Novel pathogenic mutations and further evidence for clinical relevance of genes and variants causing hearing impairment in Tunisian population

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GRAPHICAL ABSTRACT
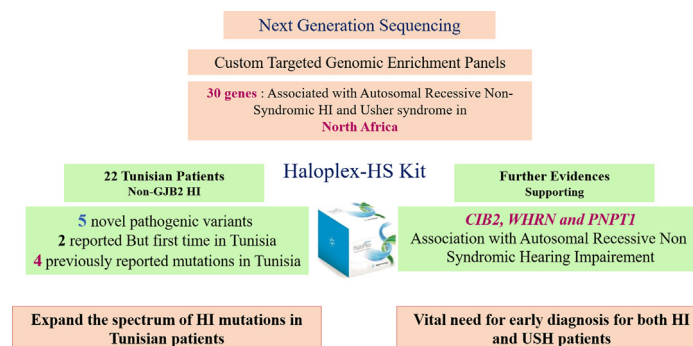
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ABSTRACT

Introduction: Hearing impairment (HI) is characterized by complex genetic heterogeneity. The evolution of next generation sequencing, including targeted enrichment panels, has revolutionized HI diagnosis.
Objectives: In this study, we investigated genetic causes in 22 individuals with non-*GJB2* HI.
Methods: We customized a Haploplex[HS] kit to include 30 genes known to be associated with autosomal recessive nonsyndromic HI (ARNSHI) and Usher syndrome in North Africa.
Results: In accordance with the ACMG/AMP guidelines, we report 11 pathogenic variants; as follows; five novel variants including three missense (*ESRRB*-Tyr295Cys, *MYO15A*-Phe2089Leu and *MYO7A*-Tyr560Cys) and two nonsense (*USH1C*-Gln122Ter and *CIB2*-Arg104Ter) mutations; two previously reported mutations (*OTOF*-Glu57Ter and *PNPT1*-Glu475Gly), but first time identified among Tunisian families; and four other identified mutations namely *WHRN*-Gly808AspfsX11, *SLC22A4*-Cys113Tyr and two *MYO7A* compound heterozygous splice site variants that were previously described in Tunisia. Pathogenic variants

in *WHRN* and *CIB2* genes, in patients with convincing phenotype ruling out retinitis pigmentosa, provide strong evidence supporting their association with ARNSHI. Moreover, we shed lights on the pathogenic implication of mutations in *PNPT1* gene in auditory function providing new evidence for its association with ARNSHI. Lack of segregation of a previously identified causal mutation *OTOA*-Val603Phe further supports its classification as variant of unknown significance. Our study reports absence of otoacoustic emission in subjects using bilateral hearing aids for several years indicating the importance of screening genetic alteration in *OTOF* gene for proper management of those patients.

Conclusion: In conclusion, our findings do not only expand the spectrum of HI mutations in Tunisian patients, but also improve our knowledge about clinical relevance of HI causing genes and variants.

Introduction

According to the World Health Organization 2019 report, 466 million people around the globe (over 5% of the world population) have disabling hearing impairment (HI) of whom 34 million are children. It is expected that this number will double by 2050 (https://www.who.int/en/news-room/fact-sheets/detail/deafness-and-hearing-loss). HI is characterized by complex heterogeneity in both genetic and clinical aspects with over 121 implicated genes in non-syndromic HI (Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage. https://hereditaryhearingloss.org. Last assessed on 4/20/2020) which complicates genetic screening process [1]. It is imperative to understand the molecular basis of HI as genetic factors are responsible for over 50% of HI cases [2] meanwhile environmental and age related HI account for the remaining percentage [3–5]. Isolated and syndromic HI account for 70% and 30% of prelingual HI cases respectively [6]. Autosomal recessive non-syndromic HI (ARNSHI) accounts for nearly 80% of hereditary HI cases [7]. ARNSHI is the most common genetic pattern for HI specifically in countries with a high rate of consanguinity as in North African Mediterranean countries. To date, 76 genes have been associated with ARNSHI (Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage. https://hereditaryhearingloss.org. Last assessed on 4/20/2020). On the other side, Usher syndrome (USH) is the most common form of syndromic HI with an estimated prevalence varying from 3 to 6.2 per 100,000 [8]. USH is inherited in an autosomal recessive pattern and is characterized by combined HI and retinitis pigmentosa (RP). USH has three main classifications namely USH1, USH2 and USH3 that differ clinically in terms of auditory and retinal phenotypes and also genetically with at least 10 associated genes. Genetic variants in those genes could also cause nonsyndromic hearing impairment (NSHI).

Identification of HI causal mutations is crucial for early diagnosis, clinical follow-up and genetic counseling. For a long time, molecular diagnostic tests for ARNSHI and USH in North Africa have been carried out using mainly routine screening techniques such as RFLP, microarray and Sanger sequencing for candidate genes [8–10]. Addressing HI complex heterogeneity for ARNSHI and USH necessitates sequencing multiplex cascade of all coding regions in large number of genes for the identification of causative mutations. Next-generation sequencing (NGS) methods provide the best alternative approach as they could analyze large number of gene positions in a single experiment offering faster and more cost-effective diagnosis. Nowadays and since many genes associated with hereditary HI have already been identified, the more appropriate choice for comprehensive genetic diagnosis of HI would be targeted exome customized panels combined with NGS sequencing [11]. The effectiveness of this technology for diagnosis of HI has been successfully evaluated with the identification of novel genes and variants associated with HI and therefore HI panels became widely used [8,11–14]. Given the large number of novel genes and variants of HI identified using NGS methods, a major interpretation challenge is imposed for precise genetic diagnosis.

The American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) published recommendations and guidelines for the interpretation of sequence variants [15]. Although these guidelines were intended to be used universally for all Mendelian disorders, certain criteria require gene- or disease-specific knowledge. For this reason, the ClinGen Hearing Loss Clinical Domain Working Group was established as expert panel to evaluate gene disease associations and to standardize variant interpretation in hereditary HI and related syndromes [16].

In the current study, in order to improve our knowledge about clinical relevance of HI causing genes and variants, we performed targeted enrichment with focused gene panels and high-throughput sequencing for 22 patients. The custom designed Halo-plex[HS] (high sensitivity) panel for hereditary diseases provided an efficient and effective tool for molecular diagnosis for known HI genes in Tunisian families.

Material and methods

Patients

22 unrelated patients with HI were enrolled in the present study. We recruited additional family members for co-segregation analysis. Based on family pedigree analysis, HI inheritance pattern was recessively transmitted. Audiological tests through air and bone conduction pure tone audiometry as well as ocular fundus examination (OFE) were performed for all the 22 patients. Moreover, the otoacoustic emissions test (OAEs) which detects response of the outer hair cells (OHCs) to environmental sound was also carried out for one of our patients. In order to confirm USH cases, the Ganzfeld electroretinogram (ERG) and the optical coherence tomography (OCT) ophthalmological investigations were performed.

Peripheral blood samples, from all affected and unaffected subjects, were obtained in EDTA- tubes and genomic DNA was extracted using standard phenol–chloroform technique. All patients were firstly screened for *GJB2* mutations through Sanger sequencing using the following primers; Forward: 5′-TCTTTTCCAGAGCAAACCGC-3′ and Reverse: 5′-CTGGGCAATGCGTTAAACTGG-3′.

