have identified a total of eleven recessive MYO3A mutations associated with variable age of onset and severity of ARNSHI (Choi et al., 2013; Li et al., 2018; Liu et al., 2019; Miyagawa et al., 2013; Qu et al., 2016; Sommen et al., 2016; Wu et al., 2015); of which only three have been linked to the kinase domain (KD).

It is noteworthy that mutations in MYO3A gene are associated to both autosomal recessive and dominant form of HI. Three dominant mutations have been identified, of which two are located in the motor-head domain and are thought to affect the ATPase activity of the gene: the c.2090T > G (p.Leu697Trp) in patients with late onset, with average age of onset of 30 to 32 years, progressive HI (Bueno et al., 2021; Dantas et al., 2018) and the c.1463G > A (p.Gly488Glu) responsible of a progressive post-lingual HI with onset in the early childhood (Grati et al., 2016).

The only autosomal dominant (AD) mutation c.716T > C, p.(Leu239Pro) in the KD was identified in a patients with progressive AD prelingual, moderate-to-profound HI (Doll, 2020). No apparent clustering of recessive and dominant variants was observed in either the kinase or the motor-head domain (Doll, 2020).

The KD of vertebrate class III myosin belongs to the Sterile-20 (STE-20) kinases family (Lin-Jones et al., 2004). Generally, two major conformational changes are associated with most protein kinases. It could either require the formation of a complete regulatory spine to form active enzymes, or they may occur in active kinases since they switch between open and closed conformations during the catalytic cycle (Roskoski, 2015). Although significant progress has been made in understanding the structure and function of pathogenic variants affecting protein kinases, in the STE-20 family, there is still a lack of systematic analysis of the structural and evolutionary characteristics of kinase activation and inactivation mutations in most human kinases. It was previously proposed that MYO3A-KD allows the autoregulation of myosin III motor-head domain (Quintero et al., 2010, 2013). In fact, MYO3A, when present with its active dephosphorylated motor-head domain, translocate to the tips of actin bundle-based structures. Once reaching the tip of the actin bundle, the auto-phosphorylation activity is more likely to occur as MYO3A become abundant in the tip compartment (Quintero et al., 2013). MYO3A influences stereocilia lengths and restricts the growth of microvilli within the formed auditory hair bundles thus it contributes to the hair bundle architecture that includes its staircase pattern required for normal hearing.

In this study, Clinical-Exome and Sanger sequencing of eight members from a consanguineous Tunisian family, allowed the identification of a novel MYO3A p.(Lys50Arg) variant. Previously, this variant was artificially generated and functional in vitro study supported the damaging effect of this ‘kinase-dead’ mutant (Quintero et al., 2010, 2013). Our findings represent the first study to report this ‘kinase-dead’ substitution as a pathogenic variant in human explaining the congenital profound ARNSHI phenotype.

Interestingly, three other causative MYO3A-KD variants have been previously reported with variable HI phenotypic severity. For a better understanding of the correlation between the MYO3A variations and the HI phenotypic severity, we provided a detailed computational structural analysis to predict the impact of these four KD pathogenic variants.

2. Materials and methods

2.1. Subjects

Tunisian consanguineous family was recruited and informed consents from both parents were obtained according to the guidelines of the Regional Committee of the Protection of Persons, Sfax, Tunisia (CPP SUD N°28/2019). Based on clinical questionnaire and family pedigree analysis, we retained the diagnosis of ARNSHI. Audiological tests through air and bone conduction pure tone audiometry were performed to identify the degree of HI. Peripheral blood samples of all subjects were collected in EDTA-containing tubes and genomic DNA was extracted using standard phenol-chloroform technique from the probands, both parents as well as all affected and unaffected siblings. All affected members were prescreened for the common HI mutations within GJB2 using PCR amplification with the following primers: Forward: 5'-TCTTTTCCAGAGCAAACCGC-3' and Reverse: 5'-CTGGGCAATGCGTAACTGG-3' followed by Sanger sequencing.

