Comprehensive analysis via exome sequencing uncovers genetic etiology in autosomal recessive nonsyndromic deafness in a large multiethnic cohort

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### Purpose:
Autosomal recessive nonsyndromic deafness (ARNSD) is characterized by a high degree of genetic heterogeneity, with reported mutations in 58 different genes. This study was designed to detect deafness-causing variants in a multiethnic cohort with ARNSD by using whole-exome sequencing (WES).

### Methods:
After excluding mutations in the most common gene, GJB2, we performed WES in 160 multiplex families with ARNSD from Turkey, Iran, Mexico, Ecuador, and Puerto Rico to screen for mutations in all known ARNSD genes.

### Results:
We detected ARNSD-causing variants in 90 (56%) families, 54% of which had not been previously reported. Identified mutations were located in 31 known ARNSD genes. The most common genes with mutations were MYO15A (13%), MYO7A (11%), SLC26A4 (10%), TMPRSS3 (9%), TMC1 (8%), ILDR1 (6%), and CDH23 (4%). Nine mutations were detected in multiple families with shared haplotypes, suggesting founder effects.

### Conclusion:
We report on a large multiethnic cohort with ARNSD in which comprehensive analysis of all known ARNSD genes identifies causative DNA variants in 56% of the families. In the remaining families, WES allows us to search for causative variants in novel genes, thus improving our ability to explain the underlying etiology in more families.

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**Key Words:** autosomal recessive; deafness; exome; next-generation sequencing

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### INTRODUCTION

Deafness is a global public health concern that affects 1 to 3 per 1,000 newborns. In more than half of the cases of congenital or prelingual deafness, the cause is genetic, and most demonstrate an autosomal recessive inheritance pattern. Mutations in 58 different genes have been reported to cause autosomal recessive nonsyndromic deafness (ARNSD) (http://hereditaryhearingloss.org).

Except for one relatively common gene, GJB2 (MIM 121011), most reported mutations are present in only a single or a small number of families. Whole-exome sequencing (WES) allows resequencing of nearly all exons of the protein-coding genes in the genome. A growing number of research and clinical diagnostic laboratories are successfully using WES for gene/variant identification because of its comprehensive analysis advantages. In this study, we...
present the results of WES in a large multiethnic cohort consisting of 160 families with ARNSD who were negative for GJB2 mutations.

**MATERIALS AND METHODS**

**Statement of ethics**

This study was approved by the University of Miami Institutional Review Board (USA), the Ankara University Medical School Ethics Committee (Turkey), the Growth and Development Research Ethics Committee (Iran), the Bioethics Committee of FFAA (HE-1) in Quito (Ecuador), and the Ethics Committee of National Institute of Rehabilitation (Mexico). A signed informed-consent form was obtained from each participant or, in the case of a minor, from the parents.

**Subjects**

We included 160 families with at least two members with nonsyndromic sensorineural hearing loss with a pedigree structure suggestive of autosomal recessive inheritance (affected siblings born to unaffected parents with or without parental consanguinity); GJB2 mutations were negative. Hearing loss was congenital or prelingual-onset with a severity ranging from mild to profound. The study comprised 101 families from Turkey, 54 from Iran, 2 from Mexico, 2 from Ecuador, and 1 from Puerto Rico. Sensorineural hearing loss was diagnosed via standard audiometry in a soundproof room according to standard clinical practice. Clinical evaluation of all affected individuals by a geneticist and an otolaryngologist included a thorough physical examination, otoscopy, and ophthalmoscopy. Tandem walking and the Romberg test were used for initial vestibular evaluation, with more detailed tests if needed based on symptoms and findings. Laboratory investigation included, but was not limited to, an electrocardiogram, urinalysis, and, when available, a high-resolution computed tomography (CT) scan of the temporal bone or magnetic resonance imaging (MRI) to identify inner ear anomalies. DNA was extracted from peripheral leukocytes of each member of the family by standard protocols.

**Whole-exome sequencing**

Agilent SureSelect Human All Exon 50 Mb versions 3, 4, and 5 (Agilent Technologies Santa Clara, CA) were used for in-solution enrichment of coding exons and flanking intronic sequences following the manufacturer’s standard protocol. The enriched DNA samples were subjected to standard sample preparation for the HiSeq 2000 instrument (Illumina San Diego, CA). The Illumina CASAVA v1.8 pipeline was used to produce 99-bp sequence reads. The Burrows-Wheeler Aligner† (http://bio-bwa.sourceforge.net) was used to align sequence reads to the human reference genome (hg19), and variants were called using the GATK (https://www.broadinstitute.org/gatk/) software package. All single-nucleotide variants (SNVs) and insertion/deletions (INDELs) were submitted to