Ethics statement

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 N°28/2019).

Custom HaloPlex[HS] panel design, library preparation and next generation sequencing

We customized HaloPlex targeted sequencing approach in order to develop an efficient platform for the detection of mutations in HI

genes. We limited our design to the most common causative genes previously reported in ARNSHI and Usher syndrome in North-African region [9]. Probes were designed to capture all coding exons and additional 10 bp flanking the intronic sequence using Agilent's SureDesign software (www.agilent.com/genomics/sure-design). Table 1 lists names and theoretical coverage for the 30 genes responsible for HI that were included in this panel.

Sequence capture was performed using the HaloPlex[HS] Target Enrichment System (Agilent Technologies, Inc. Santa Clara, CA, Version C1) according to manufacturer's standard protocol for Illumina Sequencing. Precise DNA concentrations of the 22 samples were verified with fluorometry-based Qubit dsDNA HS Assay kit. The gDNA library was created by digesting 50 ng gDNA with 16 different restriction enzymes. An Enrichment Control DNA (ECD) sample was used. The HaloPlex probe capture library was then added and hybridized to the targeted fragments. During this step, each sample was uniquely indexed. The Illumina sequencing motifs incorporated in the targeted fragments include molecular barcode sequences. The target DNA-HaloPlex probe hybrids were circularized and then captured using streptavidin beads. Elution of captured DNA libraries was achieved through addition of NaOH. Only circular DNA targets were amplified and purified using AMPure XP beads. At the end of this step, regions of interest were ready for sequencing.

High throughput sequencing was carried out using Illumina MiSeq instrument (Illumina Inc., San Diego, CA) which is designed for paired-end sequencing by synthesis chemistry. Samples were pooled in equimolar amounts. Each amplicon in our prepared library already contained Illumina adaptor sequences required for multiplexed sequencing. The instrument was set to generate FASTQ files only, without adapter trimming. HaloPlex HS index sequences were manually added through editing the sample sheet.

Data analysis and variant interpretation

FASTQ files were analyzed using Agilent's SureCall workflow-based application version 4.0.1.46 which carries out alignment, annotation and variants categorization in HaloPlex enriched NGS sample data from Illumina sequencing. Prior to alignment, sequence reads were trimmed to remove low quality bases towards the ends, Illumina adaptor sequences and to mask enzyme footprints. Burrows-Wheeler Aligner algorithm (BWA MEM) for Illumina data was used to align raw sequencing reads to the human reference genome (build hg19/GRCh37) while the SNPPET SNP was used for variant calling. We used filters in SureCall to reduce number of false positive single nucleotide polymorphisms (SNPs), multiple nucleotide polymorphisms (MNPs) and indels. We selected minimum variant call quality threshold of 100 and

allele frequency (AF) of 0.1. Variants should be also observed in both direct and reverse strands. Annotated variants were then classified according to their impact's severity (nonsense, frame-shift indels, splice sites, missense and inframe indels).

Evaluating and understanding the clinical significance of the obtained variants are crucial steps towards proper diagnosis with the greatest clinical sensitivity. In order to achieve this goal, the Hearing Loss Variant Curation Expert Panel was created within the Clinical Genome Resource. This panel adapted the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines [15] for the proper interpretation of variants in HI genes [16]. According to those guidelines, variants were classified based on typical types of variant evidence (e.g. population data, computational data, functional data, segregation data, etc.) into five categories namely "Pathogenic or Likely Pathogenic", "Benign or Likely Benign" and "Uncertain significance". The AF in the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org/) and the genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/) were used to evaluate variant's frequency. The pathogenicity of novel missense variants was analyzed with prediction algorithms such as SIFT (http://sift.bii.a-star.edu.sg/) and Provean (http://provean.jcvi.org).

Segregation validation by Sanger sequencing

Segregation analysis for detected variants was performed in cases where DNA samples from relatives were available. Variants selected and suspected to be pathogenic were amplified at optimal condition for each primer pair and then validated by Sanger sequencing on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems by Life Technologies, Thermo Fisher Scientific, Inc) using primers listed in Table S1.

Molecular modelling and functional analysis for the novel missense mutations

To analyze the structural and functional impact of the three novel missense mutations ESRRB-Tyr295Cys, MYO15A-Phe2089Leu and MYO7A-Tyr560Cys, the sequence of the wild-type ESRRB (UniProtKB - O95718-3), MYO15A (UniProtKB - Q9UKN7) and MYO7A (UniProtKB Q13402-1) were 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 performed on the mutant version to allow local regional changes for full-length ESRRB (4 3 3), MYO15A (3530) and MYO7A (2215) amino acids. Energy minimization and analysis were conducted using the Yasara virtual

Table 1
The coverage of target HI genes included in custom HaloPlex-HS panel.

Target ID	Regions	Coverage	Target ID	Regions	Coverage
CDH23	68	99.83%	OTOF	47	99.88%
CIB2	6	100.00%	PCDH15	39	99.47%
COL11A2	66	99.89%	PJVK (DFNB59)	6	100.00%
DCDC2	10	99.88%	PNPT1	28	99.62%
EPS8	20	100.00%	PTPRQ	42	99.24%
EPS8L2	20	100.00%	SLC22A4	10	100.00%
ESPN	13	99.86%	TBC1D24	7	100.00%
ESRRB	8	100.00%	TECTA	23	99.96%
GIPC3	6	100.00%	TMC1	20	100.00%
GPR98 (ADGRV1)	90	99.73%	TMPRSS3	12	99.82%
LHFPL5	3	99.72%	TPRN	4	100.00%
LRTOMT	10	99.59%	USH1C	28	99.58%
MYO15A	64	99.87%	USH1G	3	100.00%
MYO7A	48	100.00%	USH2A	71	99.87%
OTOA	30	100.00%	WHRN (DFNB31)	12	99.63%

reality workstation (http://www.yasara.org). Visualization and generation of high quality 3D graphics were performed using PyMOL program.

Results

Patients clinical data

According to audiological tests, all the 22 patients exhibited bilateral severe (70–89 dB) to profound (>90 dB) sensorineural HI with presence of HI family history. The OAEs test performed showed preserved OAEs indicating a normal outer hair cell function. For the ophthalmological examinations, the OFE test revealed normal eye fundi in almost all patients except for two patients (Family.10_10 and Family.11_11) who showed typical retinal degeneration confirming USH cases. ERG examination has as well confirmed significant bilateral retinopathy in patient (Family.10_10). For patient (Family.3_P3) no signs in favor of retinitis pigmentosa were shown in the ERG examination. OCT scans of our 3-years-old patient (Family.12_P12) showed alterations of the retinal pigment epithelium (RPE) confirming that this patient suffers from USH condition.

Targeted NGS data quality and variants detection

Using the custom HaloPlex[HS] panel diagnostic tool, we managed to screen for 30 HI genes in 22 patients. The raw data quality was checked using MiSeq Sequencing Analysis Viewer software. Approximately 6 Gbp sequences were obtained from the Haloplex capture assay. We obtained cluster densities from 800,000 to 900,000 clusters/mm2. More than 89% of generated bases were of Q30 value.