2.2. Clinical-exome sequencing

We performed NGS using a commercialized Clinical-Exome Sequencing TruSight™ One Gene Panel (Illumina Inc., San Diego, CA) for the identification of pathogenic variants in the ARNSHI family. Library preparation was performed according to the TruSight™ One Sequencing Panel Library Preparation Guide (Part #15046431 Rev. A). Then, libraries were loaded onto flow cell for a paired-end 121-bp sequencing with Illumina MiSeq platform (Illumina Inc., San Diego, CA). The Burrows-Wheeler (BWA) aligner was used to perform reads alignment to the human reference genome GRCh37/hg19.

2.3. Next generation sequencing data analysis and variant filtration

Sequence data was exported as variant calling files (.vcf) and imported into Variant Studio Data Analysis Software v3.0 (Illumina Inc., San Diego, CA) for analysis. Variants were filtered according to the following criteria: located on regions harboring known HI-causing genes, potentially functional missense, nonsense, indel variants and splice-site and absent or showing minor allele frequencies less than 0.07% (Oza et al., 2018) in genome aggregation database (gnomAD) (https://gnomad.broadinstitute.org/), dbSNP (https://www.ncbi.nlm.nih.gov/snp/) and Exome Variant Server (https://evs.gs.washington.edu/EVS/). Variants were further filtered based
on predicted pathogenicity through functional predictions algorithms. According to the familial history and pedigree, and based on an autosomal recessive pattern of inheritance, only homozygous variants were selected as candidates. Classification of the identified variants was performed according to the recommendations of standards and guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG & AMP).

2.4. Segregation analysis by Sanger sequencing

Sanger sequencing was performed to confirm the co-segregation of the novel variant c.149A > G p.(Lys50Arg) in patients and all available family members. To amplify the mutation’s region, we used the following primers: MYO3A-Forward: 5’-TTCCCATGGGTTGTGTG -3’ and MYO3A-Reverse: 5’-ATACTAAGCACAGAAATTGTGAA-3’. PCR products were sequenced on an ABI PRISM® 3500 (Thermo Fisher Scientific, Inc., Waltham, MA) according to the manufacturer’s instructions.

2.5. Molecular dynamics investigation

The association between the in-silico approach and wet lab experimentations has been rather evidenced by several previous studies (Mosaelli et al., 2017; Panchal et al., 2020; Zaki et al., 2017). CABS-flex 2.0, is an open access webserver (http://biocomp.chem.uw.edu.pl/CABSflex2) for the simulation of globular proteins. The updated provided the option to customize the restraints and parameters related to simulation (Kurcinski et al., 2019; Kuriata et al., 2018). It can be used for the study of dynamics of disordered proteins, structural flexibility and folding mechanism of proteins (Kurcinski et al., 2019). In our study, this webserver was used for a fast simulation of structural flexibility of the native and the Lys50Arg mutant form of MYO3A-KD protein using a PDB file of the query protein structure as an input (Jamroz et al., 2013). For a best possible convergence, our results were obtained based on the optimal simulation length of CABS flex; 10 ns molecular dynamics simulations, 1000 cycles, and 100 Cycles between trajectory frames and the other parameters were at default settings. The system equilibration and mean fluctuations of both MYO3A-WT and MYO3A-K50R residues were calculated by RMSF (Root Mean Squared Fluctuation), a numerical measurement, that calculates individual residue flexibility, or how much a particular residue fluctuates during a simulation. RMSF is useful to assess local flexibility in protein structures, allowing the identification of flexible and rigid regions (Craveur et al., 2015).

2.6. Variant collection and pathogenicity prediction

11 causative variants, located in both MYO3A motor and kinase domains, were collected from different research publications (Table 1). Only four KD-missense variants were included in our study for further analysis.

2.7. Structure prediction and in silico mutagenesis

To analyze the structural impact of the missense mutations on the MYO3A-KD, the sequence of the wild-type MYO3A (UniProtKB - Q8NEV4) coding for an actin-based motor-head domain with a protein kinase activity was submitted to the Phyre2 server (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index).

The 3-D structure of the MYO3A-KD was modeled based on the solved crystal structure of the KD of human Traf2- and Nck- interacting Kinase (PDB ID: 2X7F). The computer assisted modeling and Spdb viewer simulations were performed on the mutant allowing local regional changes for full-length 267 amino acids. The structure of the MYO3A kinase was relaxed to the YASARA/Amber force field through knowledge-based potentials within YASARA. The superposition and subsequent refinement of the overlapping regions yields a complete model for the MYO3A-KD. The final structures were subjected to energy-optimization by PR-conjugate gradient with an R-dependent dielectric. All structural representations in this paper were prepared with PyMOL (http://www.pymol.org).