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**Figure 1** Overall workflow of our WES pipeline. CNV, copy-number variation; INDEL, insertion/deletion; SNV, single-nucleotide variant; WES, whole-exome sequencing.
### Table 1 Mutations identified in known ARNSD genes

| Family ID | Country of origin | Genotype | cDNA | Protein | NM transcript | Gene | Reference |
|-----------|-------------------|----------|------|---------|---------------|------|-----------|
| 543       | Turkey            | Homozygous | c.4441T>C | p.S1481P | NM_016239.3 | MYO15A | Cengiz et al.23 |
| 724       | Turkey            | Homozygous | c.4652C>A | p.A1551D | NM_016239.3 | MYO15A | Diaz-Horta et al.4 |
| 765       | Turkey            | Homozygous | c.4273C>T | p.Q1425X | NM_016239.3 | MYO15A | Diaz-Horta et al.4 |
| 723       | Turkey            | Homozygous | c.8307_8309delGGA | p.E2770del | NM_016239.3 | MYO15A | Novel |
| 795       | Turkey            | Homozygous | c.5808_5814delCCGTG | p.R1937QfsX10 | NM_016239.3 | MYO15A | Cengiz et al.21 |
| 793       | Turkey            | Homozygous | c.5808_5814delCCGTG | p.R1937QfsX10 | NM_016239.3 | MYO15A | Cengiz et al.21 |
| 1209      | Puerto Rico      | Heterozygous | c.7226delC | p.P2409QfsX8 | NM_016239.3 | MYO15A | Novel |
| 1083      | Turkey            | Homozygous | c.5183T>C | p.L1728P | NM_016239.3 | MYO15A | Novel |
| 1332      | Turkey            | Homozygous | c.10361delT | p.V3454GfsX5 | NM_016239.3 | MYO15A | Novel |
| 489       | Turkey            | Homozygous | c.5288_5289delTC | p.R1788DfsX13 | NM_016239.3 | MYO15A | Novel |
| 1023      | Iran             | Homozygous | c.8638_8641delCCTG | p.P2880RfsX19 | NM_016239.3 | MYO15A | Novel |
| 862       | Turkey            | Heterozygous | c.7894G>T | p.V2632L | NM_016239.3 | MYO15A | Novel |
| 974       | Iran             | Homozygous | c.6478G>A | p.G2163S | NM_016239.3 | MYO15A | Cremers et al.25 |
| 1370      | Turkey            | Homozygous | c.1708C>T | p.R570X | NM_016239.3 | MYO15A | Novel |
| 786       | Turkey            | Homozygous | c.1001G>T | p.G334V | NM_016239.3 | MYO15A | Yoshimura et al.27 |
| 777       | Turkey            | Homozygous | c.919-2A>G splice | p.S530X | NM_016239.3 | MYO15A | Novel |
| 629       | Turkey            | Homozygous | c.399G>C | p.W133C | NM_016239.3 | MYO15A | Novel |
| 1368      | Turkey            | Homozygous | c.1126G>A | p.G376S | NM_016239.3 | MYO15A | Novel |
| 1410      | Turkey            | Homozygous | c.436G>A | p.G146S | NM_016239.3 | MYO15A | Novel |
| 911       | Turkey            | Homozygous | c.1534C>T | p.R512X | NM_016239.3 | MYO15A | Kurima et al.33 |
| 490       | Turkey            | Homozygous | c.1959C>G | p.Y653X | NM_016239.3 | MYO15A | Novel |
Table 1 Continued

| Family ID | Country of origin | Genotype | cDNA | Protein | NM transcript | Gene | Reference |
|-----------|-------------------|----------|------|---------|---------------|------|-----------|
| 393       | Turkey            | Heterozygous | c.63 + 2T>A | splice | NM_138691.2 | TMC1 | Duman et al. |
| 988       | Iran              | Homozygous | c.3215C>A | p.A1072D | NM_022124.5 | CDH23 | Duman et al. |
| 1165      | Mexico            | Heterozygous | c.2959G>A | p.D987N | NM_022124.5 | CDH23 | Novel |
| 1015      | Iran              | Homozygous | c.5851G>A | p.D1951N | NM_022124.5 | CDH23 | Novel |
| 1032      | Iran              | Heterozygous | c.7822C>T | p.R2608C | NM_022124.5 | CDH23 | Novel |
| 968       | Iran              | Homozygous | c.820C>T | p.Q274X | NM_001199799.1 | ILDR1 | Diaz-Horta et al. |
| 725       | Turkey            | Homozygous | c.3918T>G | p.R1494X | NM_144612.6 | LOXHD1 | Diaz-Horta et al. |
| 1289      | Turkey            | Heterozygous | c.3101G>A | p.G1034H | NM_001042702.3 | DFNB59 | Collin et al. |
| 1366      | Turkey            | Homozygous | c.1018G>T | p.R339W | NM_004999.3 | MYO6 | Yang et al. |
| 63        | Turkey            | Homozygous | c.387_388insC | p.K130QfsX5 | NM_173477.2 | USH1G | Novel |
| 23        | Turkey            | Homozygous | c.2335_2336delAG | p.R7855fsX50 | NM_001039141.2 | TRIOBP | Diaz-Horta et al. |
| 5         | Turkey            | Homozygous | c.387_388insC | p.K130QfsX5 | NM_173477.2 | USH1G | Novel |

Families with compound heterozygous mutations are italicized.

