


ORIGINAL ARTICLE

Comprehensive molecular analysis of 61 Egyptian families with hereditary nonsyndromic hearing loss

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Abstract

Nonsyndromic hearing loss is an extremely heterogeneous disorder. Thus, clinical diagnostics is challenging, in particular due to differences in the etiology of hearing loss between populations. With this study, we wanted to elucidate the genetic basis of hearing loss in 61 consanguineous Egyptian families. In 25 families, linkage analysis was used as a prescreening to identify regions for targeted sequencing of candidate genes. Initially, the coding regions of 12 and later of 94 genes associated with hearing loss were enriched and subjected to massively parallel sequencing (MPS) with diagnostic yields of 36% and 75%, respectively. Causative variants were identified in 48 families (79%). They were found in 23 different genes with the majority being located in *MYO15A* (15.3%), *SLC26A4* (9.7%), *GJB2* (8.3%), and *MYO7A* (6.4%). As many as 32 variants were novel ones at the time of detection. Five variants were shared by two, three, or even four families. Our study provides a first survey of the mutational spectrum of deaf patients in Egypt revealing less *GJB2* variants than in many European populations. It underlines the value of targeted enrichment of well-selected deafness genes in combination with MPS in the diagnostics of this frequent and genetically heterogeneous disorder.

KEYWORDS

clinical diagnostics, gene panel sequencing, genetic heterogeneity, hearing loss, massively parallel sequencing, nonsyndromic, sensorineural

1 | INTRODUCTION

Hearing loss (HL) is the most common birth defect in developed countries and the most prevalent sensorineural disorder. One of every

500 newborns has bilateral permanent sensorineural hearing loss ≥ 40 dBHL. Before the age of 5, the prevalence increases to 2.7 per 1000 and to 3.5 per 1000 during adolescence.¹ In more than 50% of affected neonates, sensorineural hearing loss (SNHL) is caused by genetic factors. Genetic hearing loss can either occur as an isolated condition (nonsyndromic, 70%) or with additional phenotypic features (syndromic,

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30%). Eighty percent of nonsyndromic hearing loss (NSHL) follow an autosomal recessive (AR) mode of inheritance, <20% an autosomal dominant one (AD), and 1% are either X-linked or mitochondrial forms.²

Inherited nonsyndromic hearing loss is characterized by a remarkable genetic heterogeneity. According to recently published data, more than 1000 causative variants have been identified in more than 90 genes.³ Despite this enormous heterogeneity, there is one very common cause of severe-to-profound autosomal recessive nonsyndromic hearing loss (ARNSHL) in populations of Europe, the Middle East, and North America, namely variants of the *GJB2* gene, which codes for Connexin 26. In particular, the variant NM_004004.5:c.35delG has been found to be the most common cause of ARNSHL in the mentioned populations, being responsible for up to 63% of cases.⁴

Previous studies on the cause of deafness in African populations or countries, including the Egyptian population, were mainly focused on the analysis of *GJB2*. Snoeckx et al. analyzed the coding sequence of *GJB2* in 111 Egyptian families (159 individuals) with nonsyndromic hearing loss. They found that in only 14.4% of them ARNSHL was due to mutations in *GJB2*, yet similar to Caucasian populations NM_004004.5:c.35delG was the most frequently found mutation in those families.⁵ Another study, performed on 97 families (155 individuals) from Southern Egypt, revealed an allele frequency of 8.7% for c.35delG and one other *GJB2* mutation in single family—the novel missense variant NM_004004.5:c.T212A (p.I71N).⁶ A further study reported the mitochondrial variant in *MTRNR1* NC_012920.1:m.1555A>G to rarely cause SNHL in Egyptian patients.⁷

This is the first study aimed at a comprehensive detection of gene variants causing hearing loss in Egyptian families by taking advantage of massively parallel sequencing (MPS) of beforehand-enriched DNA targets. In total, we analyzed 61 consanguineous families from Southern Egypt. In a first step, 25 families without causal variants in *GJB2* were genotyped on a 250 K SNP array for homozygosity mapping resulting in 12 candidate genes analyzed by MPS. In a second step, the remaining 36 families plus 16 families that could not be solved in the first step were analyzed using targeted enrichment of 94 genes and MPS. With this partially two-tiered approach, we identified candidate variants in 48 out of the 61 families under investigation, which we confirmed by segregation analysis. This corresponds to a diagnostic yield of 78.7%. The largest number of causal variants per gene was found in *MYO15A*.

2 | MATERIALS AND METHODS

2.1 | Subjects

The study group included 61 families with hereditary nonsyndromic hearing loss recruited from Governorates of South Egypt (for pedigrees see Figure S3). The families with A-ID numbers represent a subset of an earlier study on the frequency of *GJB2* variants in 97 families from Southern Egypt.⁶ We selected 25 families from the negatively tested ones for further analysis in this study. The 36 families with B-ID numbers had not been screened for *GJB2* variants prior to inclusion into this study. Informed consent was obtained from all participants in the study.

The study was approved by the ethical committee, Faculty of Medicine, Assiut University, Egypt and performed in compliance with national legislation and international standards (Declaration of Helsinki).

2.2 | Clinical evaluation

All participants had no history or manifestation suggestive of environmental causes of hearing loss, and no active or recent history of an otological problem. Clinical evaluation of all individuals included history taking, otoscopic examination and basic audiological evaluation using a dual channel clinical audiometer (Obiter 922) and immittance measurement with Impedance Audiometer Interacoustic AZ 26. When reliable responses via conventional audiometry were not possible an Auditory Brainstem Response (ABR) was used for threshold estimation (Nicolet Spirit OS/2).