Based on the recessive inherited model and the filtration criteria, detailed in methods section, we were able to detect a set of 11 potential pathogenic variants (Table 2): five novel mutations (*ESRRB*-Tyr295Cys, *MYO15A*-Phe2089Leu, *MYO7A*-Tyr560Cys, *USH1C*-Gln122Ter and *CIB2*-Arg104Ter), two previously reported mutations but first time to be identified in Tunisian families (*OTOF*-Glu57Ter [17–19] and *PNPT1*-Glu475Gly [20]), and four other variants previously reported in Tunisia (*WHRN*-Gly808AspfsX11 [21], *SLC22A4*-Cys113Tyr [22] and two splice site variants found in a compound heterozygous state in *MYO7A* gene [18,22–30]).

- **Patients with non-syndromic HI**

Three novel variants have been identified to cause non-syndromic HI.

The homozygous truncating mutation c.310C > T in *CIB2* leads to the replacement of Arginine amino acid (AA) in position 104 respectively into stop codon. *CIB2*-Arg104Ter has been reported in dbSNP database (rs1054728914). This variation was also found in Exome Aggregation Consortium (ExAC) and Genome Aggregation Database (gnomAD) with AF of 0.000008242 and 0.000004, respectively **(Family.1_P1)**.

The homozygous c.884A > G change in *ESRRB* gene is a mutation that results in codon change of tAt/tGt and AA change of Tyr295Cys. The *ESRRB*-Tyr295Cys is a very rare SNP (dbSNP ID rs780275423) with an AF of 0.00002537 and 9.02e-6 in ExAC and gnomAD databases respectively **(Family.2_P2)**.

The c.6265 T > C in the *MYO15A* gene leads to the replacement of the phenylalanine aa in position 2089 with leucine aa p. (Phe2089Leu) and this was not annotated in neither dbSNP nor in gnomAD databases **(Family.5_P5)**.

Furthermore, we have also identified two previously identified variants but first time found in Tunisian families. The homozygous variant c.169G > T, detected in gene *OTOF* **(Family.4_P4)**, has the dbSNP ID rs397515591. The codon change Gag/Tag results in an AA change of *OTOF*-Glu57Ter with a high impact and stop gained for transcript NM_194322. This variant was reported in Saudi siblings and Libyan families [17–19] and is classified as likely pathogenic by the ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/).

The homozygous PNPT1 **(Family.7_P7)** missense mutation (c.1424A > G) was previously found in a consanguineous Moroccan family with three siblings affected by severe HI. This alteration leads to an exchange, at the protein level, of a negatively charged glutamic acid at position 475 with glycine. The c.1424A > G variant was reported as pathogenic in ClinVar, annotated in dbSNP (ID rs397514599) and not reported in the gnomAD database.

Two other identified pathogenic variants were previously reported in consanguineous Tunisian families, the homozygous frameshift mutation c.2423delG in the *WHRN* **(Family.3_P3)** gene and the c.338G > A variant in *SLC22A4* gene **(Family.6_P6)** [21,31]. The homozygous frameshift mutation c.2423delG in the *WHRN* gene, reported by Tlili et al. 2005, changes the reading frame at position 808 with introduction of a novel stop codon p.(Gly808AspfsX11).

The c.338G > A variant causes missense change in the protein from cysteine to Tyrosine aa p.(Cys113Tyr) [32]. This *SLC22A4*-Cys113Tyr has the dbSNP ID rs768484124 and presents an AF of 0.00002185 in gnomAD databases. A well-established functional study supported the pathogenicity and the damaging effect of this *SLC22A4* alteration [31].

- **Patients with USH**

Two novel variants have been identified to cause syndromic HI, USH. The novel homozygous truncating mutation c.360C > T in *USH1C* **(Family.10_P10)** leads to the replacement of Glutamine AAs in position 122 into stop codon.

For **family.11_P11**, NGS allowed the identification of a novel missense mutation in *MYO7A* gene at exon 14 (c.1679A > G). This variation causes the change of tyrosine to cysteine (Tyr560Cys). This variant was not found neither in gnomAD nor in dbSNP data bases.

Two previously identified mutations in Tunisia have been detected in **family.12_P12** in a compound heterozygous state. The known variant, c.470 + 1G > A, was detected in both dbSNP database, with ID rs797044510 and gnomAD, with AF of 6.37e-5. This substitution was predicted to alter the splice donor site of intron 5 resulting in exon 5 skipping in the mature transcript. This variant is predicted as pathogenic in ClinVar. The second splice acceptor variant, c.2283-1G > T, was annotated in dbSNP (ID rs397516295) but not found in gnomAD.

Segregation analysis and pathogenicity assessment

All variants reported in this study were either absent or having low MAF ≤ 0.00007 in the SNP database, the 1000 Human Genome Database, ExAC and gnomAD databases. Absence of those genetic variants from controls in various databases represents a moderate evidence for pathogenicity (PM2). For loss of function (LOF) variants we followed the detailed guidelines for interpreting the pathogenic criterion PVS1 [32]. The co-segregation study, for genes definitively known to cause HI, in multiple affected family members supports deleterious effect for genetic variants (PP1 strong or moderate, depending on the number of affected members). Affected members were found to be homozygous for 11 identified variants. Both parents were carriers for those variants. The

Table 2

Potential pathogenic mutations identified in Tunisian families.

ID	Variant	Transcript	cDNA Position	Effect	Previous reports of the mutation	AF In EXAC (Global)	dbsnp	AF In gnomAD (Global)	SIFT	Provean	Clinvar
P1	CIB2-Arg104Ter	NM_006383	c.310C > T	Stop_gained	**Novel**	8.244e-06	rs1054728914	3.98e-6	D	–	NR
P2	ESRRB-Tyr295Cys	NM_004452	c.884A > G	Non_synonymous	**Novel**	2.537e-05	rs780275423	9.02e-6	T	De	NR
P3	WHRN-Gly808AspfsX11	NM_001083885	c.2423delG	Frame_shift Premature stop codon	Tunisia [17]	–	–	–	–	–	NR
P4	OTOF-Glu57Ter	NM_194322	c.169G > T	Stop_gained	Saudi Arabia [18,20] Libya [21]	–	rs397515591	4e-6	–	–	P
P5	MYO15A-Phe2089Leu	NM_016239.3	c.6265 T > C	Non_synonymous	**Novel**	–	–	–	D	De	NR
P6	SLC22A4-Cys113Tyr	NM_003059.2	c.338G > A	Non_synonymous	Tunisia [32]	0.00005	rs768484124	0.000121	D	De	NR
P7	PNPT1-Glu475Gly	NM_033109.5	c.1424A > G	Non_synonymous	Morocco [20]	–	rs397514599	–	D	De	P
P8	OTOA-Val603Phe	NM_144672.3	c.1807G > T	Non_synonymous	Algeria [19]	4.947e-05	rs775686301	3.98e-6	D	N	VUS
P9	USH1C-Gln122Ter	NM_001297764	c.360C > T	Stop_gained	**Novel**	–	–	–	–	–	NR
P10	MYO7A-Tyr560Cys	NM_000260.4	c.1679A > G	Non_synonymous	**Novel**	–	–	–	D	De	NR
P11	MYO7A - SS	NM_000260.4	c.470 + 1G > A	Splice donor variant	Tunisia [30,9,23] Algeria [22] Saudi Arabia [52]	–	rs797044510	6.37e-5	–	–	P
			c.2283-1G > T	Splice acceptor variant	Algeria [29], Morocco [28], France [27] Tunisia [24]	–	rs397516295	–	–	–	P

D: Damaging, T: Tolerated, De: Deleterious, N: Neutral.
NR: Not Reported, P: Pathogenic, VUS: Variant with Uncertain significance.
AF: Allele frequency.
*Novel mutations globally.

unaffected members were either wild type or carrier (Figure S1). According to our results, these 11 variants met the pathogenic or likely pathogenic classification criteria (Table 3).

