Custom Next-Generation Sequencing Identifies Novel Mutations Expanding the Molecular and clinical spectrum of isolated Hearing Impairment or along with defects of the retina, the thyroid, and the kidneys

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Abstract

Background: In the Tunisian population, the molecular analysis of hearing impairment remains based on conventional approaches, which makes the task laborious and enormously expensive. Exploration of the etiology of Hearing Impairment and the early diagnosis of causal mutations by next-generation sequencing help significantly alleviate social and economic problems.

Methods: We elaborated a custom SureSelectXT panel for next-generation sequencing of the coding sequences of 42 genes involved in isolated hearing impairment or along with defects of the retina, the thyroid, and the kidneys.

Results: We report eight pathogenic variants, four of which are novel in patients with isolated hearing impairment, hearing impairment, and renal tubular acidosis, Usher syndrome and Pendred syndrome. Functional studies using molecular modeling showed the severe impact of the novel missense mutations on the concerned proteins. Basically, we identified mutations in nuclear as well as mitochondrial genes in a Tunisian family with isolated hearing impairment, which explains definitely the phenotype detected since 2006.

Conclusion: Our results expanded the mutation spectrum and genotype-phenotype correlation of isolated and syndromic hearing loss and also emphasized the importance of combining both targeted next-generation sequencing and detailed clinical evaluation to elaborate a more accurate diagnosis for hearing impairment and related phenotypes especially in North African populations.

KEYWORDS

hearing impairment, high-throughput targeted sequencing, Pendred syndrome, renal tubular acidosis, Usher syndrome

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1 | INTRODUCTION

Miscellaneous epidemiological studies yielded that one of the highest rates of consanguinity in the world is found in the North African region (Hamamy, 2012). In Tunisia, the offspring of consanguineous unions may be at an increased risk for recessive disorders because of the expression of autosomal recessive (AR) mutations inherited from a common ancestor. In particular, among the AR disorders, our central focus is upon hearing impairment (HI) in Tunisia with a high prevalence of isolated HI ranging between 2% and 8% in some of the isolates in the northern villages (Ben Arab et al., 2004). In 2015, a diagnostic oligonucleotide array using multiplex-PCR coupled with dual-color arrayed primer extension and targeting 58 mutations was undertaken by our laboratory allowing a rapid, an accurate, and a cost-effective method to screen common mutations in the Tunisian population (Chakhchouk et al., 2015). Nowadays, 76 genes have been associated with isolated ARHI all over the word (https://hereditaryhearingloss.org/). There are no accurate statistical figures of HI in Tunisia. However, worldwide investigations revealed that approximately 466 million people throughout the world (more than 5% of the world population) have disabling HI (Schmucker et al., 2019). In addition to its complex genetic heterogeneity, HI is characterized by clinical heterogeneity mainly if HI appears together with defects of other organs (e.g., eye, kidney). Usher syndrome is a recessive disorder, characterized by sensorineural HI, retinitis pigmentosa and in some cases, vestibular dysfunction with at least 10 associated genes: MYO7A (OMIM#276903), USH1C (OMIM#605242), CDH23 (OMIM#605516), PCDH15 (OMIM#605514), US1G (OMIM#607696), USH2A (OMIM#608400), ADGRV1 (OMIM#602851), WHRN (OMIM#607928), CLRN1 (OMIM#606397), HARS (OMIM#142810). (https://hereditaryhearingloss.org/usher). Always among recessive disorders, we are interested mainly in Pendred syndrome which is typically characterized by the combination of HI and inner ear malformations (enlarged vestibular aqueduct (EVA) and Mondini dysplasia), goiter and an abnormal organification of iodide with or without hypothyroidism. Pendred syndrome is caused by biallelic mutations in three genes: SLC26A4 (OMIM#605646), FOXI1 (OMIM#601093), KCNJ10 (OMIM#602208) (https://hereditaryhearingloss.org/pds). In addition, HI and distal renal tubular acidosis (dRTA) with a recessive autosomal pattern can be triggered by mutations in two genes: ATP6V0A4 (OMIM#602722), ATP6V1B1 (OMIM#267300) (Karet et al., 1999; Smith et al., 2000; Stover et al., 2002). dRTA is a kidney tubulopathy characterized by a metabolic acidosis with normal anion gap, hypokalemia, hypercalciuria, hypocitraturia, and nephrocalcinosis.

The diagnosis of such genetically heterogeneous diseases by simple techniques is quite difficult in view of the involvement of several genes. The elaboration of high-throughput sequencing technologies can enhance clinical care following genetic risk identification in a time sensitive and cost-effective manner (Neveling et al., 2013). Within this framework, several researchers are using exome or whole genome sequencing technologies to better explore the genetic basis of various diseases. Yet, in terms of diseases for which candidate genes are described, the use of targeted sequencing may be a favorite method for choice. In fact, a targeted approach increases the reading depth of the sequences, facilitates the analysis of data generated by the sequencing, reduces the cost of sequencing, and does not involve ethical considerations in terms of the return results.