3. Results

3.1. Hearing and novel pathogenic variant data in a consanguineous Tunisian family

In this study, we recruited a consanguineous Tunisian family, where parents I:1 and I:2 are first cousins and have normal-hearing (Figure 1). The three affected kids (II:1, II:2 and II:3) with congenital ARNSHI have no response to any pure tone audiometry signals. Hearing level for both ears of all affected individuals exceeds 95 dB. Ocular and vestibular analyses showed a normal vision and absence of balance abnormalities in all affected individuals.

Using Sanger sequencing, we firstly screened GJB2, a common HI gene in North African population, and no mutation has been found. We therefore proceeded to target sequence capture and NGS using Illumina Clinical-Exome Sequencing TruSight™ One Gene Panel on sample (II:3) to identify the possible pathogenic variant in this family. Given the large number of variants resulting from the high-throughput sequencing reaction, variant filtration was performed according to well-defined criteria, detailed in materials and methods section. Considering known HI genes, we finally identified a novel homozygous variant c.149A > G within
Table 1. Biallelic pathogenic mutations within MYO3A (NM_017433.4) responsible for recessive non-syndromic hearing impairment.

| Human recessive mutation | cDNA   | Genomic position | Protein  | Domain   | Age of onset and degree of hearing impairment | Origin | Reference          |
|--------------------------|--------|------------------|----------|----------|---------------------------------------------|--------|--------------------|
|                          | c.149A > G | 26241188        | p.(Lys50Arg) | Kinase   | Congenital                                  | Tunisian | Current study      |
|                          |         |                  |          |          | Hearing level of left and right ear of affected individuals exceed 95 dB |        |                    |
| 1                        | c.386A > G | 26285501        | p.(Tyr129Cys) | Kinase   | Progressive                                 | –       | (Li et al., 2018)  |
| 2                        | c.1841C > T | 26409669        | p.(Ser614Phe) | Motor    | Congenital                                  | Chinese | (Qu et al., 2016)  |
| 3–4                      | c.4462A > G | 26482157        | p.(Lys1488Glu) | –        | –                                            | Chinese | (Wu et al., 2015)  |
|                          | c.4861C > T | 26491987        | p.(Arg1561Ter) | –        | –                                            | –       |                    |
| 5–6                      | c.580C > A  | 26305920        | p.(Pro194Thr) | Kinase   | Profound                                    | Korean  | (Choi et al., 2013) |
|                          | c.1582_1583insT | 26385330      | p.(Tyr530Leufs*9) | Motor    | –                                            | –       |                    |
| 7                        | c.4267 > G  | 26286105        | p.(His142Gln) | Kinase   | Late-onset                                  | Japanese| (Miyagawa et al., 2013) |
| 8                        | c.1324C > A  | 26359295        | p.(His442Asn) | Motor    | Early-onset                                  | –       |                    |
| 9–11                     | c.3126T > G  | 26457658        | p.(Tyr1043Ter) | Motor    | Late-onset. It begins in the second decade  | Jewish- Iraq | (T. Walsh et al., 2002) |
|                          | c.1777-12G > A | 26409593      | p.(Ser614Phe) | Splice mutation | Progressive, first affecting the high frequencies, and by age 50 is severe in high and middle frequencies and moderate at low frequencies. | –       |                    |
|                          | c.732-2A > G | 26312949        | p.(Ser614Phe) | Splice mutation | –                                            | –       |                    |

Mouse model of human recessive mutation

| cDNA   | Protein  | Domain   | Phenotype                    | Origin | Reference                  |
|--------|----------|----------|------------------------------|--------|----------------------------|
| 1      | c.410A > G | p.(Tyr137Cys) | Kinase | Progressive                 | –       | (V. L. Walsh et al., 2011) |
| 2      | c.3126T > G | p.(Tyr1041Ter) | –       | Progressive, closely reflecting the hearing impairment of the family described in T. Walsh et al. (2002) | –       | (Li et al., 2018) |

**MYO3A** gene leading to the substitution of a critical Lysine in the kinase catalytic domain with Arginine at position 50 p.(Lys50Arg). This variant has not been reported in both dbSNP and gnomAD nor in ClinVar databases.