CNV, copy-number variation.
SeattleSeq137 for further characterization and annotation. Sanger sequencing was used for confirmation and segregation of the variants in each family.

Bioinformatics analysis
We analyzed WES data using our in-house tool (https://genomics.med.miami.edu). Our workflow is shown in Figure 1. The analysis started with quality control (QC) checks, including the coverage and average read depth of targeted regions, numbers of variants in different categories, and quality scores. All variants were annotated and categorized into known and novel variants. As previously recommended, we filtered variants based on minor allele frequency of <0.005 in dbSNP141. We also filtered out variants that are present in >10 samples in our internal database of >3,000 exomes from European, Asian, and American ancestries that includes Turkish, Iranian, Mexican, Ecuadorian, and Puerto Rican samples (Figure 1). Autosomal recessive inheritance with both homozygous and compound heterozygous inheritance models and a genotype quality (GQ) score >35 for the variant quality were chosen. Missense, nonsense, splice site, in-frame INDEL, and frameshift INDELs in the known ARNSD genes (Supplementary Data online) were selected. Missense variants that remained after these filters were later analyzed for presence in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk) and for having a pathogenic prediction score at least in two of the following tools: PolyPhen2, SIFT, MutationAssessor, and MutationTaster. Finally, we used CoNIFER (Copy Number Inference From Exome Reads) and XHMM.

Table 2 Overview of mutation detection and parental consanguinity

| Countries  | Number of families | Reported parental consanguinity | Number of homozygous probands (consanguineous) | Number of compound heterozygous probands (consanguineous) |
|------------|--------------------|----------------------------------|-----------------------------------------------|----------------------------------------------------------|
| Turkey     | 101                | 82                               | 67 (59)                                       | 5 (2)                                                    |
| Iran       | 54                 | 31                               | 12 (10)                                       | 1 (1)                                                    |
| Ecuador    | 2                  | 0                                | 0                                             | 1 (0)                                                    |
| Mexico     | 2                  | 0                                | 0                                             | 1 (0)                                                    |
| Puerto Rico| 1                  | 0                                | 0                                             | 1 (0)                                                    |

Figure 2 Capture kit versions and coverages. (a-c) Overview of coverage of 58 known ARNSD genes according to three different versions (version 3 = V3, version 4 = V4, and version 5 = V5) of the exome enrichment kit. (d) Numbers of samples studied with different capture kits. ARNSD, autosomal recessive nonsyndromic deafness.
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(eXome-Hidden Markov Model) to detect copy-number variations (CNVs). After this filtering, only variants cosegregated with the phenotype in the entire family were considered pathogenic.

RESULTS

On average, each exome had 99, 95, and 88% of mappable bases of the Gencode-defined exome represented by coverage of 1X, 5X, and 10X reads, respectively. Average coverage of the mappable bases for the 58 known ARNSD genes (exons and the first and last 20 bps of introns) were 99, 95, and 87% for the 1X, 5X, and 10X reads, respectively.

We detected pathogenic or likely pathogenic variants that can explain ARNSD in 90 (56%) families. All identified variants cosegregated with deafness as an autosomal recessive trait. Fifty-four percent of the mutations were not previously reported in the Human Gene Mutation Database. Mutations were identified in 31 ARNSD genes. The genes with mutations identified in at least three families are MYO15A (MIM 602666) (13%), MYO7A (MIM 276903) (11%), SLC26A4 (MIM 605646) (10%), TMPRSS3 (MIM 605551) (9%), TMC1 (MIM 606706) (8%), ILDR1 (MIM 609739) (6%), CDH23 (MIM 605516) (4%), OTOF (MIM 603681) (4%), PCDH15 (MIM 605514) (3%), and TMIE (MIM 607723) (3%).

During the course of this study we reported mutations in OTOGL (MIM 614925) and FAM65B (MIM 611410) as novel causes of ARNSD16,17 (Figure 1; Table 1).