As far as our work is concerned, we built up a comprehensive next-generation sequencing (NGS) panel interrogating 42 genes involved in isolated HI or together with defects of other organs. The panel covered the coding regions and splice-site junctions, providing a high-throughput assay.

2 | MATERIALS AND METHODS

2.1 | Subjects and sample preparation

Whole blood was collected from eight Tunisian patients. Two patients had a clinical diagnosis of isolated HI. Two patients exhibited Usher syndrome, one patient with Pendred syndrome, and three patients with dRTA and HI. Whenever possible, samples from additional family members were used to perform segregation analysis of the sequence variants identified in the index patient. Informed consent from all participants or their legal representatives was obtained in accordance with the guidelines of the Regional Committee of the Protection of Persons, Sfax, Tunisia (CPP SUD reference number 28/2019). During the informed-consent process, investigators explained the risks and benefits of research-based high-throughput sequencing analysis to patients and their families, and an option for disclosure of medically actionable incidental findings was provided.

DNA from all samples was extracted using the phenol-chloroform standard method. The quantity of the DNA was assessed with fluorometry-based Qubit dsDNA HS and Qubit BR DNA Assay Kits according to the manufacturer's instructions. Besides, all samples underwent a 1% Agarose-TBE Blend gel so as to detect any degradation.
2.2 | Capture design

A hybridization-based, multi-disease gene panel was designed using SureDesign which is a web application from Agilent Technologies in order to create custom target enrichment library design (Agilent Technologies, Santa Clara, CA 95051, United States). Design was based on GRCh37/hg19 references sequences, with target sources obtained from the RefSeq database. In this custom design, all coding exons were targeted involving 25 bp of the flanking intronic sequence of 42 genes implicated in isolated HI or together with defects of other organs. Complete gene list and associated diseases are displayed in Table 1. The noncoding exon of the GJB2 gene where the IVS1+1G>A mutation is located, is included in the design (Riahi et al., 2013).

2.3 | Target capture sequencing

Libraries were prepared according to the “SureSelect™ QXT Target Enrichment for Illumina Multiplexed sequencing” sample preparation protocol kit (Agilent Technologies, Santa Clara, CA 95051, United States). Mainly, 50 ng of genomic DNA was enzymatically fragmented and adaptors were added to ends of the fragments in a single reaction. The adaptor-tagged DNA library was purified and amplified. Subsequently, 750 ng of each library was hybridized using SureSelect™ QXT capture library for 90 minutes. The resulting libraries were recovered using streptavidin magnetics beads, and a post-capture PCR amplification was carried out. Libraries were pooled following the manufacturer’s protocol. Resting on the “Illumina MiSeq System Denature and Dilute Libraries” Guide, 10 pM of the pooled library was next loaded onto a MiSeq (Illumina) for 2 x 151 paired-end sequencing using a Standard v2 cartridge and flow cell. The MiSeq Reporter software settings (Illumina) were adjusted to generate FASTQ files for index reads.

2.4 | Bioinformatics pipeline

For NGS data analysis, we followed the methods of Souissi, Ben Said, Ben Ayed, et al., 2021 (Souissi, Ben Said, Ben Ayed, et al., 2021). Primary data analysis was performed using MiSeq Reporter so as to generate a pair of FASTQ files for each sample and for adaptor trimming from all reads. Generated FASTQ files were analyzed using SureCall NGS data analysis software version 3.0.3.1 (Agilent Technologies). SureCall allows data analysis from alignment to categorization and annotation of mutations. Nonsense variants and small deletions or insertions inducing a frameshift of the coding sequence were considered the most damaging, as they necessarily altered the amino acid sequence of the protein. The pathogenicity of missense variants was estimated using the SIFT and PROVEAN algorithms (Choi & Chan, 2015). Furthermore, in accordance with the ACMG (American College of Medical Genetics and Genomics) standards and guidelines, variants were classified into five types: “Pathogenic”, “Likely Pathogenic”, “Benign”, “Likely Benign”, and “Uncertain significance”. We also sought the pathogenicity of the different variants in the ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) and varsome databases. Moreover, the AF in the genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/) was invested to evaluate variant’s frequency.

From those sequence variants predicted to be pathogenic, candidate variants were chosen provided that they were lying within genes matching with the clinical diagnosis.

For Phosphorylation site prediction, we used NetPhos 3.1 Server (Blom et al., 1999) to predict potential phosphorylation sites from the protein sequence. This server (http://www.cbs.dtu.dk/services/NetPhos/) produces neural network predictions for serine, threonine, and tyrosine phosphorylation sites in eukaryotic proteins. The probability score indicates the chance of each potential

| TABLE 1 | Complete list of diseases and associated genes in the panel |
|-------------|-----------------|-----------------|
| Diseases | Genes | Coverage of target regions |
| Isolated hearing loss | GJB2, MYO15A, TMC1, TMPRSS3, ESRRB, ESPN, PJVK, LRTOMT, LHFP5, PNPT1, TPRN, EYA1, SIX1, GIPC3, OTOA, OTOF, PTPRF, COL11A2, DCDC2a, ENSL2, ENSL8, SLC22A4, TECTA, MYO3A, TBC1D24 | 100% |
| Usher Syndrome | MYO7A, USH1C, CDH23, PCDH15, USH1G, CI2B, USH2A, ADGRV1, WHRN, CLRN1, PDZD7, HARS | |
| Pendred Syndrome | SLC26A4, FOXI1, KCNJ10 | |
| Hearing loss with renal tubular acidosis | ATP6V1B1, ATP6V0A4 | |
phosphorylation site within the peptide to be phosphorylated. The score range from 0.0 to 1.0 and everything above 0.5 is marked as a potential P-site, but obviously, the higher score the better.