We have as well detected a previously identified missense variant OTOA-Val603Phe **(Family.8_P8)** that found in an Algerian family with severe-to-profound HI [33], as well as a non-previously reported variation TMC1-Met195Arg **(P9)**; both of them did not segregate with the disease since they were absent or found in heterozygous state in other patients of the studied families (Table 3).

Discussion

Tunisia is among North African countries characterized by a high consanguinity rate which increased autosomal recessive HI

Table 3

Variants segregation and pathogenicity assessment according to the ACMG guidelines.

Patient ID	Mutation	Segregation	Pathogenic criteria				Benin criteria	ACMG Classification
			Very Strong (PVS1)	Strong (PS1-PS4)	Moderate (PM1-PM6)	Supporting (PP1-PP5)	Strong (BS1-BS4)	
P1	CIB2-Arg104Ter	Yes	PVS1	–	PM2-PP1*	PP3	–	Pathogenic
P2	ESRRB-Tyr295Cys	Yes	–	–	PM2-PP1*	PP3	–	Likely Pathogenic
P3	WHRN-Gly808AspfsX11	Yes	PVS1	PP1**	PM2	–	–	Pathogenic
P4	OTOF-Glu57Ter	Yes	PVS1	PP1** PP5***	PM2	PP3	–	Pathogenic
P5	MYO15A-Phe2089Leu	yes	–	PP1**	PM1-PM2	PP2-PP3	–	Pathogenic
P6	SLC22A4-Cys113Tyr	yes	–	PS3-PP1**		PP3	–	Pathogenic
P7	PNPT1-Glu475Gly	yes	–	PP1**	PM2	PP3-PP5	–	Pathogenic
P8	OTOA-Val603Phe	No	–	–	PM2	–	BS4	Uncertain significance
P9	TMC1- Met195Arg	No	–	–	PM2	PP3	BS4	Uncertain significance
P10	USH1C- Gln122Ter	Yes	PVS1	PP1**	PM2	–	–	Pathogenic
P11	MYO7A-Tyr560Cys	yes	–	PP1**	PM1-PM2	PP2-PP3	–	Pathogenic
P12	MYO7A - SS	Yes	PVS1	PP5***	PM2	PP1-PP3	–	Pathogenic
	MYO7A - SS	yes	PVS1	PP5***	PM2	PP1-PP3	–	Pathogenic

* PP1 Moderate: CIB2 and ESRRB (2 affected members).
** PP1 Strong: WHRN, OTOF and USH1C (3 affected members).
***PP5 using strength "Strong" because ClinVar classifies this variant as Pathogenic.
SS : Splice Site Variant.

frequency in Tunisian population [34]. In this study, 22 patients were screened for genetic variants in 30 known autosomal recessive HI causative genes using the HaloPlex[HS] custom-designed kit. All patients' data as well as all identified variants are summarized in Table 4. Custom target-enrichment and high throughput parallel sequencing technologies have been widely used for molecular diagnosis of both syndromic and isolated HI [8,12,13,35]. Our study is the first of its kind to use a custom HaloPlex[HS] kit for the detection of various HI causing variants. With its ultra-high sensitivity, Agilent's HaloPlex[HS] customized target enrichment panel enables the detection of genetic variants including rare ones. A comparative study on custom targeted NGS panel [36] revealed that HaloPlex platform has the lowest workflow complexity and the highest alignment rate to target regions (over 90% of raw sequencing files were aligned to the human genome) in addition to the highest on-target specificity (over 99% of its mapped reads were aligned to targeted regions). Moreover, this targeted-capture NGS kit enables deeper sequencing coverage with the greatest average normalized coverage. The aim of our study was not only to assess the feasibility of this tool to identify inherited mutations, but also to extend our knowledge about the clinical relevance of HI genes and mutations. In fact, the spectrum of HI-causing genes shows huge genetic and phenotypic diversities. One example that best describes this diversity is the presence of a group of genes in which mutations can cause either NSHI or USH. It is therefore essential to provide proper diagnosis prior to offering optimal patient care. ClinGen gene curation process is publicly available and is designed to help in evaluating gene-disease strength relationship (https://www.clinicalgenome.org/).

Expanding the spectrum of HI mutations in Tunisian patients

In this study, we successfully identified and confirmed the segregation of five novel pathogenic mutations and two previously reported pathogenic variants but first time identified in Tunisian population.

The Arg104Ter mutation in *CIB2* gene was found in homozygous state in our patient (Family.1_P1) as well as a second affected member, absent in unaffected members, while both parents were carriers for this particular variant (Figure S1). According to ACMG this variant is predicted as pathogenic since it represents a nonsense variant that affects the *CIB2* gene, which is a known mechanism of HI. Furthermore, this variation has a low MAF in ExAC and

Table 4
Summary of all recruited patients' information.

Patient ID	Pedigree ID	Consanguineous Relations	Pathogenic Variant	Age	Gender	Affected Members	Segregation Analysis	Ophthalmological investigation Tests / Figures / Outcomes		
Patients with non-syndromic Hearing Impairment										
P.1	Family 1	First Cousins	*CIB2*-Arg104Ter	24	Male	2	+	OFE	Data not shown	No signs in favor of RP
P.2	Family 2	First Cousins	*ESRRB*-Tyr295Cys	22	Male	2	+	OFE	Data not shown	No signs in favor of RP
P.3	Family 3	First Cousins	*WHRN*-Gly808AspfsX11	34	Male	3	+	OFE ERG	Data not shown Fig. 3	No signs in favor of RP
P.4	Family 4	First Cousins	*OTOF*-Glu57Ter	27	Male	3	+	OFE	Data not shown	No signs in favor of RP
P.5	Family 5	Third cousins	*MYO15A*-Phe2089Leu	19	Female	4	+	OFE	Data not shown	No signs in favor of RP
P.6	Family 6	Third cousins	*SLC22A4*-Cys113Tyr	33	Male	3	+	OFE	Data not shown	No signs in favor of RP
P.7	Family 7	> Third cousins	*PNPT1*-Glu475Gly	28	Male	5	+	OFE	Data not shown	No signs in favor of RP
P.8	Family 8	First Cousins	*OTOA*-Val603Phe	30	Female	5	–	OFE	Data not shown	No signs in favor of RP
P.9	Data Not Shown	First Cousins	*TMC1*-Met195Arg	22	Female	5	–	OFE	Data not shown	No signs in favor of RP
Patients with Usher Syndrome										
P.10	Family 10	> Third Cousins	*USH1C*-Gln122Ter	32	Female	3	+	OFE ERG	Data not shown Fig. 1	Significant bilateral retinopathy
P.11	Family 11	First Cousins	*MYO7A*-Tyr560Cys	40	Male	7	+	OFE	Data not shown	Typical retinal degeneration
P.12	Family 12	–	*MYO7A* c.470 + 1G > A *MYO7A* c.2283-1G > T	3	Female	1	+	OFE OCT	Fig. 2	Normal eyes fundi Alterations of the RPE and of the boundary line between IS and OS in the perimacular region