Validation analysis, using Sanger sequencing, revealed a full segregation of the new variant with the HI; all affected members were homozygous, both parents were carriers and three unaffected siblings were either wild type or heterozygous for this variant (Figure 1).

### 3.2. Pathogenicity prediction of the novel Lys50Arg KD-MYO3A missense variant

Variant pathogenicity was assessed using Varsome that displays an automated variant classification based on the standard and adapted ACMG/AMP guidelines (Oza et al., 2018; Richards et al., 2015). Classification of our newly identified variant (Lys50Arg) was performed based on the Hearing Loss specific rules outlined by Oza et al 2018 (Lin-Jones et al., 2004). The c.149A > G, presented PM2 (Variant not found in both dbSNP and gnomAD genomes) and PP3 ACMG-criteria. In fact, according to the Varsome output, the p.(Lys50Arg) was predicted as pathogenic using 11 computational prediction algorithms (PROVEAN, SIFT, REVEL, PrimateAI, DANN, M-CAP, MVP, Mutation Assessor, EIGEN, FATHMM-MKL and Mutation Taster).

By validating the segregation in eight family members, in MYO3A, a definitively known HI gene, we added the PP1 criteria supporting the deleterious effect for this genetic variant. This criteria represents, in this case, a strong pathogenic evidence since this variant segregated in three affected relatives with recessive HI. A well-established in vitro study supported the damaging effect of the identified p.(Lys50Arg) variation (Quintero et al., 2010) which represents an additional PS3 pathogenic criteria. Based on these criteria (PM2, PP3, PP1 and PS3), this variant was classified as likely pathogenic. The classification rules are detailed in Supplementary Table S1.

Through sequence alignment analysis (Mutation Taster) and conservation scores indicated in Varsome (6.01 in Genomic Evolutionary Rate Profiling: GERP) we confirmed that Lys50 residue is highly conserved within different species including Ptroglodytes, Mmulatta, Fcatus, Mmusculus, Ggallus, Trubripes, Drerio, Dmelanogaster and Xtropicalis (Figure 2).

### 3.3. Molecular dynamics simulation of the novel Lys50Arg variant

The novel pathogenic variant Lys50Arg was taken for further comprehensive analysis. Therefore, we ran MD simulations for both MYO3A-WT and MYO3A-K50R proteins for a standard time step of 10 nanosecond. A comparative analysis of the structural deviation between WT and mutant structure were computed using the CABS-flex protocol that generates structural protein models, a contact map of the residue-residue interaction pattern and a RMSF of atoms. Figure 3 shows the superimposition of the structure as well as the contact...
map of 10 models of flexibility simulation of both wild type and Lys50Arg MYO3A mutant as well as a fluctuation profile of amino acid residues that shows residue fluctuation profiles. Our findings showed that the complex has many fluctuations, the highest amplitude being of residue 298 of around 6 Å. In order to investigate how our mutant affected the dynamic behavior of the residue we based our analysis on the calculation of RMSF of the Cα atom of the native and mutant protein. In fact, the RMSF per residue is typically plotted vs. residue number, and indicate structurally which amino acids in the protein contribute the most to a molecular motion. We deduced from Figure 3 that residue level fluctuations for mutant structure were rather high comparing to the native structure, especially for residues located between 200 and 250 positions. It is important to note that the RMSF data of this novel pathogenic missense variant in a highly conserved AA manifesting a change in residue the fluctuation pattern compared to the wild type MYO3A protein that shifted from 2.441 to 0.532 Å. The RMSF analysis pointed to a flexibility decrease in the Lys50Arg within the KD region of the MYO3A protein (Figure 3) confirming our previous predictions.

3.4. Functional prediction and molecular modeling of MYO3A-KD

In this study, in order to explain the phenotypic variability induced by pathogenic variants within the MYO3A-KD, we built a structural model based on the KD structure of human Traf2- and Nck- interacting Kinase (PDB ID: 2X7F) sharing a high sequence identity with the KD of human MYO3A with a resolution of 2.8 Å in an open conformation refinement over best sequence identity. The modeled structure was validated to evaluate the structural properties in the proposed model including the packing effects and bumps, bond length, bond angles. First, the analyzed stereochemical quality with PROCHECK shows that there are 98.5% of residues in the allowed region for structures. It is worth noting that our results are within the quality score range.