For Sequence comparison of the proteins orthologs, we used HomoloGene database (https://www.ncbi.nlm.nih.gov/homologene/).

2.5 Validation by Sanger sequencing

Variants suspected to be pathogenic were analyzed by Sanger sequencing as described (Tani et al., 2002). The DNA fragments containing the variants were amplified by PCR with specific primers and were sequenced on both strands using the Big Dye 3.1 Terminator Sequencing Kit. The purified sequence products were analyzed on a 3100 ABI instrument (Applied Biosystems, Foster City, CA). Segregation analysis was carried out in cases where DNA samples of relatives were available.

2.6 Molecular modeling and functional analysis for the novel missense mutations

To address the structural and functional impact of the two novel missense mutations CDH23-Tyr2209Cys (c.6626A>G), and SLC26A4-Ile71Asn (c.212T>A), we followed the methods of Souissi, Ben Said, Ben Ayed, et al., 2021 (Souissi, Ben Said, Ben Ayed, et al., 2021). The sequence of the wild-type CDH23 (UniProtKB - Q9H251), and SLC26A4 (UniProtKB-O43511) proteins was submitted to the Phyre2 server (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index). The computer-assisted molecular modeling and Spdb viewer simulations were conducted on the mutant version to permit local regional changes for full-length CDH23 (3354) and SLC26A4 (780) proteins amino acids. Energy minimization and analysis were undertaken investing the Yasara virtual reality workstation (http://www.yasara.org). Visualization and generation of high quality 3D graphics were carried out through the use of PyMOL program.

3 RESULTS

Capture of NGS using our customized panel was performed in eight patients. The quality scores obtained using “MiSeq Sequencing Analysis Viewer” were high, with 96% of the sequenced bases >Q30. On average, 86% of all targeted bases were covered by at least 20 reads. The mean read length was approximately 92–98 bp. To identify rare possibly pathogenic variants in each sample, we applied parameters according to SNP Caller in the SureCall, namely: minor allelic frequency<1%, a minimum quality for base = 30, a minimum number of reads supporting variant allele = 10. Relying on our personalized approach, we managed to report several variants per sample. This list was then sorted according to (1) the extent of the variant’s negative impact on the gene product, (2) the zygosity of the variant, and (3) the location of the variant within a gene that is consistent with the phenotype. We identified a total of eight variants predicted to be pathogenic in the eight samples (two frameshift, four missense, and two nonsense variants) (Table 2). Four out of the eight different mutations identified in this study were not previously reported. Sanger sequencing on available family members revealed that these variants segregate with the disease in each family (Figure 1). Variants interpretation using dbSNP, gnomAD, ClinVar, SIFT, Provean, and varsome databases are illustrated in Table 3. In addition, the co-segregation study, for genes definitively known to cause a disease in multiple family members, supports the presence of deleterious effect for genetic variants (PP1). According to the adapted ACMG/AMP standards and guidelines, we classified the three variations CDH23-Tyr2209Cys (c.6626A>G), CDH23-Ser384Arg (c.1152C>A), and SLC26A4-Ile71Asn (c.212T>A), as likely pathogenic, pathogenic and likely pathogenic, respectively (Table 4).

3.1 Patients with isolated hearing impairment

Family 1: A novel missense variation (c.6626A>G; p. Tyr2209Cys) was identified in CDH23 gene (NM_022124.5) leading to the change of tyrosine AA at position 2209 into cysteine (Table 2). The pathogenicity of p. Tyr2209Cys mutation is supported by the homozygous state in two affected members and the heterozygous state in their parents (Family 1, Figure 1). The variant was predicted to be damaging by SIFT and deleterious by Provean (Table 3). This variant was classified as Likely Pathogenic based on ACMG guidelines (Table 4). Moreover, NetPhos 3.1 predicted that tyrosine 2209 may be a potential phosphorylation site with a score of 0.949. In addition, based on our molecular modeling analysis, this variation may disturb the correct folding of CDH23 protein.

Family 2: A previously frame-shift mutation was identified in the homozygous state in WHRN gene (c.2423delG; p. Gly808Asps*11) (NM_015404.4) (Table 2). Segregation analysis revealed that the mutation segregated with the disease phenotype and was present in the homozygous state in only affected members with congenital and
profound HI (Family 2, Figure 1). The c.2423delG mutation was present in heterozygous state in IV1 individual with high frequency HI and was absent in V5 individual with unilateral and high frequency HI (Figure 2). The mutation was described as pathogenic in ClinVar and varsome (Table 3).