2.3 | DNA extraction and prescreening

DNA was extracted from peripheral blood using standard methods. In the first set of families (A-ID numbers) a prescreening for *GJB2* variants by restriction assays, single strand conformational polymorphism analysis and Sanger sequencing had been performed as part of a previous project.⁶ A subset of 25 families without HL associated variants in *GJB2* was transferred to this study. The second set of 36 families (B-ID numbers) was not part of the previous project and had not been subjected to a prescreening for *GJB2* variants.

2.4 | Linkage analysis

Genome-wide linkage analysis of 51 individuals from the prescreened 25 families was carried out using GeneChip Human Mapping 250 K SNP Array data (Thermo Fisher Scientific). Genotypes were called by the GeneChip DNA Analysis Software (GDAS v3.0, Thermo Fisher Scientific). Subsequent data handling was performed using the graphical user interface ALOHOMORA.⁸ Relationship errors were identified using the program Graphical Relationship Representation.⁹ The program PedCheck was applied to find Mendelian errors¹⁰ and data for SNPs with such errors were removed from the data set. Non-Mendelian errors were identified using the program MERLIN¹¹ and unlikely genotypes for related samples were deleted. Linkage analysis was performed assuming autosomal recessive inheritance, full penetrance, consanguinity and a disease allele frequency of 0.0001. Multi-point LOD scores were calculated using the programs ALLEGRO¹² or MERLIN.¹¹ Also haplotypes were reconstructed with either of these programs and presented graphically with HaploPainter.¹³

2.5 | Estimation of variant age by IBD

DNA samples of 14 individuals belonging to five families were genotyped on the Axiom Precision Medicine Research Array (Thermo

Fisher Scientific). Genotypes were called by the Axiom Analysis Suite v4.0 (Thermo Fisher Scientific). Genotype data were checked for relationship errors, Mendelian errors and unlikely genotypes, as described before. Haplotypes in linkage regions covering the causing variant were reconstructed in order to determine the length of the shared haplotype between two or more families. The minimum variant age was estimated from the shared haplotype length as described elsewhere.¹⁴

2.6 | Targeted enrichment of 12 genes (gene panel 1, GP1) and MPS

The RainDance enrichment assay was designed to enrich all exons including the exon-intron boundaries of 12 genes known to be associated with hearing loss or located in linkage regions (for gene list see Table S1). The assay comprised 373 exons to capture 377 targets with a total target size of 63 633 bp. 36 patient DNA samples (including eight positive control samples with known mutations) were enriched according to the manufacturer's protocol (RainDance Technologies). Following the enrichment, RainDance fragments had to be concatenated and sheared prior to library preparation. 500 ng of RainDance amplification products were used for the concatenation step according to Illumina's protocol. Chloroform-extraction steps were replaced by a clean-up step using the QIAquick PCR Purification Kit (Qiagen). After concatenation the RainDance samples were sheared according to Illumina's protocol using a Covaris machine (Duty Cycle: 10%, Intensity Cycle: 5, Cycles per burst: 200, Mode: frequency sweeping, treatment time: 180 s, volume: 100 μ L, temperature: 4°C-6°C). Sample volumes for all library preparations were adjusted to 200 μ L and completely loaded onto the SPRIworks system from Beckman Coulter. The Illumina protocol for 200 to 400 bp libraries was used for this automated library production. After library production, the samples were subjected to PCR enrichment following Illumina's protocol (16 cycles of PCR). Afterwards products were purified to remove PCR-primers using AmPure beads. For sequencing on a Genome Analyzer GAIx (Illumina), pools of four or five samples were loaded per lane. Cluster generation was performed on the cluster station according to Illumina's protocol. Subsequent sequencing was also performed following Illumina's protocol with a 1 \times 36 bp run generating on average 129 Mb of raw data per sample.

2.7 | Targeted enrichment of 94 genes (gene panel 2, GP2) and MPS

The SureSelect enrichment assay (Agilent Technologies) was designed to enrich all exons, including the exon-intron boundaries, of 94 genes known to be associated with nonsyndromic and syndromic forms of hearing loss (for gene list see Table S1). GP2 was designed with the SureDesign online tool covering 1504 targets with a total probe size of 496 kb and finally ordered from Agilent. In total, 62 patient DNA samples of 52 families were analyzed together with four patient DNA

control samples. Library preparation and subsequent enrichment were performed using the SureSelect custom XT enrichment protocol (Agilent Technologies). For this, 3 μ g of genomic DNA underwent fragmentation using the Bioruptor (Diagenode) sonication method. Fragments were end-repaired, A-tailed and adapter-ligated and individually enriched according to the standard protocol. A pool of 16 libraries was loaded on a MiSeq, HiSeq2000 or HiSeq4000 sequencer (Illumina). Runs of 2 \times 100 bp or 2 \times 75 bp, respectively, generated on average 152 Mb of raw data per sample.

