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***TBC1D24* Mutation Causes Autosomal Dominant Non-Syndromic Hearing Loss**

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Abstract

Hereditary hearing loss (HHL) is extremely heterogeneous. Over 70 genes have been identified to date, and with the advent of massively parallel sequencing, the pace of novel gene discovery has accelerated. In a family segregating progressive autosomal dominant non-syndromic hearing loss (ADNSHL) we used OtoSCOPE® to exclude mutations in known deafness genes and then performed segregation mapping and whole exome sequencing (WES) to identify a unique variant, p.Ser178Leu, in *TBC1D24* that segregates with the hearing loss phenotype. *TBC1D24* encodes a GTPase-activating protein expressed in the cochlea. Ser178 is highly conserved across vertebrates and its change is predicted to be damaging. Other variants in *TBC1D24* have been associated with a panoply of clinical symptoms including autosomal recessive NSHL (ARNSHL), syndromic hearing impairment associated with onychodystrophy, osteodystrophy, mental retardation and seizures (DOORS syndrome), and a wide range of epileptic disorders.

Keywords

TBC1D24, autosomal dominant; non-syndromic; hearing loss; hearing impairment; pleiotropy; OtoSCOPE ®

Hereditary Hearing loss (HHL) is the most common sensory deficit in humans. It is also extremely heterogeneous with 64 genomic loci associated with autosomal dominant non-syndromic hearing loss (ADNSHL). Thirty of these genes have been identified (Hereditary Hearing Loss website; <http://hereditaryhearingloss.org>; last access in January 2014). Despite this heterogeneity, the advent of targeted genomic enrichment (TGE) and massively parallel sequencing (MPS) has changed the clinical evaluation of the deaf and hard-of-hearing person by making it possible to screen most genes implicated in non-syndromic hearing loss

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simultaneously. A variety of high throughput sequencing platforms have been developed for this purpose that vary in the number of targeted exons and genes and the type of TGE and MPS used (Baek, et al., 2012; Brownstein, et al., 2011; Shearer, et al., 2010; Sivakumaran, et al., 2013).

The TGE platform we have developed is called OtoSCOPE®. It has a solve rate of ~30% for families with presumed ADNSHL. OtoSCOPE®-negative ADNSHL families are valuable for novel deafness gene discovery especially with the availability of whole exome sequencing (WES). Because WES generates a huge number of plausible variants, when used alone it may be impossible to reduce the number of variants to establish single-variant causality. In this study, we used OtoSCOPE®, segregation mapping and WES to identify a novel cause of deafness in a four-generation family of European descent segregating bilateral post-lingual progressive ADNSHL (Figure 1A).

After obtaining written informed consent from all participants with approval by the Institutional Review Board of the University of Iowa, clinical examination of the subjects was completed to exclude any additional and/or syndromic findings. Pure tone audiometry was performed (Supp. Methods) and blood samples were obtained from 21 family members. The age of onset of hearing loss in patients was in the third decade. Audiograms of affected individuals had a gently down-sloping configuration. Significant progression occurred at 0.25-2 KHz and 8 kHz (Figure 1B), with the resulting age-related typical audiograms (ARTA) demonstrating progression with an Annual Threshold Deterioration (ATD) of ~0.8 dB per year at 2-4 kHz and ~1.6 dB per year at the other frequencies (Figure 1C, Supp. Methods).

We used TGE and MPS to screen one affected family member (IV.1) for disease-causing variants in most known NSHL genes, including the non-syndromic mimics like Usher Syndrome, with OtoSCOPE®.v4 (66 known deafness genes are included on v4), as previously described (Shearer, et al., 2013). We have adopted this approach as the first step because it is simple, inexpensive and quick. Our 'solve' rate using this strategy varies with mode of inheritance and is 39%, 29% and 32% respectively for autosomal recessive, autosomal dominant and sporadic HL (Shearer, et al., 2013). The OtoSCOPE® run on individual IV.1 generated 2,611,594 mapped reads with an average coverage depth of 249; 98% of targeted regions were covered at 10× (Supp. Table S1). Analysis using a customized Galaxy pipeline identified a single variant that passed all filtering criteria (Supp. Methods): c.3845A>G, p.Asn1282Ser in *CDH23* (MIM# 605516; Refseq NM_001171930), which was present in a heterozygote state. This variant has a minor allele frequency (MAF) of 0.18% and 0.47% in 1000 Genomes Project database (October 2011 edition) (<http://www.1000genomes.org>) and EVS (<http://evs.gs.washington.edu/EVS/>), respectively, and was excluded from further consideration.