In our previous work, we identified additional variants in genes known to be responsible for HI in family 2 (Mkaouar-Rebai et al., 2006). In fact, we detected the c.109G>A and c.35delG variants in GJB2, the c.798C>T variant in GJB3 as well as the mitochondrial A1555G mutation in the 12S rRNA gene (Figure 2). However, the presence of these variants uniquely does not explain the phenotypic heterogeneity reported in this family.

### 3.2 Patients with Usher syndrome

Family 3: A novel frame-shift mutation was identified in USH2A gene (c.5340_5344dup; p. Leu1782Profs*37) (NM_206933.3) (Table 2). Segregation analysis portrayed that the mutation segregated with the disease

| Clinical information | Gene | Validated mutation | Clinical features |
|----------------------|------|--------------------|------------------|
| Hearing loss         | CDH23| c.6626A>G, p. Ty2209Cys | - Consanguinity (+)  
- Bilateral profound hearing loss  
- Other abnormalities: Absence |
| Usher syndrome       |      | c.1152C>A, p. Ser384Arg | - Consanguinity (+)  
- Bilateral Severe hearing loss  
- Retinitis pigmentosa  
- Familial history of congenital Usher syndrome |
| Hearing loss         | WHRN | c.2423delG, p. Gly808Aspfs*11 | - Consanguinity (+)  
- Age of examination: 33 years  
- Bilateral profound hearing loss  
- Other abnormalities: Absence |
| Usher syndrome       | USH2A| c.5340_5344dup, p. Leu1782Profs*37 | - Consanguinity (+)  
- Bilateral Profound hearing loss  
- Retinitis pigmentosa  
- Balance Problems  
- Familial history of congenital Usher syndrome |
| Pendred syndrome     | SLC26A4| c.212T>A, p. Ile71Asn | - Consanguinity (+)  
- Bilateral profound hearing loss  
- Presence of goiter  
- Enlarged vestibular aqueduct |
| Hearing loss with renal tubular acidosis | ATP6V1B1 | c.1243C>T, p. Gln415* | - Consanguinity (+)  
- Dehydration and vomiting with failure to thrive  
- Metabolic acidosis with normal anion gap, evidence of renal potassium wasting and no evidence of secondary causes of distal renal tubular acidosis, hypercalciuria  
- Bilateral sensorineural profound hearing loss |
| Hearing loss with renal tubular acidosis | ATP6V0A4 | c.387C>A, p. Tyr129* | - Consanguinity (+)  
- Metabolic acidosis  
- Alkaline urine pH  
- Hypokalemia  
- Grade III medullary nephrocalcinosis  
- Bilateral sensorineural mild hearing loss |
| Hearing loss with renal tubular acidosis | ATP6V1B1 | c.2227C>T, p. Arg743Trp | - Consanguinity (+)  
- Metabolic acidosis  
- Alkaline urine pH  
- Hypokalemia  
- Hypercalcicria with polyuria  
- Grade III medullary nephrocalcinosis  
- Bilateral sensorineural mild hearing loss |
phenotype and was present in the homozygous state in only affected members (Family 3, Figure 1). Using the PROVEAN and SIFT softwares, the mutation was predicted to be damaging. The mutation was not reported in ClinVar and was predicted to be pathogenic in varsome (Table 3).
**TABLE 3** Interpretation of variants using databases

| Gene   | Mutation                  | Transcript | Effect           | Previous reports of the mutation | dbsNP | AF In gnomAD (Global) | ClinVar | SIFT   | Provean | varsome   |
|--------|---------------------------|------------|------------------|----------------------------------|-------|-----------------------|---------|--------|---------|-----------|
| CDH23  | c.6626A>G, p. Tyr2209Cys  | NM_022124.6| Missense variant | Novel                            | —     | —                     | NR      | Damaging | Deleterious | VUS       |
|        | c.1152C>A, p. Ser384Arg   | NM_022124.5| Missense variant | Bonnet et al. (2016)             | —     | —                     | NR      | Damaging | Deleterious | Likely pathogenic |
| WHRN   | c.2423delG, p. Gly808Asfs*11 | NM_015404.4| Frame_shift variant | Tlili et al. (2005)              | rs869320674 | Pathogenic        | —       | —       | Pathogenic |
| USH2A  | c.5340_5344dup, p. Leu1782Profs*37 | NM_206933.3| Frame_shift variant | Novel                            | -     | —                     | NR      | —       | —       | Pathogenic |
| SLC26A4| c.212T>A, p. Ile71Asn     | NM_000441.2| Missense variant | Novel                            | rs774808319 | —                     | NR      | Damaging | Deleterious | VUS       |
| ATP6V1B1| c.1243C>T, p. Gln415*    | NM_001692.4| Stop_gained variant | Novel                            | rs977545513 | —                     | NR      | —       | —       | Pathogenic |
| ATP6V0A4| c.387C>A, p. Tyr129*     | NM_020632.2| Stop_gained variant | Vargas-Poussou et al. (2006)    | rs763834363 | —                     | NR      | —       | —       | Pathogenic |
|        | c.2227C>T, p. Arg743Trp  | NM_020632.2| Missense variant | Escobar et al. (2016)            | rs770052600 | 0.000007961 | NR      | Damaging | Deleterious | VUS       |