RP: Retinitis Pigmentosa / OCT: Optical Coherence Tomography.
RPE: Retinal Pigment Epithelium / ERG: Ganzfeld Electro-Retinogram.
OFE: Ocular fundus examination / IS / OS: Inner and Outer Segments.

gnomAD databases. To the best of our knowledge, this novel mutation also represents the second *CIB2*-mutation identified in Tunisian family.

The novel homozygous missense variation (c.884A > G) in *ESRRB* gene; which is a well-known gene associated with HI, leads to the change of tyrosine AA at position 295 into cysteine (Family.2_P2). The pathogenicity of p.(Tyr295Cys) mutation is supported by the homozygous state in two affected members and the heterozygous state in their parents (Figure S1) in addition to various prediction programs (SIFT and Provean) used to validate this finding. This variant has low MAF in the SNP, ExAC and gnomAD databases. According to the adapted ACMG/AMP standards and guidelines concerning HI, we classified this novel variation as likely pathogenic (Table 3). Mutations in *ESRRB* seem to be a rare cause for ARNSHI in Tunisian population. To the best of our knowledge, we believe that this novel missense variation represents the second *ESRRB* mutation reported in Tunisia since 2011 [37]. Mutations in *ESRRB* gene were also reported in population of Pakistan, Turkey and Czech Republic [38–40]. According to UniProt, the ESRRB-Tyr295Cys variant is located in an essential region for ESRRB transcriptional activity and interaction with other proteins such as the Nuclear Receptor Coactivator 3 protein (NCOA3). Our molecular modeling analysis showed that ESRRB-Tyr295Cys is located in a loop region, known as patternless regions, which connect two regular secondary structures. The substitution of the aromatic amino acid Tyr, located on the ESRRB solvent exposed areas within the protein's surface, with Cys amino acid may cause loss of hydrogen bonds in the protein core and/or disturbing correct folding (Figure. S2.A). Therefore, this variation would interrupt the interaction between ESRRB and RNA polymerase II complexes, blocking the NCOA3 co-recruitment to ESRRB, KLF4, NANOG and SOX2 enhancer regions that should trigger ESRRB-dependent gene activation involved in self-renewal and pluripotency.

We have as well identified a novel variant Phe2089Leu (Family.5_P5) in *MYO15A* gene, the third most critical HI causing gene**.** In fact, *MYO15A* has been associated with HI for the first time as early as 1998 [41]. Over the 192 recessive variations previously reported in this particular gene in HI patients from all over the world, 82 variants were reported to be pathogenic, 73 reported to be likely pathogenic and 17 variants of unknown significance VUS [42,43]. In Tunisian population, the regional prevalence of *MYO15A* mutations in GJB2 negative HL patients is around 2.3%. The *MYO15A*-Phe2089Leu variant was not found in any database. According to UniProt protein this variation is located in the MYO15_HUMAN Tail which represent a crucial domain for normal hearing function by maintaining normal structure and function of vestibular hair cells [44] and also encompass over 64 previously identified pathogenic variants [43]. Based on our analysis, the Phe2089 is located in a beta sheet and its substitution with Leu in this position may disturb the secondary rigid structure (Figure. S2.B). Our segregation analysis confirmed the pathogenicity of this novel variant in *MYO15A* gene (Figure S1).

The c.360C > T variant was reported in this study affecting two transcripts of *USH1C* gene (namely NM_005709 and NM_153676) in a Tunisian patient (Family.10_P10). This mutation was detected in 3 siblings (Figure S1). It was previously discussed that the shorter *USH1C* transcript (NM_005709) is expressed in both retina and inner ear whereas the longer transcript (NM_153676) which includes the PDZ domain, a region thought to cause NSHI, is exclusively expressed in inner ear [45]. Variants in common exons of both transcripts lead to USH type 1C whereas variants in unique exons of the longer transcript lead to NSHI [46,47]. Based on ClinGen gene curation process, mutations in this gene were definitely classified as USH type 1 causing mutations. Detailed ophthalmologic examinations in two affected members in this family were performed to determine the phenotype associated with the

Gln122Ter mutation. The ERG recorded in both patients showed almost absence of responses in scotopic and in photopic with absence of 30 Hz flicker in both eyes. These traces confirm the evidence of a significant bilateral retinopathy in both affected members (Data of Family.10_P10 is shown in Fig. 1).

Herein, we also identified and established the segregation of a novel homozygous pathogenic variant in five patients with USH1B (Figure S1). The c.1679A > G (Family.11_P11) is localized in the motor domain of the *MYO7A* gene resulting in a substitution of Tyr560, a highly conserved residue, to Cys (*MYO7A*-Tyr560Cys). Clinical exploration in this family (an audiometric examination, an ophthalmological examination and a caloric test) confirmed that all 7 patients suffered from congenital profound deafness, retinitis pigmentosa as well as a vestibular dysfunction (data not shown). In fact, according to genetics home reference (https://ghr.nlm.nih.gov/gene/MYO7A#resources), over 200 pathogenic variants have been previously detected in Usher syndrome type I patients, precisely USH1B, accounting for more than 50% of USH1 cases [48]. Among those variants, 124 pathogenic variants have been located in the MYO7A motor domain which supports the pathogenicity of our newly discovered variant (PM1-ACMG according to Varsome). Over 20 mutations have been previously identified in North African population, particularly in the Tunisian population, where molecular studies showed that mutations in *MYO7A* gene represents the major cause for USH1 [23]. Our data further confirms the heterogeneity in *MYO7A* HI-causing mutation among the Tunisian population. The MYO7A-Tyr560Cys variant is located in the Myosin motor domain which represents a hot spot region with 84 pathogenic non-VUS variants with only 9 benign variants (Pathogenicity rate approaching 90.3%). This mutation is located in a non-structured region. Tyrosine560 is a highly conserved amino acid which establishes two hydrogen bonds with both His553 and Asp455 (Figure.S2.C). The former His aa belongs to the same strand while the latter Asp aa lies upstream of the relay in the HP helix. These two residues, as well as the two hydrogen bonds, are conserved in all myosin motor domains. Replacement with cysteine will disrupt those hydrogen bonds and would probably affect the Myosin head motor domain stability. Moreover, this substitution is likely to prevent phosphorylation as this highly conserved Tyr residue is located in the surface and it is therefore totally exposed to the solvent.