2.8 | Controls

We analyzed eight patient DNAs with already known mutations as positive controls in the GP1 enrichment experiments.¹⁵⁻¹⁸ In the GP2 enrichment experiments, three patient DNA samples of the GP1 enrichment experiments carrying confirmed mutations were re-analyzed together with one Usher type 2A patient DNA. The DNA of CEPH individual NA12878 served as an internal control and was received from the Coriell Institute for Medical Research. The detected variants of NA12878 were compared with the GIAB (Genome in a Bottle) reference genome data.¹⁹

2.9 | Analysis of MPS data

For data analysis, the in-house VARBANK pipeline and corresponding filter interface was used (versions 1.0 and 2.0, <https://varbank.ccg.uni-koeln.de/> and <https://varbank.ccg.uni-koeln.de/varbank2>, respectively). Primary data were filtered according to signal purity by the Illumina Realtime Analysis (RTA) software v.1.8. Subsequently, the reads were mapped onto the human genome reference builds GRCh37 or GRCh38 using the BWA alignment algorithms v.0.6.2 or v.0.7.15, respectively.²⁰ Duplicates were marked with Picard v.2.1.1 (<https://broadinstitute.github.io/picard/>) and GATK v.3.6²¹ was used to perform local realignments around short insertion and deletions, recalibrate the base and variant quality scores, and to call SNPs and short indels. Scripts developed in-house at the Cologne Center for Genomics were applied to detect protein changes, affected donor and acceptor splice sites, and overlaps with known variants. Acceptor and donor splice site variants were analyzed by maximum entropy²² as well as position weight modeling.²³ In case of GP1, we filtered for rare variants (minor allele frequency <0.01 based on dbSNP build 134, the 1000 Genomes database build 20110521, the public Exome Variant Server, US National Heart, Lung, and Blood Institute, build ESP5400 and an in-house database of 511 epilepsy patients). In case of GP2, we applied additionally to the filters mentioned above the default GATK best practice filter of VARBANK because of the higher quality of data.

We focused on variants with predicted changes in the protein sequence and major effects on core splice sites while inspecting the filtered data received from the enrichment and sequencing of both panels. The resulting lists of genes were then prioritized based on

scores obtained by Polyphen2²⁴ and Sorting Intolerant From Tolerant (SIFT) algorithms²⁵ or the MedPred Score of VARBANK 2.0. In case of missense variants, we used the NCBI's HomoloGene database (<http://www.ncbi.nlm.nih.gov/homologene/>) to scrutinize the conservation of the affected amino acid residues among mammals or other species. All variants were checked for their presence in the gnomAD database (<http://gnomad.broadinstitute.org/>), ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) and the Deafness Variation Database.²⁶

2.10 | Sanger sequencing analysis

Primer pairs were designed using Primer 3 software.²⁷ Primer sequences and PCR conditions are available upon request. PCR products were directly sequenced using an ABI PRISM 3730 DNA Analyzer and the BigDye Terminator Cycle Sequencing Kit version 1.1 (Thermo Fisher Scientific).

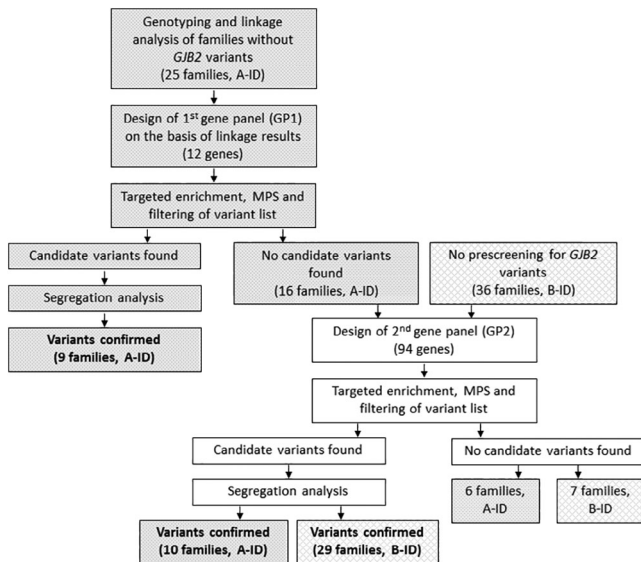
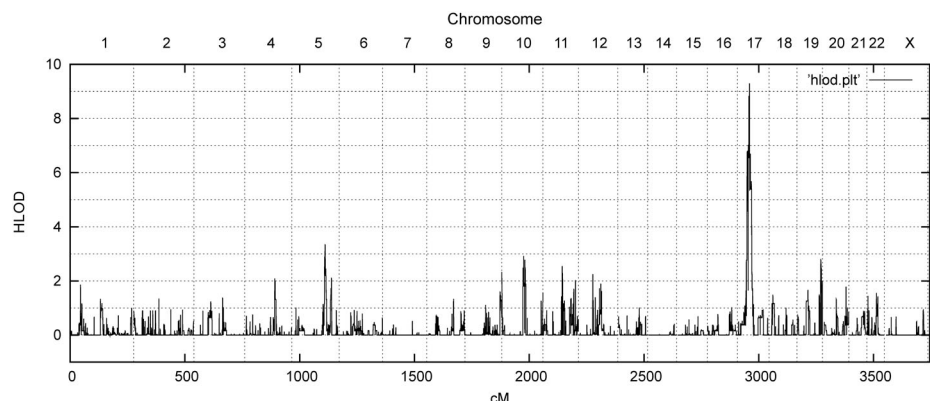


FIGURE 1 Outline of the study. The different experimental steps, included family sets and obtained results are summarized in the flow chart

FIGURE 2 Genome-wide HLOD plot of 25 families. MERLIN with LD modeling was used for Linkage analysis assuming autosomal recessive inheritance, full penetrance, consanguinity, and a disease allele frequency of 0.0001.¹¹ Chromosome numbers are given on the top of the plot; genetic distance and HLOD are displayed on the x- and y-axis, respectively



3 | RESULTS

3.1 | Linkage Analysis

After exclusion of *GJB2* variants,⁶ 25 consanguineous Egyptian families, in the majority of cases with 2 to 3 affected members suffering from severe or profound ARNSHL, were selected for further genetic work-up (Figure 1). In a first step, 51 individuals of the 25 families were genotyped with a GeneChipHuman Mapping 250 K SNP array.