Abbreviations: NR, Not Reported; VUS, Variant of Uncertain Significance.
| Gene   | Mutation                        | Segregation | Pathogenic criteria | ACMG classification |
|--------|---------------------------------|-------------|---------------------|---------------------|
|        |                                 |             | Very Strong | Strong | Moderate | Supporting |                   |
|        |                                 |             |            |        |          |            |                   |
| CDH23  | c.6626A>G, p. Tyr209Cys         | Yes         | —          | —      | PM2      | PP2-PP3-PP1* | Likely Pathogenic |
|        | c.1152C>A, p. Ser384Arg         | Yes         | —          | —      | PM2      | PP2-PP3     | Pathogenic        |
| WHRN   | c.2423delG, p. Gly808Asps*11    | Yes         | —          | —      | PM2      | PP3-PP5     | Pathogenic        |
| USH2A  | c.5340_5344dup, p. Leu1782Profs*37 | Yes         | —          | —      | PM2      | PP3         | Pathogenic        |
| SLC26A4| c.212T>A, p. Ile71Asn           | Yes         | —          | —      | PM2      | PP2-PP3-PP1* | Likely Pathogenic |
| ATP6V1B1| c.1243C>T, p. Gln415*           | Only, the patient's DNA is available | —          | —      | PM2      | PP3         | Pathogenic        |
| ATP6V0A4| c.387C>A, p. Tyr129*           | Yes         | —          | —      | PM2      | PP3         | Pathogenic        |
|        | c.2227C>T, p. Arg743Trp        | Yes         | —          | —      | PM2      | PP2-PP3     | Variant of uncertain significance |

Note: PP1* supporting: as one or two affected members.
PP1**: strong as four affected members.
Family 4: A previously reported missense variant was identified in *CDH23* gene (c.1152C>A; p. Ser384Arg) (NM_022124.5) in a patient with Usher syndrome (Table 2). The c.1152C>A mutation was found in the homozygous state in sample 4 and in the heterozygous state in the parents (Family 4, Figure 1). With the predictions of PROVEAN and SIFT softwares, this variant turned out to be severe. The mutation was not reported in ClinVar and was predicted to be Likely pathogenic in Varsome (Table 3). This variant was classified as Pathogenic based on ACMG guidelines (Table 4). Moreover, NetPhos 3.1 predicted that Serine 384 may be a potential phosphorylation site with a score of 0.581.

### 3.3 | Patients with Pendred syndrome

Family 5: A novel homozygous missense variation (c.212T>A; p. Ile71Asn) was identified in *SLC26A4* gene (NM_000441.2) leading to the change of isoleucine AA at position 71 into asparagine (Table 2). The pathogenicity of (c.212T>A; p. Ile71Asn) mutation was confirmed by the homozygous state in two affected members as well as the heterozygous state in their parents (Family 5, Figure 1). Prediction of the consequence of variant was damaging by SIFT and deleterious by Provean (Table 3). This variant was classified as Likely Pathogenic based on ACMG guidelines (Table 4). Relying on our molecular modeling analysis, this variation may disturb the correct folding of SLC26A4 protein.

### 3.4 | Patients with hearing impairment and distal renal tubular acidosis

Family 6: A novel mutation was identified in *ATP6V1B1* gene (c.1243C>T, p. Gln415*) (NM_001692.4) (Table 2). This mutation was present in the homozygous state in the patient and Sanger sequencing confirmed it...
(Family 6, Figure 1). The mutation is predicted to be pathogenic in varsome (Table 3).

Family 7: A previously reported mutation was identified in ATP6V0A4 gene (c.387C>A, p. Tyr129*) (NM_020632.2) (Table 2). We demonstrated that this variant is segregating with the disease in this family by investigating normal carrier parents (Family 7, Figure 1). The c.387C>A mutation was predicted to be pathogenic in varsome (Table 3).

Family 8: A previously reported mutation was identified in ATP6V0A4 gene (c.2227C>T, p. Arg743Trp) (NM_020632.2) (Table 2). We highlighted that this variant is segregating with the disease in this family by investigating normal carrier parents (Family 8, Figure 1). The c.2227C>T mutation was described as a variant with uncertain significance in varsome and with the predictions of PROVEAN and SIFT softwares, this variant turned out to be severe (Table 3).