In addition to those five novel pathogenic variants, we detected two previously reported mutations but identified for the first time in Tunisian families. The *PNPT1*-Glu475Gly variant identified in (Family.7_P7) segregated in 5 siblings born from consanguineous parents with ARNSHI (Figure S1). This variant which leads to substitution of the residue Glu475 located at the functional domain of the protein, precisely in the second RNase-PH domain, was identified in a consanguineous Moroccan family experiencing severe HI [20]. This missense variant was not annotated in both dbSNP and gnomAD databases. It was also absent in over 400 control individuals analyzed in the published study from Germany, Morocco and Turkey [20]. It was proven that congenital severe HI in the Moroccan family caused by this hypo-functional variation, would be explained by the disruption of the mitochondrial-RNA-import [20]. Even though the genetic data reported the functional importance of this residue (Glu475) suggesting the implication of this missense variation in HI, the *PNPT1*-ARNSHI relationship was classified as limited, when reviewed by the ClinGen Hearing Loss Working Group. According to the ACMG criteria, we classified this variant as likely pathogenic. ClinVar and UniProt classified this variant as pathogenic. Our findings provide further solid evidence for the *PNPT1* gene implication in the auditory function and thus its association with ARNSHI.

The second previously reported homozygous mutation in *OTOF* gene (Family.4_P4) resulted in p.(Glu57Ter) change. To our
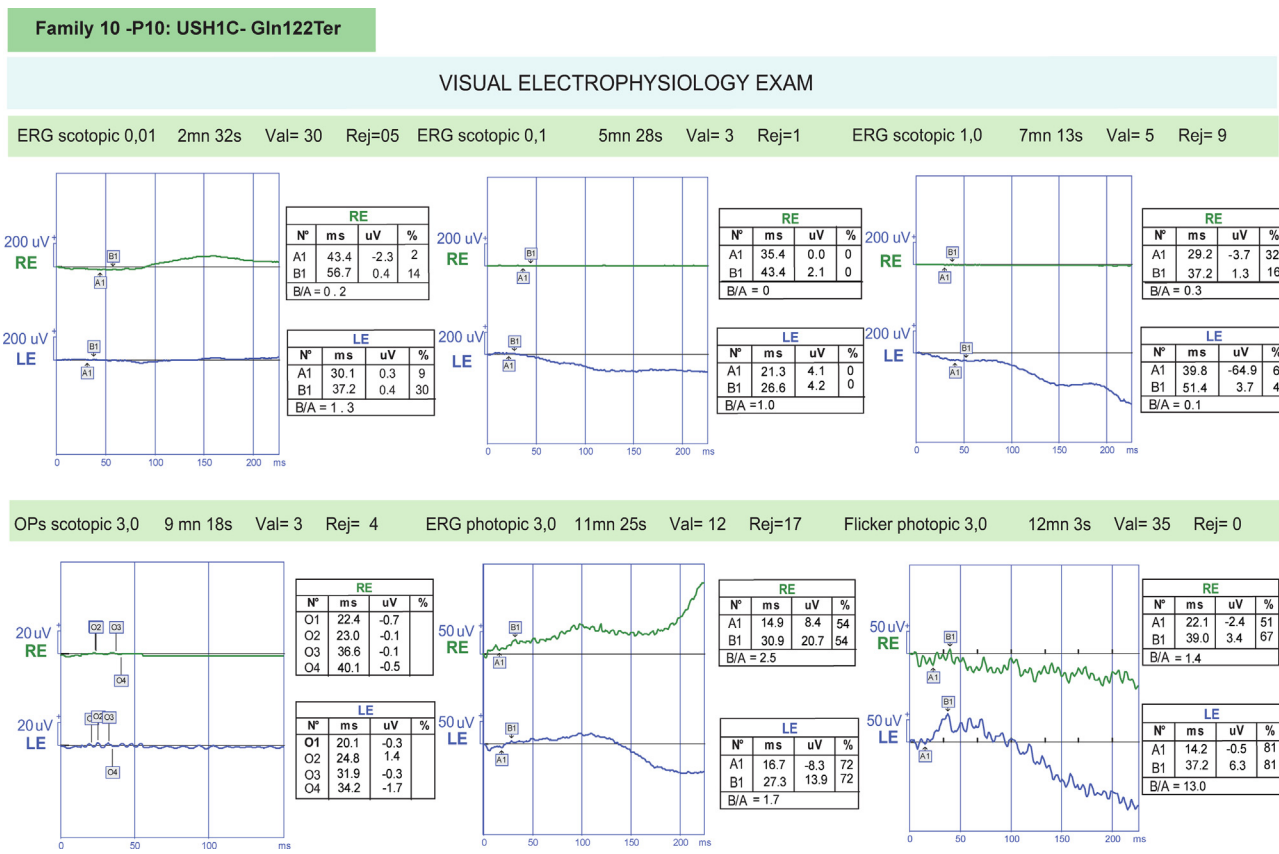
Family 10 -P10: USH1C- Gln122Ter

VISUAL ELECTROPHYSIOLOGY EXAM

ERG scotopic 0,01   2mn 32s   Val= 30   Rej=05   ERG scotopic 0,1   5mn 28s   Val= 3   Rej=1   ERG scotopic 1,0   7mn 13s   Val= 5   Rej= 9

RE

N°	ms	uV	%
A1	43.4	-2.3	2
B1	56.7	0.4	14
B/A = 0 . 2			

LE

N°	ms	uV	%
A1	30.1	0.3	9
B1	37.2	0.4	30
B/A = 1 . 3			

RE

N°	ms	uV	%
A1	35.4	0.0	0
B1	43.4	2.1	0
B/A = 0			

LE

N°	ms	uV	%
A1	21.3	4.1	0
B1	26.6	4.2	0
B/A =1.0			

RE

N°	ms	uV	%
A1	29.2	-3.7	32
B1	37.2	1.3	16
B/A = 0.3			

LE

N°	ms	uV	%
A1	39.8	-64.9	6
B1	51.4	3.7	4
B/A = 0.1			

OPs scotopic 3,0   9 mn 18s   Val= 3   Rej= 4   ERG photopic 3,0   11mn 25s   Val= 12   Rej=17   Flicker photopic 3,0   12mn 3s   Val= 35   Rej= 0

RE

N°	ms	uV	%
O1	22.4	-0.7	
O2	23.0	-0.1	
O3	36.6	-0.1	
O4	40.1	-0.5	

LE

N°	ms	uV	%
O1	20.1	-0.3	
O2	24.8	1.4	
O3	31.9	-0.3	
O4	34.2	-1.7	

RE

N°	ms	uV	%
A1	14.9	8.4	54
B1	30.9	20.7	54
B/A = 2.5			

LE

N°	ms	uV	%
A1	16.7	-8.3	72
B1	27.3	13.9	72
B/A = 1.7			

RE

N°	ms	uV	%
A1	22.1	-2.4	51
B1	39.0	3.4	67
B/A = 1.4			

LE

N°	ms	uV	%
A1	14.2	-0.5	81
B1	37.2	6.3	81
B/A = 13.0			

Fig. 1. ERG recordings in Family.10_P10 patient with retinitis pigmentosa. Severely reduced scotopic and photopic ERG flash responses with attenuated 30 Hz flicker in both eyes.

knowledge this is the first *OTOF* mutation identified in a Tunisian family (Figure S1). This variant is labeled as likely pathogenic by the ClinVar database and has an AF of 4e-6 in gnomAD database. This mutation has been already reported in two Saudi siblings [18] as well as three other families from various regions in Saudi Arabia with severe to profound HI being described [17]. It was also reported in a single Libyan family with ARNSHI and other HI probands [19]. All families reported in Almontashiri et al. (2018) study had tribal origin, so they argued that pathogenic variants identified only once in this cohort may either have drifted further with tribal migration, along the same trade and tribal migration routes, or into neighboring Middle Eastern countries (Syria, Lebanon, Jordan, Palestine, Libya, and Tunisia to the north).