The subsequent linkage analysis of all 25 families under the assumption of heterogeneity revealed a significant HLOD score of 9.3 (alpha = 0.36) on chromosome 17 (Figure 2). Nine families contributed to the HLOD peak. Haplotype construction revealed a common homozygous region spanning 0.5 cM that did not harbor any known deafness gene or locus. Six out of the nine families (A14, A20, A36, A40, A41, and A50) shared an expanded homozygous region of 15.3 cM that included the genes *MYO15A* and *RNF135*. Apart from the main HLOD peak mentioned above, four families (A19, A29, A36, and A40) contributed to a second peak (HLOD = 3.15, alpha = 0.184), which was located on chromosome 5. Since the shared homozygous region on chromosome 5 did not include any known deafness gene or locus, we searched within the homozygous region of each family separately and assigned *DIAPH1*, *POU4F3*, and *TCOF1* as candidate genes.

Analyzing family A12 alone, we detected a significant linkage signal on chromosome 10 with a maximum LOD score of 3.6 (Figure S1A). Haplotype construction revealed a homozygous region shared by all affected family members. It spans 13.5 cM and is flanked by the markers rs10999893 and rs11200759 (Figure S1B). The linkage interval includes *CDH23*. Three other families, namely families A14, A17, and A21, shared different parts of the linkage region of family A12 on chromosome 10.

3.2 | Targeted capture of HL genes and MPS

Based on the linkage results, we designed a small gene panel (GP1) for ARNSHL. It included six candidate genes located in the linkage regions on chromosomes 5, 10 and 17 (*MYO15A*, *RNF135*, *CDH23*, *DIAPH1*,

TABLE 1 HL associated variants detected in the present study

Gene	cDNA change	Amino acid change	Variant effect	No. of causal alleles	No. of causal alleles per gene	% of causal alleles of the respective gene	% of all causal alleles	References
ADGRV1	NM_032119.3:c.3508del	p.(Tyr1170MetfsTer36)	Frameshift	1	4	25	1.02	
ADGRV1	NM_032119.3:c.8809_8816del	p.(Phe2937GlnfsTer14)	Frameshift	1	4	25	1.02	
ADGRV1	NM_032119.3:c.6500A>G	p.(Tyr2167Cys)	Missense	1	4	25	1.02	
ADGRV1	NM_032119.3:c.7839_7840del	p.(Gly2615GlnfsTer18)	Frameshift	1	4	25	1.02	
BSND	NM_057176.2:c.107C>A	p.(Thr36Asn)	Missense	4	4	100	4.08	
CDH23	NM_001171931.1:c.1152C>A	p.(Ser387Arg)	Missense	2	4	50	2.04	
CDH23	NM_001171931.1:c.2595del	p.(Arg865SerfsTer4)	Frameshift	2	4	50	2.04	
GJB2	NM_004004.5:c.35delG	p.Gly12ValfsTer2	Frameshift	8	8	100	8.16	28, 29
GRXCR1	NM_001080476.2:c.568C>T	p.(Arg190Ter)	Stop gain	2	2	100	2.04	3
ILDR1	NM_175924.3:c.357_361del	p.(Arg120AspfsTer13)	Frameshift	2	2	100	2.04	
LOXHD1	NG_016646.1(NM_144612.6): c.3350+1G>A		Splice donor	1	4	25	1.02	
LOXHD1	NM_144612.6:c.3727C>T	p.(Gly1243Arg)	Missense	1	4	25	1.02	
LOXHD1	NM_144612.6:c.4465G>C	p.(Gly1489Arg)	Missense	2	4	50	2.04	
MYO15A	NM_016239.3:c.4310A>G	p.(Tyr1437Cys)	Missense	2	20	10	2.04	3
MYO15A	NM_016239.3:c.5330C>A	p.(Ser1777Ter)	Stop gain	2	20	10	2.04	
MYO15A	NM_016239.3:c.6100C>T	p.(Gln2034Ter)	Stop gain	2	20	10	2.04	
MYO15A	NM_016239.3:c.6340G>A	p.(Val2114Met)	Missense	4	20	20	4.08	30
MYO15A	NG_011634.2(NM_016239.3): c.8601+2T>G		Splice donor	6	20	30	6.12	31
MYO15A	NM_016239.3:c.8899dup	p.(Arg2967ProfsTer33)	Frameshift	2	20	10	2.04	
MYO15A	NM_016239.3:c.8899C>T	p.(Arg2967Ter)	Stop gain	2	20	10	2.04	
MYO6	NM_004999.3:c.2302C>T	p.(Gln768Ter)	Stop gain	2	2	100	2.04	
MYO7A	NM_000260.3:c.3659C>T	p.(Pro1220Leu)	Missense	2	10	20	2.04	32
MYO7A	NM_000260.3:c.5581C>T	p.Arg1861Ter	Stop gain	2	10	20	2.04	33, 34
MYO7A	NM_000260.3:c.3997C>T	p.(Gln1333Ter)	Stop gain	2	10	20	2.04	
MYO7A	NM_000260.3:c.4111G>A	p.(Val1371Met)	Missense	2	10	20	2.04	
MYO7A	NM_000260.3:c.5501G>C	p.(Trp1834Ser)	Missense	2	10	20	2.04	
OTOF	NM_194248.2:c.1492C>T	p.(Gln498Ter)	Stop gain	2	2	100	2.04	
PCDH15	NM_001142769.1:c.4542dup	p.Pro1515ThrfsTer4	Frameshift	2	2	100	2.04	35
POU3F4	NM_000307.4:c.346dup	p.(Ala116GlyfsTer77)	Frameshift	1	1	100	1.02	
PTPRQ	NG_034052.1(NM_001145026.1): c.6193-2A>C		Splice acceptor	2	2	100	2.04	
SLC26A4	NG_008489.1(NM_000441.1): c.164+1delG		Splice donor	2	12	17	2.04	36, 37
SLC26A4	NM_000441.1:c.346G>A	p.(Gly116Ser)	Missense	2	12	17	2.04	38
SLC26A4	NM_000441.1:c.691G>A	p.(Val231Met)	Missense	2	12	17	2.04	39
SLC26A4	NM_000441.1:c.1198delT	p.(Cys400ValfsTer32)	Frameshift	6	12	50	6.12	40
STRC	NM_153700.2:c.3851T>A	p.(Val1284Glu)	Missense	2	2	100	2.04	
TECTA	NM_005422.2:c.5870_5884del	p.(Asp1957_Val1961del)	In-frame deletion	2	3	67	2.04	
TECTA	NG_011633.1(NM_005422.2): c.[6156_6162 + 3del;6162+4A>G]		Deletion in splice region	1	3	33	1.02	
TMC1	NM_138691.2:c.420delA	p.(Lys140AsnfsTer8)	Frameshift	2	2	100	2.04	
TMIE	NM_147196.2:c.247C>T	p.(Pro83Ser)	Missense	2	2	100	2.04	
TMPRSS3	NM_024022.2:c.1029G>C	p.(Trp343Cys)	Missense	2	2	100	2.04	