4 | DISCUSSION

In Tunisia, genetic techniques applied in the medical field remain confined to conventional molecular techniques which are extremely laborious, time-consuming, and enormously expensive and their positive diagnostic rate remains low, essentially for genetically heterogeneous pathologies. From this perspective, the majority of patients with a genetic disease do not have a molecular diagnosis. Similarly, accurate genetic counseling and potential prenatal diagnosis cannot be specified, which further aggravates the social and economic consequences of these pathologies. In recent years, the explosion of high-throughput sequencing technologies has made it possible to overcome this shortcoming, and to develop strategies for systematic and parallel sequencing of several genes at the same time. However, for HI analysis in Tunisia as well as in the other North African countries, the application of high-throughput sequencing techniques remains restricted to a dozen of cases based on whole exome sequencing (WES) rather than targeted sequencing (Abdi et al., 2016; Behlouli et al., 2016; Bousfiha et al., 2017; Dahmani et al., 2015; Elhrarchi et al., 2018; Hamdi et al., 2018; Kim et al., 2017; Nagar et al., 2018; A. Riahi et al., 2018; Z. Riahi et al., 2015; Salime et al., 2017). However, WES approach is not always feasible in the clinical setting, taking into account the challenging, time-consuming analysis, interpretation of the results, and financial limitation in certain countries. Human disease panels like the one we present here, allows identifying genetic variants that may explain the cause of HI. Besides, custom gene panels have the merit that can be modified by adding or removing genes.

4.1 | Expanding the spectrum of isolated hearing Impairment or associated with retinitis pigmentosa, thyroid goiter, or renal tubular acidosis in Tunisian patients

4.1.1 | Identified variants within CDH23 gene

We have identified a novel variant (c.6626A>G; p. Tyr2209Cys (Family 1, Figure 1)) in CDH23 gene. Currently, more than 700 variants in this gene have been described (https://databases.lovd.nl/shared/variants/CDH23/unique). To the best of our knowledge, we believe that this novel missense variation corresponds to the first CDH23 mutation reported in HI Tunisian patients. The amino acid alignment of CDH23 protein orthologs demonstrates that 2209 residue is a conserved tyrosine, suggesting that a substitution (i.e., Tyr>Cys) at this position may have deleterious effects on the function of CDH23 protein (Figure 3b). According to UniProt, the Tyr2209Cys (c.6626A>G) variant is located in the Cadherin 21 domain of the CDH23 protein. This is an extracellular cadherin (EC) repeat domain among 27 in CDH23 protein. In the inner ear, CDH23 protein is implicated in the perception of sound. The EC domains interact with other cadherin molecules and the stability of these interactions is achieved by binding of Ca²⁺ ions to cadherin-specific amino acids motifs located in each EC domain (Nollet et al., 2000). Our molecular modeling analysis showed that CDH23-Tyr2209Cys (c.6626A>G) is located in a structurally disordered region. Tyrosine2209 is a highly conserved amino acid which establishes two hydrogen bonds with both Ala2158 and Gly2004 (Figure 3a) and its replacement with cysteine will create new rigid bonds in the protein core. This may disturb correct folding or even the stability of the protein. In addition, the new residue S-methyl-cysteine does not exist in the alpha helical form owing to the around existing steric factors in that region, which seems to be responsible for the non-helical nature of this amino acid. It is also related to the proximity of the heteroatom of Cys residue to the peptide backbone relatively to the aromatic polar Tyr residue. Therefore, this variation might impair the interactions between cadherin proteins. Here, we also identified the CDH23-Ser384Arg (c.1152C>A) variant in Family 4. This variant was identified in European patients with Usher Syndrome but details about the severity of HI and retinitis pigmentosa were not recorded (Bonnet et al., 2016) (Table 5). It is noteworthy that in our study, we identified two missense mutations in the CDH23 gene, one in a patient with isolated HI (c.6626A>G;p. Tyr2209Cys) and the other in a patient with Usher syndrome (c.1152C>A; p. Ser384Arg). Our results further support previous studies showing that missense mutations of CDH23 have been
FIGURE 3  (a) Structural modelling and functional analysis for CDH23-Tyr2209Cys mutation (b) Sequence comparison of CDH23 orthologs. Tyr2209 is marked in gray. Accession numbers of the various protein sequences: NP_071407.4: H.sapiens; XP_507839.3: P.troglodytes; XP_002805748.1: M.mulatta; XP_003434519.1: C.lupus; NP_001178135.1: B.taurs; NP_075859.2: M.musculus; NP_446096.1: R.norvegicus; XP_421595.3: G.gallus; XP_002933158.2: X.tropicalis; NP_001159387.1: D.rerio. (c) Structural modelling and functional analysis for SLC26A4-Ile71Asn mutation (d) Sequence comparison of SLC26A4 orthologs. Ile71 is marked in gray. Accession numbers of various protein sequences: NP_000432.1: H.sapiens; XP_519308.2: P.troglodytes; XP_001094049.1: M.mulatta; XP_002686849.2: B.taurs; NP_001159387.1: M.musculus; XP_425419.3: G.gallus; XP_002933158.2: X.tropicalis; NP_001159387.1: D.rerio.
observed in families with isolated HI as well as in families with Usher syndrome. It was also shown that nonsense, five splice-site, and frame-shift mutations produce Usher syndrome type I phenotype (Astuto et al., 2000, 2002; Bolz et al., 2001; Bork et al., 2001).