Correlation between OTOF mutations and hearing aids

More than 90 pathogenic variants were reported in *OTOF* gene [33] with over 50% of subjects having *OTOF* mutations causing non-syndromic auditory neuropathy (AN) commonly with normal OAEs [19,49,50]. One of our patient (27-year-old) had preserved OAEs indicating a normal outer hair cell function and suggesting that AN would be the most likely diagnosis in this case. However, concerning her sister (18-year-old) who has been using bilateral hearing aids for eleven years, OAEs were absent. OAEs disappearance might be caused by damage from hearing aid use. Since patient care differs between AN and non-AN groups, finding its genetic cause is important. Genetic screening for mutations in *OTOF* are needed when no neurological syndrome is present with preserved OAEs for an effective genetic counseling and medical guidance [51,52].

Importance of genetic testing for early USH-diagnosis

In this study, NGS enabled the identification of two compound heterozygous mutations previously reported in the *MYO7A* gene, the c.470 + 1G > A, a splice donor site variant, and the c.2283-1G > T a splice acceptor site variant in a three-years-old girl (Family.12_P12). Their parents were carriers for the c.470 + 1G > A (mother) and the c.2283-1G > T (father) pathogenic alterations (Figure S1). The splicing defect c.470 + 1G > A was first reported in 1997 in Tunisia [30]. This mutation was later detected in other Tunisian families [9,23], in Algerian population [22] as well as in a Saudi family [53]. The second splice site variant, c.2283-1G > T was first identified in a family from Algeria [29], a patient from Morocco [28], France [27] and then using whole exome sequencing, Riahi et al. identified this splice acceptor site mutation for the first time in Tunisian families with USH1B [24]. Pathogenicity of this variant was confirmed by different bioinformatics tools and functional analysis [26].

A recent study of patients suffering from USH1B caused by the homozygous splice site mutation (c.470 + 1G > A) indicated that the age of onset of visual symptoms was between five and six years old [53]. Therefore, it is important to note that children with USH1 are most likely clinically misdiagnosed and considered as patients with NSHI since visual defect is only detectable in later years [54] such was the case of our three years-old patient. Based on our genetic diagnosis result, we asked the parents for an extensive ophthalmological examination. OFE of this patient was normal in both eyes, whereas optical coherence tomography (OCT) showed alterations of the retinal pigment epithelium (RPE) and of the
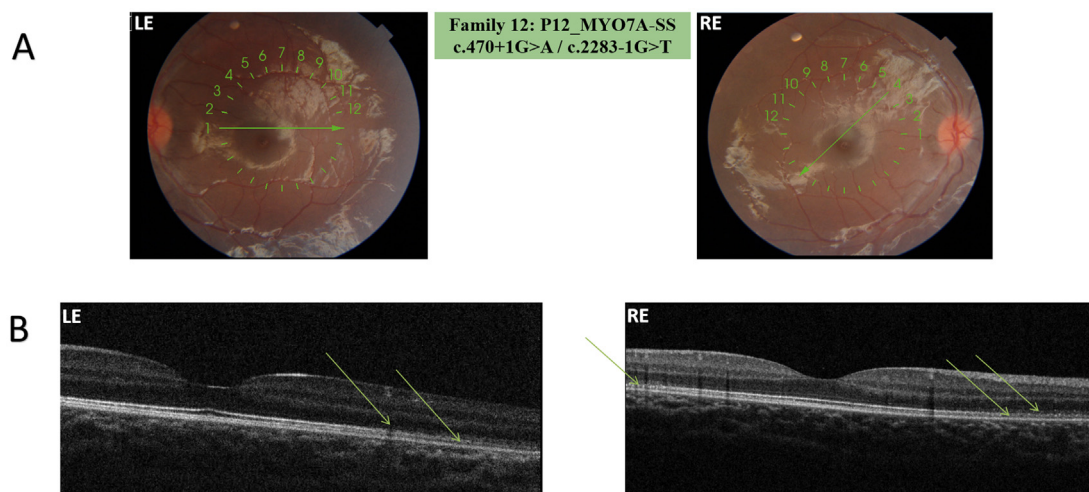
Fig. 2. Ophthalmologic examinations of a 3-years-old patient with compound heterozygous SS-*MYO7A* (Family.12_P12). (A) Fundus photographs of both the left eye (LE) and the right eye (RE) are normal. (B) Optical coherence tomography (OCT) scans showing alterations of the retinal pigment epithelium (RPE) and of the boundary line between inner and outer segments (IS and OS) in the perimacular region. Lesions are indicated with arrows.

boundary line between inner and outer segments (IS and OS) in the perimacular region (Fig. 2). A recent study of the retinal findings in a group of patients with *MYO7A*-associated USH1 showed that the most common qualitative retinal abnormality was the external layer damage in macular area, which represents an early and closely symptom associated with USH1 [55]. Our findings would assist in offering adapted educational orientation for this patient.

Further evidence supporting the association of WHRN and CIB2 genes with ARNSHI

WHRN gene was previously described to be associated with autosomal recessive Usher syndrome type 2 [56–58] and the role of this gene in USH has been scored as having a "definitive" clinical validity with highly supportive evidence (https://www.clini-calgenome.org/). Mutations in *WHRN* were also described as ARN-SHI causing variants [21,56,59,60]. Variants affecting the C-terminal region of the *WHRN* protein [21,61] have been reported to segregate with HI. However, based on ClinGen Hearing Loss Working Group updates, there is currently moderate evidence to support the *WHRN*-ARNSHI gene-disease relationship. In our study, high throughput sequencing in one consanguineous Tunisian family with ARNSHI revealed a 1-bp deletion (c.2423delG, NM_015404.1) in exon 16 of the *WHRN* gene leading to frameshift with a premature stop codon Gly808AspfsX11. Subsequent genotyping of this deletion in related family members confirmed its co-segregation with the disease. The c.2423delG was found in homozygous state in three more affected members and in heterozygous state in both unaffected parent and siblings (Figure S1). This mutation has been previously reported by Tlili et al. (2005) in a different Tunisian family with ARNSHI. To determine the phenotype associated with the c.2423delG mutation, we performed detailed ophthalmologic examinations in affected members. Ocular fundus examination was normal and no night blindness was reported either. Ganzfeld-ERG recorded particularly in two patients (34 and 44 years of age) showed normal response flash visual-evoked potential in both eyes. Plot in photopic and scotopic with ample flicker 30 Hz and normal latency indicate that there are no signs in favor of retinitis pigmentosa in both patients. Ophthalmological examination's results of our patient (Family.3_P3) are shown in Fig. 3. Our findings present strong evidence supporting the *WHRN*-ARNSHI relationship.