Because a plausible genetic cause of hearing loss was not identified with OtoSCOPE®, the family was carried forward for WES and simultaneous segregation mapping, using the latter to identify regions of identity-by-descent (IBD) to reduce the search space relevant in variant filtering. Genotyping was carried out using the HumanOmniExpressExome BeadChip Kit (Illumina, Inc., San Diego, CA) (Supp. Methods) on seven individuals (4

affected individuals - III.8, III.12, IV.4 and IV.9; 3 unaffected individuals - III.10, IV.8 and IV.11) (Figure 1A). Genotypic data were used as input for PEDIBD to infer regions of IBD, using R to analyze the probability of IBD between paired family members to generate lists of candidate regions consistent with dominant inheritance (Li and Li, 2011). Three genomic regions: chr8:1-2324136 (2.3Mb); chr11:132826084-135006516 (2.2Mb); and chr16:1-5961636 (6Mb) were identified (Figure 2B, Supp. Table S2).

Whole exome capture was performed with the Agilent SureSelectXT Human All Exon V5 (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol on 3 affected individuals (III.4, IV.1 and IV.12) (Figure 1A). All enriched libraries were sequenced on the Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA) (Supp. Methods). Data analysis was performed on a local installation of Galaxy using the Burrows-Wheeler Alignment (BWA) for read mapping to the reference genome (hg19, NCBI Build 37), Picard for removal of duplicate reads, GATK for local re-alignment and variant calling, and ANNOVAR and a custom workflow for variant annotation. Variants were annotated for conservation and predicted pathogenicity using dbNSFP v2.0 (<http://sites.google.com/site/jpopgen/dbNSFP>) (Liu, et al., 2013), and for variant frequency using the 1000 Genomes Project database and EVS. Variants with a MAF >0.0005 were removed. Remaining exonic variants were filtered based on coding effect, heterozygosity and allele sharing amongst the three sequenced affected individuals. We did not perform WES as the initial step because, as compared to OtoSCOPE®, WES makes the simultaneous analysis of multiple families more expensive and is therefore time- and resource-inefficient. Indeed, there are multiple reports in which WES has been used up front only to discover that the selected families segregate known deafness-causing variants or novel variants in known deafness-causing genes (Gao, et al., 2013; Kim, et al., 2013; Park, et al., 2013).

WES yielded an average depth of coverage of 251, with 92% of targeted regions covered at 10× (Supp. Table S1). The average number of high quality variants in the three samples was 37,900, but after filtering for coding effect, MAF and heterozygosity, the final candidate variant list was reduced to 320, 308 and 328 for individuals III.4, IV.1 and IV.12, respectively (Figure 2A, Supp. Table S2). 46 variants were shared across the 3 sequenced exomes (7 of these variants were novel). Integrating the three genomic regions consistent with dominant inheritance with the WES data identified an average of 33, 21 and 431 high quality variants for genomic intervals chr8:1-2324136 (2.3Mb), chr11:132826084-135006516 (2.2Mb) and chr16:1-5961636, respectively (Supp. Table S2). Within these genomic intervals, the three exomes had only a single variant in common. The variant, c.533C>T; p.Ser178Leu in *TBC1D24* (TBC1 domain family, member 24; MIM# 613577; RefSeq NM_001199107.1) is a novel non-synonymous variant absent from 1000 Genomes Project and EVS. The variant substitutes a highly conserved serine for a leucine at position 178 (Figure 2D) and is predicted to be pathogenic and disease-causing by Polyphen2, LRT and MutationTaster. Cosegregation of c.533C>T with the ADNSHL phenotype in this family was confirmed by Sanger sequencing of all family members for whom DNA samples were available (10 affected and 11 unaffected) (Figure 1A and Figure 2C). This variant was not observed in 300 chromosomes from CEPH controls (Supp. Methods). It was submitted to the LOVD database (<http://www.lovd.nl/TBC1D24>).