The molecular model showed that MYO3A-KD exhibits an overall typical kinase fold, with N- and C-lobes (Figure 4). The upper part the N-lobe comprises residues 1 to 90 and contains five-stranded, antiparallel β-sheet (strands β1-β5) that packs against the αC-helix while the C-lobe is composed of six α-helices, a small two stranded β-sheet (strands β6-β7) and two small α-helices (αH- αJ). We also noticed the presence of several highly conserved residues that are important for kinase function: the glycine-rich loop, the DFG motif, the position of the αC-helix, and the catalytic and regulatory hydrophobic spines (C- and R-spine) (Figure 5). In fact, the glycine-rich loop localizes the c-phosphate of the ATP in a specific orientation facilitating phosphoryl transfer during catalysis (Taylor & Kornev, 2011).

As a second step, SwissPDBV was used to induce the four variants p.(Lys50Arg), p.(Tyr129Cys), p.(His142Gln) and p.(Pro194Thr) thus obtaining the mutant structures for MYO3A-KD. The steepest descent method and the Gromacs force field were used to evaluate the structure’s energy and...
Figure 2. Alignment analysis of the four conserved residues among different species including Ptroglodytes, Mmulatta, Fcatus, Mmusculus, Ggallus, Trubripes, Drerio, Dmelanogaster and Xtropicalis. (A) Lys50, (B) Tyr129, (C) His142 and (D) Pro194.

Figure 3. Molecular Dynamics simulation of MYO3A wild type and Lys50Arg mutant models through CAB-flex 2.0 server. (A) The ribbon view represents the superimposition of the top 10 simulated structures of MYO3A wild type and Lys50Arg mutant; (B) The correlation map represents contact maps of superimposition of top 10 simulated structures of wild type and Lys50Arg mutant that defines the contact in between the amino acids value (between [0; 1]), The color of square depends on the frequency of occurrence in this particular interaction; (C) Fluctuation plot that shows the residue-wise fluctuations recorded throughout the simulation of both WT and mutated structure of MYO3A-KD; (D) Root-mean-square fluctuations (RMSF) scores of atoms in the MYO3A Lys50Arg Mutant and wild type amino acid residue.
repair the distorted geometry, thereby minimizing the energy of all mutant structures.

3.5. Correlation between genotypes and protein structure/function

In this study, we noticed that two groups of variants could be responsible of HI phenotype variability.

3.5.1. Mutations specifically affecting the catalytic activity of the MYO3A-KD

According to our model, the c.149A>G pathogenic variant identified in this study caused the substitution of the highly conserved Lys at position 50 within the β3-strand of the N-lobe (Figures 5 and 6). The Ala-Xxx-Lys sequence in typically present in the β3-strand of all protein kinases hindering its catalytic capacity. Therefore, the MYO3A-Lys50 represents...
the key amino acid that forms a salt bridge with the conserved glutamate near the center of the αC-helix (E88) (Figure 5). The Lys50 also interacts with the terminal phosphate groups of ATP by stabilizing them in the correct position for catalysis which is essential for maintaining the catalytically active conformation of the small lobe.

The second conserved Pro194 residue plays a mandatory structural role in the maintain of the active form of the kinases since the activation segment starts from a DFG-motif and ends with Alanine–Proline–Glutamic consensus sequence in the APE-motif. The Tyr129 and His142 residues are both located within the αE-helix that connects the N-terminal subdomain and the C-terminal lobe.

3.5.2. Mutations probably affecting the whole MYO3A protein structure

As for the second group of variants, the structural model, in this study, showed that Tyr129 and His142 residues are both located within the αE-helix that connects the N-terminal subdomain and the C-terminal lobe (Figures 5 and 6). The αE-helix plays a critical role in transmitting structural changes within the KD, the missense variants from a polar amino acid to neutral hydrophobic cysteine p.(Tyr129Cys) as well as the variation from a cyclic Histidine residue into branch-chain Glutamine p.(His142Gln) thus would possibly mediate the disruption of the stability of the helix at those positions. They are expected to distort the structure and the function of the αE helix and thereby disrupt the function of MYO3A-KD and may even alter the motion of the whole protein MYO3A.