DISCUSSION

Identifying causative variants in ARNSD is challenging because of the following: (i) the extreme genetic heterogeneity of ARNSD; (ii) the presence of different categories of genetic variants such as SNVs, INDELs, and CNVs; (iii) the presence of a high proportion of nonrecurrent mutations; and (iv) the variability in mutation frequencies in individual ARNSD genes across ethnicities. Consequently, we performed a comprehensive analysis to detect pathogenic SNVs, INDELs, and CNVs in the ARNSD genes.

Targeted resequencing allows identification of mutations in the interested gene sets. Recent studies pioneered by Shearer et al.8,19 have shown the effectiveness of the targeted resequencing of deafness genes. Advantages of the targeted sequencing over WES are having better coverage with higher depth and significantly lower costs, which is suitable for clinical diagnostic laboratories. However, a main limitation of the targeted sequencing is the need for revalidation of the panel after adding each new gene. By contrast, many laboratories around the world offer WES as a diagnostic tool requiring validation only when a new WES version is introduced. Our analysis using three different versions of an exome capture kit during the 4-year period shows that the depth of coverage of WES has improved to reliably identify most mutations in known ARNSD genes (Figure 2; Supplementary Tables S1 and S4 online). Recently developed WES approaches provide more coverage for genes that are known to cause Mendelian disease. They are
expected to cover deafness genes more efficiently. In addition, adding in baits to improve coverage over poorly covered regions may be considered if better coverage is desired. It was recently shown via targeted sequencing that CNVs are a common cause of deafness. Although CNV analysis of the WES data is still being optimized for clinical usage, we integrated two currently available tools—XHMM and CoNIFER—into our WES analysis pipeline and identified large OTOA (MIM 607038), STRC (MIM 606440), and PCDH15 (exon 27–28) homozygous deletions in our cohort, supporting a significant role of CNVs in deafness etiology.

In this study, after excluding GJB2 mutations we detected pathogenic variants in the known ARNSD genes in 56% of the families. The advantage of this study is having large multiplex autosomal recessive families (including affected and unaffected children) who can be tested for cosegregation of all variants. While we identified more novel variants than those reported in Table 1 through WES, only those variants cosegregated in the family with deafness were considered pathogenic. Similarly heterogeneous variants did not explain the phenotype because they did not cosegregate with deafness and were not included. WES facilitates the cataloging of mutations in different populations. Population characteristics such as the rate of consanguineous marriages may affect the distribution of deafness mutations in different populations. As expected, the vast majority of Turkish and Iranian probands from consanguineous marriages are homozygous for the pathogenic variants (Table 2). However, there is a marked difference between the rates of solved families in Turkey (73%) versus Iran (24%) (Figure 3). As shown in Figure 3, the distribution of genes is also different between the two countries. In our study, the top five genes explain 39 of 101 families (39%) in Turkey, whereas they explained only 10 of 54 families (19%) in Iran. Moreover, our analysis of the WES data in the unsolved Iranian families shows that there are no common mutations in genes that are not known to be deafness genes (data not shown). Unless there are common mutations in regions that are not well covered by WES, our data suggest that many rare genes are responsible for the majority of hereditary deafness in the Iranian cohort. It is likely that there are undetected rare variants specific to certain ethnicities in Iran.

Another advantage of WES is that surveying of mutations for founder effects is possible. We detected TMIE c.250C>T (p.R84W) in three unrelated Turkish families that all shared a flanking haplotype as noted previously. Furthermore, MYO15A, MYO7A, SLC26A4, TMIPRSS3, ILDR1, OTOF, ESRRB (MIM 602167), and GIPC3 (MIM 608792) genes had recurrent mutations with shared haplotypes, indicating founder effects (Supplementary Table S2 online).

There is no correlation between the size of transcript and number of mutant alleles (Supplementary Table S3 online). There may be some deafness genes that are more prone to having mutations. Founder effects appear to play a role because some small genes such as TMIE, ESRRB, and GIPC3 ranked high in mutation frequency because of founder mutations. Some discrepancy between the size of a gene and number of mutations can be explained by the fact that only certain mutations cause nonsyndromic deafness for some genes. For instance, CDH23, PCDH15, and MYO7A are big genes, but many mutations in those genes cause Usher syndrome (MIM 276900) instead of ARNSD. An interesting example is TMC1, which ranks 20th based on size but 5th in mutation frequency. Nonsyndromic deafness is the only phenotype caused by TMC1 mutations, and none of the TMC1 mutations is recurrent in our cohort. These may suggest that TMC1 is relatively more prone to have de novo mutations or that it is a highly conserved gene and its variants are rarely tolerated.

In conclusion, WES is an effective tool for identifying pathogenic SNVs, INDELS, and CNVs simultaneously in ARNSD genes and provides further analysis of the families without mutations for novel gene discovery. Identification of two novel ARNSD genes during the course of this study testifies to its power.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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DISCLOSURE

The authors declare no conflict of interest.

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