TABLE 1 (Continued)

Gene	cDNA change	Amino acid change	Variant effect	No. of causal alleles	No. of causal alleles per gene	% of causal alleles of the respective gene	% of all causal alleles	References
<i>TPRN</i>	NM_001128228.2:c.440_444dup	p.Arg149AlafsTer303	Frameshift	2	2	100	2.04	
<i>TRIOBP</i>	NM_001039141.2:c.1039C>T	p.(Arg347Ter)	Stop gain	2	4	50	2.04	41
<i>TRIOBP</i>	NM_001039141.2:c.4984dup	p.(Thr1662AsnfsTer48)	Frameshift	2	4	50	2.04	
<i>WFS1</i>	NM_006005.3:c.972C>G	p.(Ile324Met)	Missense	2	2	100	2.04	

TABLE 2 Splice-site alterations

Gene	Variant	Predicted effect	Prediction scores	
			Maximum entropy score ²² Ref → Variant	Position weight score ²³ Ref → Variant
<i>LOXHD1</i>	NG_016646.2(NM_144612.6):c.3350+1G>A	5' splice donor LOF	9.30 → 1.12	6.70 → -6.59
<i>MYO15A</i>	NG_011634.2(NM_016239.3):c.8601+2T>G	5' splice donor LOF	6.23 → -1.42	7.75 → -5.54
<i>PTPRQ</i>	NG_034052.1(NM_001145026.1):c.6193-2A>C	3' splice acceptor LOF	8.01 → -0.03	9.16 → -4.13
<i>SLC26A4</i>	NG_008489.1(NM_000441.1):c.164+1del	5' splice donor LOF	7.38 → -7.56	5.06 → -20.86
<i>TECTA</i>	NG_011633.1(NM_005422.2): c.[6156_6162+3del;6162+4A>G]	5' splice region LOF	10.44 → -34.45	4.54 → -21.48

Abbreviations: Ref, reference allele; LOF, loss of function.

POU4F3, *TCOF1*). We added six further genes, which are all frequently mutated deafness genes (see Table S1). We enriched the coding sequences of all 12 genes using the RainDance microdroplet technique. DNA of at least one affected member of each family (a total of 28 samples) and of eight SNHL patients with known causing variants were subjected to this procedure, and subsequently, the enriched products were analyzed by MPS. In all eight positive control samples we could detect the known causal variants. In the 28 test samples, MPS revealed on average 18 variations per sample that passed the filter criteria of the processing pipeline VARBANK. Among these only one or none was homozygous, but two to five were found in a compound heterozygous state. Probably causative variants were detected in nine out of 25 families (36%). They were found in four different genes, *MYO15A*, *MYO7A*, *CDH23*, and *TECTA*. We confirmed all variants by Sanger sequencing. For segregation analysis additional family members were included. In the six families with expanded homozygous regions on chromosome 17 as revealed by linkage analysis, variants in *MYO15A* could be identified and confirmed. Furthermore, a variant in *CDH23* located in the linkage interval of family A12 was demonstrated to cosegregate with SNHL (Figure S1B). The verified variants are listed in Table S2.

Since we could not uncover the genetic cause of ARNSHL in 16 out of the 25 analyzed families, a more comprehensive analysis was required. Therefore, we designed a panel of 94 hearing loss genes (GP2, see Table S1) and analyzed at least one member of each of the 16 remaining families with unknown mutations and of 36 additional, mainly consanguineous families, which were not prescreened for

GJB2 variants. Thus, a total of 52 families was subjected to the Agilent SureSelect custom enrichment and MPS procedure (Figure 1). As positive controls, we included four patient samples with already known causal variants and the CEPH sample NA12878 into the large gene panel analysis. The disease-causing variants of all four control patient samples were present in the sequencing data, likewise, all known variations of the CEPH sample NA12878 as published by the GIAB consortium. For the samples from the 52 Egyptian families, we found that on average seven variations (range of 2-11 variations) passed the filter criteria of VARBANK. Candidate variants were re-sequenced by Sanger sequencing including samples of additional family members. All candidate variants were found to co-segregate with hearing loss in each of the analyzed families. Our approach yielded a total of 37 different probably damaging variants in 23 genes in 10 out of the 16 remaining families (62.5%) and 29 of the 36 new families (80.5%). Most variants were homozygous; only three compound heterozygous states were observed, concerning variants in *ADGRV1* (2x) and *LOXHD1* (1x), and one heterozygous or hemizygous state each in families with autosomal dominant or X-linked inheritance, respectively (for summary of variants see Table 1, for variants of each family see Table S2).