4. Discussion

Three different deleterious variants in MYO3A gene have been firstly reported to cause ARNSHI (DFNB30) (Walsh et al., 2002). Since then, six other reports identified additional MYO3A mutations associated with the recessive form of HI. In the present study, we identified a novel homozygous pathogenic variant p.(Lys50Arg) located in the MYO3A-KD in a DFNB30 Tunisian consanguineous family. Class III myosins uniquely contain a KD at their conserved N-terminal involved in auto-phosphorylation (Ng et al., 1996) which may influence their cellular localization and function (Dantas et al., 2018; Quintero et al., 2010, 2013).

Previous studies have reported the impact of the association between mutation and their position in the protein structure (Panchal et al., 2020; Zhang et al., 2018). Our molecular modeling results, has first helped to investigate the structural effect of our novel mutation on protein
structure. In fact, the highly conserved Lys50 within the MYO3A kinase domain interacts with the conserved glutamate near the center of the αC-helix forming the salt bridge. This was further corroborated by RMSF data which indicates that residue level variations were high for mutant protein, thereby indicating that mutation affected the protein conformation considerably leading to the increased flexibility of the protein. To apprehend the conformational changes in the mutated MYO3A-KD owing to the Lys50Arg mutation, molecular dynamics simulation was performed for 10 nanoseconds using the CABS-Flex 2.0, a promising tool for initial reconnaissance screening studies, such the effect of mutations on the protein stability (Jamroz et al., 2014). The fluctuation of the individual amino acid residues of the best hit was explained based on the RMSF values, in order to analyze the conformational stability within a nanosecond time scale of the mutated MYO3A-KD. The highest RMSF value reflects more flexibility, whereas the lowest value implies the limited motion of the system during the simulation process. This mutated structure attained a high level of fluctuation in both residue positions 218 and 289 that were discerned to range between 5 and 6 Å. The decrease of the RMSF value reflects that this Lys50Arg substituted residue form would be relatively rigid compared to the native forms and thus affecting the structural plasticity of the MYO3A protein as well as its biological function. This predicted variation in residue fluctuation pattern causing changes in flexibility of MYO3A structure would as well influence the protein-ATP binding properties. These results also suggest that Arg50 could be unstable during catalysis compared to the native form (Gerek et al., 2013).

In order to define the importance of MYO3A-KD in the cochlea, a knock-in (KI) Myo3a p.(Tyr137Cys) mouse was generated. This variant is similar to that observed in the human MYO3A p.(Tyr129Cys) within the KD. The analysis of the KI mouse showed the degeneration of inner ear hair cells after 6 months of age. This finding proved the crucial role of the KD for a normal hearing through preserving the intact structure of hair cells (Li et al., 2018).

Interestingly, we noted variability in both age of onset and severity of HI associated with homozygous kinase mutations in previously reported DFNB30 families. Using molecular modelling, we showed that variation between genotypes and their effect on either the MYO3A-KD or the whole protein would explain this phenotypic variability within these families. In fact, both KD variants; p.(Tyr129Cys) (Li et al., 2018) and p.(His142Gln) (Miyagawa et al., 2013) located within the alpha E helix are associated with HI progressive phenotype. Considering the critical role of the alpha E helix in transmitting structural changes within the KD, these variations from Tyrosine, a polar amino acid into cysteine, a neutral hydrophobic and the variation from a cyclic Histidine residue into branch-chain Glutamine at positions 129 and 142 respectively would possibly mediate the disruption of this helix normal function. Thus, these missense variants changing the secondary structure are more likely to have a partial or strong destabilizing effect on the whole protein structure (Abrusán & Marsh, 2016). Partial inactivation of the MYO3A protein would explain the progressive HI phenotype. Moreover, total inactivation of MYO3A would also result in progressive HI phenotype. Remarkably, it was shown in previous studies that reduction of MYO3A expression or a total loss of the protein function by deleterious recessive variants led to late onset progressive HI (Walsh et al., 2011). Generation of MYO3AKI/KI mice with a nonsense mutation, p.Tyr1041*, similar to the human p.Tyr1043* mutation, consolidates this finding, as the mice showed a progressive HI with age (Walsh et al., 2011). Consistent with this result, Myo3a+/− mice also showed a late-onset mild HI. By comparing these results with those obtained for Myo3b+/− and double-knockout Myo3a+/− Myo3b+/− mice it was suggested that during the auditory system development, this class III myosins work redundantly (Lelli et al., 2016). This redundancy could explain the mild observed HI phenotype caused by these two variants when the MYO3A function is partially or fully absent.