CIB2 gene was first associated with autosomal recessive Usher Syndrome Type 1 [62]. Then in 2018, a multi-ethnic cohort study on patients with *CIB2*-related-ARNSHI called into question any involvement of *CIB2* in the physiopathology of USH [63]. It was shown that the p.(Glu64Asp) variant in the Usher family previously described by Riazuddin et al., (2012) is only two AAs away from the p.(Arg66Trp) variant that has been linked to ARNSHI in three families [63]. Booth et al., (2018) argued that it would be expected to see retinal and vestibular phenotypes in families segregating those variants if *CIB2* played a role in USH, however, all patients of this study showed normal ocular phenotype with no vestibular dysfunction. According to the ClinGen Hearing Loss Working Group, *CIB2* gene was associated only with ARNSHI excluding the *CIB2*-autosomal recessive USH Type 1 relationship. Up till now, a total of 9 *CIB2* variants (missense, in-frame indels, nonsense, frameshift) were reported in patients with ARNSHI [60,62,64–66]. Here, we report an additional novel nonsense mutation Arg104Ter in *CIB2* gene in a patient with ARNSHI. Figure-logical evaluations of all affected individuals revealed absence of RP. Our results provide additional evidence and insight for *CIB2* gene-ARNSHI association.

Further evidence supporting the uncertain clinical significance of a variant previously reported as causal mutation in OTOA gene

We identified the p.Val603Phe variant in *OTOA* gene which was previously reported in an Algerian family [33]. In Ammar-Khodja et al. 2015 study, two related patients carrying a homozygous missense mutation (c.1807G > T: p.Val603Phe) in *OTOA* gene were reported. This missense mutation was predicted to be pathogenic by PolyPhen-2, SIFT and Mutation Taster programs, and also predicted to affect the acceptor site of intron 18 by NNSPLICE prediction program [33]. Furthermore, the clinical significance of this variant in ClinVar is uncertain. According to ClinGen gene curation process, cautions should be used when evaluating missense variants in this gene as it has a pseudogene. Segregation analysis of this previously reported HI variant proved that it was absent in one affected member and found in heterozygous state in two other affected members in the same family. It is important to note that genetic heterogeneity of HI is common even within the same family. However, even though a second variant in *OTOA* or a different gene could explain the deafness in this family, we believe that lack of segregation of this *OTOA* variant, further support its
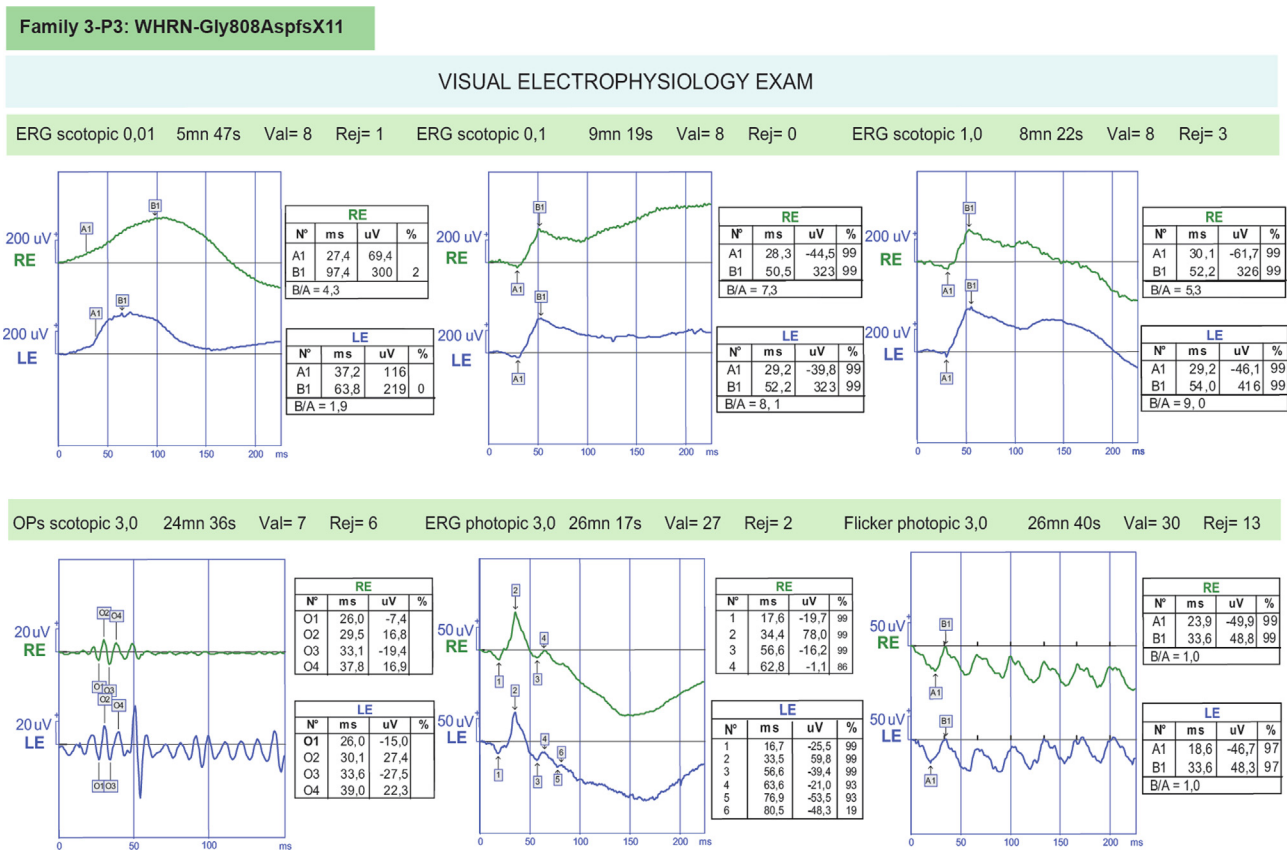
Fig. 3. Plot in photopic and scotopic with ample flicker 30 Hz and normal latency. There are no signs in favor of retinitis pigmentosa in Family.3_P3.

classification as variant of unknown significance According to the ACMG/AMP guidelines (Table 3).

In conclusion, our findings do not only exhibit the usefulness of the targeted NGS approach in the identification and diagnosis of genetic mutations affecting HI in Tunisian families (11 identified pathogenic variants, 5 of which are novel and two newly identified in Tunisian population), but would also have global impact with introduction of new evidence which improve our knowledge concerning clinical relevance of HI causing genes and variants.

Finally, we believe that our findings would be useful for the curation activities of the ClinGen Hearing Loss working group especially in re-evaluating HI gene-disease validity as well as variants pathogenicity.

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.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2021.01.005.

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