Taking the results from both gene panel sequencing analyses together, we noticed that some variants of the tested candidate genes are present in more than one family (see Table 1). For example, the already known pathogenic variants NM_004004.5:c.35del in *GJB2*, NM_000441.1:c.1198del in *SLC26A4* and NM_016239.3:c.6340G>A in *MYO15A* are shared by four, three, and two families, respectively.

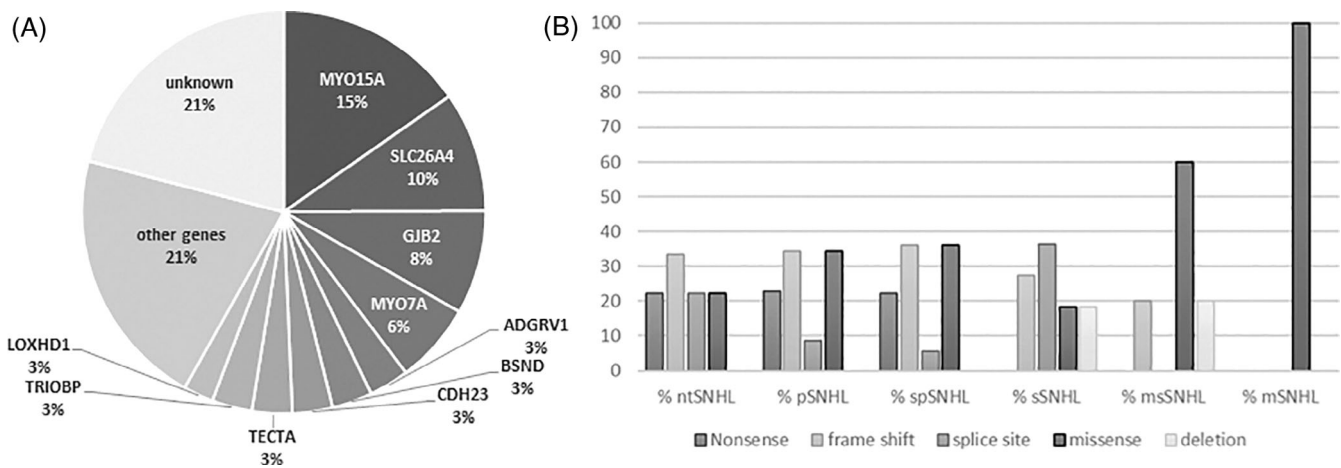


FIGURE 3 Frequency and distribution of gene variants co-segregating with hearing loss in 61 Egyptian families. A, Percentages of gene variants associated with SNHL. The frequency of families with *GJB2* variants refers to a total of 133 families merging the results of the present study for the 36 families without prescreening for *GJB2* variants with data of our previous study on *GJB2* variants in 97 families.⁶ Genes with variants in one family only are lumped together as “other genes.” In total, variants of deafness genes were detected and confirmed by segregation analysis in 48 of 61 families (79%). B, Distribution of putative consequences at the protein level within subgroups of patients showing the same phenotype. Abbreviations: ntSNHL, near total SNHL; pSNHL, profound SNHL; spSNHL, severe to profound SNHL; sSNHL, severe SNHL; msSNHL, moderately severe SNHL; mSNHL, moderate SNHL

Also, the splice donor variant NG_011634.2(NM_016239.3):c.8601+2T>G in *MYO15A*, originally considered as novel, is shared by the families A40, B27, and B84. Haplotype analysis of the three families revealed a shared region of 6.01 Mb, which is suggestive of a common founder origin (results not shown). Additionally, a novel missense variant in *BSND*, NM_057176.2:c.107C>A, was detected in two families (A38 and B91). According to haplotype data, the affected individuals of these families have a region of 4.96 Mb surrounding the *BSND* variant in common. We estimated the age of both variants based on a calculation described earlier.¹⁴ It yielded a minimum age between five and six generations for the *MYO15A* variant and between nine and 10 generations for the *BSND* variant.

4 | DISCUSSION

Hearing loss is extremely heterogeneous, yet mutations in *GJB2* have been described as the most frequent cause of ARNSHL in many populations of the world. Interestingly, previous studies revealed that disease-causing variants in *GJB2* are not that common in the Egyptian population.⁴² To date, comprehensive studies on the mutational spectrum associated with hearing loss in Egypt have been missing. Our study is the first one performed in Egyptian families that is aimed at the detection of causal variants in a large number of genes associated with hearing loss. The study group comprised 61 families recruited from Southern Egypt. The first set of 25 families had been tested negative for *GJB2* variants in a previous project.⁶ In four of the remaining 36 families, we found a variant in *GJB2*, namely the well-known NM_004004.5:c.35delG mutation. This represents a frequency of 11.1%, which matches data of previous studies.^{5,6} When we combine the new results with our previous data,⁶ where seven of 97 families

showed variants in *GJB2*, we have seen *GJB2* variants in total in 11 out of 133 Egyptian HI families, which is a frequency of 8.3%.