4.1.2 | Identified variants within WHRN gene

The c.2423delG mutation in WHRN gene was previously reported in patients with profound HI in the Tunisian population (Souissi, Ben Said, Frikha, et al., 2021; Tlili et al., 2005) (Table 5). In this research, we detected this variant in another Tunisian family, which presents HI with a phenotypic heterogeneity (Figure 2). Indeed, all patients with profound HI had the c.2423delG mutation in the homozygous state (subjects IV4, IV7, V3, V4). Subject IV1 exhibits three mutations in the homozygous state: c.2423delG in WHRN, c.109G>A and c.35delG in GJB2, with the mitochondrial A1555G mutation, which explains the high frequency HI phenotype which is less severe than that of the subjects carrying the c.2423delG mutation in the homozygous state. Subject V5 only displays the c.109G>A mutation in GJB2 in the heterozygous state with the A1555G mitochondrial mutation, which explains the phenotype that is also less severe than that of the subjects carrying the c.2423delG mutation in the homozygous state (Figure 2). Since 2015, the synonymous variant c.798C>T (p. Asn265Asn) of the GJB3 gene has been considered benign. From this perspective, we excluded it from our analysis (Richards et al., 2015). The identification of the c.2423delG mutation in a HI family clearly shows that the phenotypic heterogeneity observed in hearing-impaired families may refer to the presence of mutations in both nuclear and mitochondrial genes.

4.1.3 | Identified variants within USH2A gene

We have also identified a novel mutation (c.5340_5344dup; p. Leu1782Profs*37) in USH2A gene that is responsible for Usher syndrome, standing an autosomal recessive disorder causing hereditary deaf-blindness in humans (Family 3, Figure 1). So far, more than 1200 different variants of USH2A have been reported (https://databases.lovd.nl/shared/variants/USH2A/unique). Although the
genotype–phenotype correlation in USH2A variants remains unclear, a specific combination of USH2A variants causing truncating proteins in both alleles has elicited a more severe phenotype (Hartel et al., 2016). It is noteworthy that, this seems to go in good consistency with our results. Indeed, our patient presents Profound HI, retinitis pigmentosa, and balance problems. Basically, one nonsense mutation (c.14586T>A; p.Tyr4862*) and one CNVs were previously identified in Tunisian families (Yan et al., 2016).

4.1.4 | Identified variants within SLC26A4 gene

We have as well identified a novel homozygous missense variation (c.212T>A) in SLC26A4 gene; which is a well-known gene associated with Pendred syndrome. The amino acid alignment of the SLC26A4 protein orthologs demonstrates that residue 71 is a conserved isoleucine suggesting that a substitution (i.e Ile>Asn) at this position may have deleterious effects on the function of SLC26A4 protein (Figure 3d). To the best of our knowledge, we believe that this novel missense variation represents the fourth SLC26A4 mutation reported in Pendred syndrome-Tunisian patients since 2000 (Charfeddine et al., 2010; Masmoudi et al., 2000; Rebeh et al., 2010; Ben Said et al., 2012). The association between SLC26A4 and autosomal recessive Pendred syndrome is well-established and over 500 variants have been described (https://clinicalgenome.org/). According to UniProt, the Ile71Asn variant is located in the first cytoplasmic domain of SLC26A4 protein. This domain corresponds to the NH2-terminal tail of the protein (Everett et al., 1997). It has been demonstrated that the cytoplasmic NH2-terminal domain of anion exchangers is significant for their binding to multiple cytoskeletal proteins, glycolytic enzymes, and hemoglobin (Zhang et al., 2000). For the SLC26A4 protein, its main role is in the inner ear and in the thyroid as a chloride-bicarbonate-iodide exchanger (Scott et al., 1999; Yoshida et al., 2002). Based on our molecular modeling analysis, the SLC26A4-Ile71Asn (c.212T>A) is located in a loop region, known as patternless region, connecting two regular secondary structures. In addition, isoleucine is a highly conserved amino acid which interacts with Glu42, Val285, and Arg79. The substitution of the hydrophobic amino acid Ile, located on the SLC26A4 protein solvent exposed areas within the protein’s surface, with an uncharged
4.1.5 | Identified variants within ATP6V1B1 gene

The novel p. Gln415*(c.1243C>T) mutation in ATP6V1B1 gene was found in a homozygous state in our patient presenting dRTA with profound HI (Family 6, Figure 1). To the best of our knowledge, this novel mutation represents the first nonsense and the fifth ATP6V1B1-mutation identified in Tunisian families (Elhayek et al., 2013; Nagara et al., 2014). Owing to ATP6V1B1 protein, having a relatively high expression in human inner ear besides kidneys, recessive dRTA patients usually suffer from HI (Boualla et al., 2016). ATP6V1B1 mutations were associated with early sensorineural HI. The nonsense variant c.1243C>T (p. Gln415*) identified in this study affects exon 12 of ATP6V1B1 gene. Indeed, a recurrent mutation (c.1155dupC; p. Ile386Hisfs*56), affecting the same exon, had already been characterized as a founder mutation reported in Tunisian patients with dRTA and HI (Nagara et al., 2014). Moreover, the (c.1169dupC; p. Ser391Phe*51) mutation affecting exon 12 has been reported in North African patients (Boualla et al., 2016; Elhayek et al., 2013). Our data may provide more evidence and more thorough information to confirm that exon 12 is a potential “hotspot”. The presence of the three mutations (c.1243C>T; p. Gln415*), (c.1169dupC; p. Ser391Phe*51), and (c.1155dupC; p. Ile386Hisfs*56) in exon 12 of ATP6V1B1 gene may facilitate and help rapid genetic diagnosis of HI and dRTA in North African population. Early genetic testing can improve patient management by adequate treatment with positive impact on growth and renal function.