On the other hand, both p.Lys50Arg (this study) and p.(Pro194Thr) (Choi et al., 2013) are associated with a profound HI phenotype. These pathogenic variants were predicted by modeling to seriously disturb the function of the MYO3A-KD leading to an inactive form enable to phosphorylate the motor-head domain. In fact, these highly conserved residues play key roles in ATP positioning, conserving the catalytic mechanism or stabilizing the active-conformation. The Lys50 represents a highly conserved residue from the β3-strand that typically forms a salt bridge. This salt bridge, formed with the conserved glutamate, near the center of the protein-kinase αC-helix (Gagic et al., 2019) stabilizes the ATP α- and β-phosphates interaction with the Lys residue which is required for the protein kinases activity (Roskoski, 2015, 2019, 2019). Well-established in vitro studies supported the damaging effect of the Lys50Arg mutation since the Myo3a-Lys50Arg ‘kinase-dead’ mutant does not undergo auto-phosphorylation of the motor-head domain (Quintero et al., 2010, 2013).

The second highly conserved Pro194 is essential for maintaining the active form of the kinases. In fact, the activation segment starts at the DFG-motif (Asp-Phe-Gly) and ends at a sequence with the consensus (Ala–Pro–Glu) in the APE motif (Gagic et al., 2019). Thus, the p.(Pro194Thr) mutation located in the highly conserved MYO3A-KD APE motif may lead to an inactive kinase by hindering the right positioning of the substrate thereby anchoring the spine to the αE-helix thus the hydrophobic spines cannot be fully formed in MYO3A-KD. These findings proved that these two kinase mutations are located in structurally clue sites where mutations could specifically reduce kinase activity thus preventing the motor-head domain auto-phosphorylation, a crucial mechanism for normal MYO3A function (An et al., 2014). Therefore, the motor-head domain activity remains fully active leading to the concentration of the MYO3A proteins at the inner ear hair cell stereocilia (Dosé et al., 2003; Pollock et al., 2016; Schneider et al., 2006). MYO3A tip localization would cause stereocilia elongation and the stereocilia tips bulging, thus this dysregulation of the KD function could explain the severe congenital HI in these two families.
Three additional dominant mutations have been identified in the MYO3A gene, of which two are located in the motor-head domain and a single mutation within the KD (Bueno et al., 2021; Dantas et al., 2018; Grati et al., 2016). It is important to note that dominant mutations could either be categorized as negative-dominance (ND) or haploinsufficiency mutations. Since MYO3A mutations are responsible for both autosomal recessive and dominant forms, the potentially defective MYO3A protein would interact with the existing WT protein via a DN mechanism causing HI phenotypes. Even though, the effect on the WT form cannot be easily evaluated, it is noteworthy that according to the available clinical features within the three previously described families with dominant pathogenic variants, mutations in the KD are responsible for a prelingual HI, however those with mutations in the motor-head domain suffer from a post lingual HI, which is in accordance with our described model.

In conclusion, we successfully identified a novel homozygous disease-causing variant (c.149A>G) in the highly conserved ATP binding site of MYO3A-KD. Molecular modeling and dynamics proved that the p.(Lys50Arg) may disturb the KD structure and modify the stability and flexibility of the protein, consequently impacting the MYO3A motor domain phosphorylation elucidating the profound congenital ARNSHI (DFNB30) phenotype within this family.

Our results also provide a better insight about the correlation between the MYO3A variants’ genotype and the HI phenotype suggesting that KD mutations could be responsible for both progressive and severe congenital HI form when affecting either the MYO3A function or particularly the catalytic activity of its KD respectively.

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Ethics approval

This study was approved by the Regional Committee of the Protection of Persons, Sfax, Tunisia. Ethical committee number for the study: CPP SUD N°28/2019.

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