While in some populations the prevalence of variants in *GJB2* causing ARNSHL is high, a number of studies have shown that its contribution to deafness varies considerably by ethnicity.⁴ Among individuals of African descent from for example, Cameroon, Nigeria, Sudan, and Kenya, pathogenic variants in *GJB2* are very rare.⁴³ In contrast, studies of Tunisian and Algerian families revealed a prevalence of deafness caused by *GJB2* mutations (39% and 48%, respectively) quite similar to those observed in Caucasian populations, with NM_004004.5:c.35delG being the most prominent mutation.^{44,45} A genomic study on the ancestry of North African populations revealed that they represent varying combinations of at least five distinct ancestries, namely Maghrebi, European, Near Eastern, Eastern and Western sub-Saharan African. Among them, two ancestries show opposite gradients; whereas the proportion of the autochthonous Northern African (Maghrebi) ancestry decreases from Western Sahara eastward to Egypt, the proportion of Near Eastern Arabic ancestry increases from Western Sahara to Egypt.⁴⁶ The low frequencies of *GJB2* mutations found in Egypt and Sudan^{6,47} are in concert with the higher proportion of Near Eastern Arab and sub-Saharan ancestries of north-eastern African populations, which are both showing comparably low *GJB2* mutation frequencies.⁴

In the present study, we detected putatively causative variants in 48 out of 61 analyzed families. These variants are located in 23 different genes, corroborating the enormous heterogeneity of ARNSHL known for other geographic regions also for the southern part of Egypt. Variants in *MYO15A* ranked first with 15.3% families having one followed by *SLC26A4* (9.7%) and *MYO7A* (6.4%) apart from *GJB2* with a separately determined variant frequency of 8.3% based on the analysis of 133 families (see Figure 3A and Figure S2). In total, we

found 44 different variants, 32 of which were novel ones at the time of detection. Types of variants in terms of different consequences at the protein level were widely spread over patients grouped by graduated severity of hearing loss, except that milder phenotypes seemed to be mainly due to missense variants (Figure 3B).

Splice site variants were predicted for five different genes in seven families (see Table 2). One variant in *MYO15A* (OMIM 602666, NG_011634.2(NM_016239.3):c.8601+2T>G) was detected in three unrelated families, A40, B27, and B84. Haplotype analysis revealed a common haplotype of a size suggesting a founder to have lived five to six generations ago. Maximum entropy modeling as well as position weight modeling revealed strongly lowered scores of -1.42 and -5.54 , respectively, for the variant allele as compared to 6.23 and 7.75 for the wild-type allele.^{22,23} Since RNA samples of affected family members were not available, the exact consequences of the splicing error were not analyzed.

Variants in *SLC26A4* (OMIM 605646) are known to cause Pendred syndrome and NSHL. In our study, four different variants were detected in six families, all members without thyroid defects. One of the variants is a homozygous frameshift variant, NM_000441.1:c.1198del, present in five affected members of three families. This variant was reported to cause Pendred syndrome⁴⁰ as well as NSHL (severe to profound hearing loss associated with enlarged vestibular aqueduct [EVA]).⁴⁸ Unfortunately, computerized tomography (CT) to evaluate EVA was not available at the time of assessment. Affected siblings in another family (B29) have severe to profound hearing loss and EVA, but medical history does not suggest abnormal thyroid function. The underlying homozygous missense variant NM_000441.1:c.691G>A had already been found in Pakistani families with severe to profound hearing loss associated with goiter.³⁹ The missing goiter in our family is probably due to its later manifestation, as all affected children were younger than 12 years at the time of examination. The median age of goiter appearance in a previous study was 14.9 years for patients with Pendred syndrome.⁴⁹ So a follow-up of this family will be important.

In family B89, two homozygous variants were detected in *MYO7A* (OMIM 276903). This gene is known to underlie both DFNB2 and Usher 1B syndrome. The first variant is a homozygous missense mutation, NM_000260.3:c.3659C>T, p.(Pro1220Leu), that changes an amino acid residue located in the first MyTH4 domain of the myosin VIIa tail. This mutation was reported before in a family with Usher syndrome type 2, but it was found in a heterozygous state and accompanied by other compound heterozygous mutations in the *USH2A* gene.³² The second variant c.5581C>T is a nonsense mutation, which may lead to a truncated protein that lacks >25% of the tail domain.³³ Regarding the observed phenotype, our findings are not in keeping with a previous study detecting the same mutation in a Samaritan family with Usher 1B syndrome.³³ This form of Usher syndrome is characterized by profound congenital deafness, vestibular dysfunction and retinitis pigmentosa (RP) beginning at age 10 and finally, leading to blindness. In our family, there was no hint to any vision or vestibular affection. One reason might be the early age of the two affected siblings of B89, who were 8 and 5 years old at the time of evaluation. Hence for this family, a regular follow-up is advised.

The analysis of family A12 with a significant LOD score on chromosome 10 resulted in the identification of a novel homozygous missense variant, NM_001171931.1:c.1152C>A, p.(Ser384Arg), in *CDH23* (OMIM 605516) that SIFT and PolyPhen-2 predicted as damaging. Sanger sequencing of available family members confirmed that the variant co-segregates in the fourth generation with bilateral profound SNHL without a retinal phenotype. In contrast, patient III-3 was homozygous for the wild-type allele. Since this patient suffers from a unilateral total hearing loss, the diverging phenotype is most likely due to a different genetic variant or a non-genetic cause. The variant p.(Ser384Arg) affects a highly conserved residue of *CDH23* (see Figure S1C) that resides in the fourth repeat of its N-terminal extracellular domain (EC4). The EC repeats mediate adhesion by binding to interaction partners as was shown for EC1 and EC2 that interact with PCDH15 domains.⁵⁰ Another novel variant in *CDH23* was detected in family B57. This is a frameshift variant in EC8 leading to the substitution of a serine for arginine at position 868 and a premature stop after four further amino acids. So far homozygous frameshift variants were found in *USH1D* patients only with one exception, Ganapathy et al. describe two families with NSHL and homozygous frameshift variants.⁵¹