4.1.6 | Identified variants within ATP6V0A4 gene

We recorded a previously reported missense variation (c.2227C>T; p. Arg743Trp) in ATP6V0A4 gene. This variant is reported previously only once in a Mexican patient with dRTA and without HI (Escobar et al., 2016). However, our patient presents dRTA with mild HI (Table 5). In fact, it has been revealed that ATP6V0A4 mutations are associated with variable hearing phenotypes ranging from early to late onset HI (Karet et al., 1999; Stover et al., 2002; Vargas-Pousou et al., 2006). Additionally, both the Tunisian and the Mexican patients present hypokalemia and nephrocalcinosis. Hypercalciumia with polyuria was recorded in the Tunisian patient and dehydration as well as failure to thrive were recorded in the Mexican patient (Table 5). We also recorded the nonsense mutation (c.387C>A, p. Tyr129*) in ATP6V0A4 in one dRTA patient with mild HI (Table 5). This variant was detected previously in families originating from Algeria and Morocco and all shared the same haplotype at the disease locus, suggesting that this mutation was probably inherited from a common ancestor (Vargas-Pousou et al., 2006). Vargas-Pousou et al. asserted that patients harboring the homozygous (c.387C>A; p. Tyr129*) mutation present dRTA with or without HI (Table 5). Remarkably, all dRTA patients harboring the (c.387C>A; p. Tyr129*) homozygous mutation, present alkaline urine pH, hypokalemia (K<7<.5mmol/l), and metabolic acidosis (HCO3−<22mmol/l). However, nephrocalcinosis could be present or absent (Table 5).

4.2 | Importance of early genetic testing for the diagnosis of Hearing impairment and related disorders

As far as HI is concerned, its early identification may be extremely beneficial with hearing aid or treatment of the disease for instance cochlear implantation at the earliest possible time, which can upgrade language development and speech (Zhang et al., 2018). For some syndromes, such as Usher and Pendred, the complete phenotypic spectrum is absent from the birth, but appears later during the patient’s life, further obstructing the correct diagnosis of the disorder. It is noteworthy that children with Usher and Pendred syndromes are most likely clinically misdiagnosed and considered as patients with isolated HI. Indeed, visual defect is detectable later in life in Usher patients. For most people with Pendred syndrome, goiter is manifesting in late childhood or early puberty in approximately 40% of patients. In the remaining 60%, it begins in early adult life (Malesci et al., 2020; Toms et al., 2020). In the light of the above analysis, we assert that even though an accurate clinical assessment can enable clinicians to raise a particular suspect, only the identification of the molecular cause of the disease may shun any incomplete or even incorrect diagnosis, permitting an appropriate genetic counseling. While there is currently no available cure for these syndromes, for dRTA, there is actually a therapeutic approach which aims to correct the metabolic acidosis and other biochemical abnormalities and
to prevent the evolution of nephrocalcinosis and chronic kidney disease (Soares et al., 2019). This study enabled the identification of three different mutations in three patients with dRTA aged less than 2 years. Based on our genetic diagnostic results, our patients received oral alkaline and potassium supplementation. The signs of rickets disappeared and good psychomotor development was recorded for all patients. For example, for the patient carrying the (c.1243C>T; p. Gln415*) mutation in ATP6V1B1, biochemical values were followed after treatment. It has been demonstrated that the calcemia goes from 3.3 to 2.6 mmol/L (normal values: 2.2–2.6 mmol/L) and the HCO3− goes from 4.8 to 20 mmol/L (normal values: 22–27 mmol/L). The correction of biochemical defects is crucial to foster growth and ensure the development of children.

5 | CONCLUSION

Using next-generation-targeted sequencing, we identified mutations in eight tested families and provided genetic counseling to related members of these families. Our customized panel is promising as it will help to avoid unreasonable examinations of the cohort as well as enhance the effectiveness of the diagnosis. Our findings not only exhibit the usefulness of the targeted next-generation sequencing approach in terms of the identification and diagnosis of genetic mutations affecting HI in Tunisian families, but also brush up our knowledge of genetic causes of this disease. Basically, our research is a step that may be taken further as it paves the way and lays the ground for future works to consider different therapies relying upon the identified mutant genes and the actual conditions of the patient. We equally aspire to include other genes implicated in HI in our personalized panel.

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CONFLICT OF INTEREST

The authors have no conflict of interest that are directly relevant to the content of this article.

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