Mutations affecting *BSND* (OMIM 602522) can cause Bartter syndrome type 4A or SNHL with mild renal dysfunction. We identified a homozygous missense variant of *BSND*, c.107C>A, in all affected individuals of families A38 and B91. The variant leads to a substitution of a highly conserved residue at position 36, namely NP_476517.1:p.(Thr36Asn) that is located in the second transmembrane domain (TD) of the encoded protein barttin.⁵² The variant is predicted to be probably damaging by PolyPhen-2 and damaging by SIFT. Since all affected individuals of both families were not diagnosed with renal dysfunction (normal blood urea and serum creatinine levels), the variant could be solely responsible for ARNSHL as described in other studies.⁵²⁻⁵⁴ One of the described *BSND* mutations associated with hearing loss only was also found in the second TD.⁵⁴ Interestingly, both families share a founder haplotype including the variant position.

The gene *ADGRV1* (OMIM 605472), encoding a G protein-coupled receptor, is known to be associated with Usher syndrome type IIc. The two affected brothers of family A10 (37 and 50 years old) are compound heterozygous for two frameshift variants whereas one affected distant relative is homozygous for one of these variants. In addition, two affected members of another family (B48) are compound heterozygous for a frameshift and a missense variant. The initial diagnosis of all mentioned patients did not include retinal defects as in other *USH2C* patients. In case of family B48, it is likely that the patients of our families (8 and 10 years old) were too young at the time of their initial examination, since visual symptoms usually manifest only in the second decade.⁵⁵ In contrast, the members of family A10 were clearly above the manifestation age and had no retinal defects.

Mutations in *TECTA* (OMIM 602574), encoding tectorin alpha, have been shown to be associated with dominant (DFNA8/12) and recessive nonsyndromic hearing loss (DFNB21).^{56,57} We found a homozygous 15-bp deletion in *TECTA* in family A19 leading to an in-

frame deletion of five highly conserved amino acids. It is predicted as damaging by the SIFT indel prediction algorithm. The deletion is located in the zona pellucida domain of TECTA that interacts with different proteins to assemble the tectorial membrane. An inefficient assembly of the membrane may affect the mechanotransduction process of the hair cells as it has been shown for mouse models carrying different mutations in the ZP domain.⁵⁸

In family B100 the homozygous variant NM_006005.3:c.972C>G in *WFS1* (OMIM 606201) was identified. Variants in the gene *WFS1* cause either Wolfram syndrome 1 (AR) or ADNSHL. Since the variant is located in the first transmembrane (TM1) domain of the protein adjacent to causal variants of Wolfram syndrome⁵⁹ and inherited in an autosomal recessive fashion, the phenotype Wolfram syndrome 1 could be assumed for the affected members of family B100. Wolfram syndrome 1 is a neurodegenerative disease characterized by childhood-onset diabetes mellitus, optic atrophy, diabetes insipidus, and SNHL. In family B100, the two affected siblings (30 and 31 years old) have bilateral congenital severe to profound hearing loss with no other manifestations of Wolfram syndrome. There is one other study describing a homozygous missense variant in a patient (16 years old) with NSHL only and older than the manifestation age of the other phenotypic features of Wolfram syndrome patients.⁶⁰ This and our study suggest ARNSHL to belong to the phenotypical spectrum of *WFS1* variants.

Overall, our two enrichment approaches together reached a diagnostic yield of 78.7%. Whereas the first enrichment approach (GP1), targeting at 12 genes only, yielded confirmed variants in nine out of 25 families (36%), the introduction of an enlarged gene panel (GP2) doubled the diagnostic rate reaching 75% (39/52 families). When looking at 30 studies with diagnostic rates between 10% and 83% reviewed by Shearer and Smith in 2015, the present study compares favorably.⁶¹ NSHL in the remaining unsolved families (13/61) could be due to variants in newly identified deafness genes as well as in still unknown HI genes. Additionally, copy number variations (CNVs) should be taken into account. The impact of CNVs ranges between 5.5% in regard to *STRC*, 2% in regard to *USH2A* and 18.7% for up to 90 analyzed genes.⁶²⁻⁶⁴ Since the detection of CNVs was not included in the current pipeline for gene panel data analysis, we have no data on the impact of CNVs on the variant spectrum of the analyzed Egyptian families.

In summary, we have analyzed a large number of Egyptian families with ARNSHL using MPS of two gene panels of different sizes, including 12 and 94 deafness genes, respectively. Our approach resulted in the detection of probably causative variants in 48 out of 61 families (78.7%). These variants were found in 23 different genes documenting a similarly high heterogeneity of ARNSHL in Southern Egypt as observed in other geographic regions of the world. Many of the detected variants were novel ones (32/44). The highest mutational burden was found in the gene *MYO15A* with a total of 10 families (15.3%) being affected by variants in that gene. Our findings are of great value for improved diagnostics of deaf children in Egypt. An updated version of the gene panel incorporating newly discovered genes or exome sequencing with CNV analysis options shall raise the diagnostic yield beyond 79%.

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CONFLICT OF INTEREST

P.N. is founder, CEO, and shareholder of ATLAS Biolabs GmbH. ATLAS Biolabs GmbH is a service provider for genomic analyses.

DATA AVAILABILITY STATEMENT

All pathogenic variants identified in this study are subject to submission to the Deafness Variation Database (<http://deafnessvariationdatabase.com/>). Further data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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