TBC1D24 encodes a protein with two conserved domains: a TBC domain (tre2/Bub2/Cdc16), which is shared by Rab GTPase-activating proteins (Rab-GTPs); and a TLDc domain, which is believed to be involved in oxidative stress resistance and to have catalytic activity although its substrates remain unknown (Oliver, et al., 2011). In vitro studies suggest that *TBC1D24* may play a role in neuron projection via negative regulation of the ARF6 signaling pathway (Corbett, et al., 2010; Falace, et al., 2010). ARF6 is involved in the regulation of membrane trafficking between the plasma membrane and the endocytic compartment (D'Souza-Schorey and Chavrier, 2006).

Recently, *TBC1D24* has been implicated in ARNSHL at the DFNB86 locus (MIM# 614617). Two different homozygous mutations c.208G>T (p.Asp70Tyr) and c.878G>C (p.Arg293Pro) were identified in three consanguineous families (Rehman, et al., 2014). Of note, two individuals in one family (segregating the p.Asp70Tyr mutation) have a history of seizures, and although the authors suggest that this association is coincidental, given the phenotypic variability associated with *TBC1D24* variants, it may not be. Mutations in *TBC1D24* are associated with multiple epileptic phenotypes (Figure 2E, Supp. Table S3). Two compound heterozygous missense mutations (p.Asp147His and p.Ala509Val) were identified in an Italian family with familial infantile myoclonic epilepsy (FIME; MIM# 605021) (Falace, et al., 2010); a homozygous p.Phe251Leu mutation has been identified in a consanguineous Arab-Israeli family with autosomal-recessive syndromic focal epilepsy, dysarthria, and mild-to-moderate intellectual disability (MIM# 605021) (Corbett, et al., 2010); compound heterozygous mutations p.Cys156* and p.Phe229Ser cause familial malignant migrating partial seizures of infancy (Milh, et al., 2013); and a homozygous two-base pair deletion leading to premature termination causes severe, lethal epileptic encephalopathy (EIEE16; MIM# 615338) (Güven and Tolun, 2013). Variants in *TBC1D24* also cause syndromic hearing loss. In a cohort of families from 17 countries with DOORS syndrome (Deafness, Onychodystrophy, Osteodystrophy, mental Retardation and Seizures) (MIM# 220500) Campeau and colleagues identified 8 variants either in the homozygous or compound heterozygous state in *TBC1D24* (Campeau, et al., 2014).

It is noteworthy that all reported mutations to date are recessive (Supp. Table S3). This study is the first to link a variant in *TBC1D24* to a dominant disease phenotype. In aggregate, these data highlight the significant degree of clinical variability for phenotypes associated with *TBC1D24* mutations and add another level of complexity to genotype-phenotype correlations while shedding light on *TBC1D24* genetic pleiotropy. *TBC1D24* may carry out different functions with different partners or in different biological contexts, whether it is in the ear, brain or bones. Perturbations of interactome networks may differ between complete loss of gene products ('node removal') and interaction-specific or edge-specific alterations, which might alter specific interactions (Das, et al., 2013; Zhong, et al., 2009). In addition, since the p.Ser178Leu mutation we identified segregates with an autosomal dominant phenotype, the pathological changes and underlying molecular mechanism differ from those reported with recessive mutations. Given that many of the recessive mutations are loss of function, haploinsufficiency as the mechanism of action of the p.Ser178Leu can be excluded. The p.Ser178Leu variant localizes to the TBC domain of *TBC1D24* and substitutes a polar amino acid (serine) for a hydrophobic amino acid (leucine), a change that

could impact folding or conformation thus altering protein stability or function. Although some of the recessive loss-of-function mutations in *TBC1D24* have been hypothesized to impair interaction with ARF6, if this occurs, it likely leads to node removal, and abolishes or alters all interactions of TBC1D24, which is consistent with the more severe phenotypes that are observed with recessive inheritance. Genotype-phenotype relationships for TBC1D24 remain ambiguous as there is no apparent correlation between mutation type and location and the clinical disease manifestations (Figure 2E, Supp. Table S3).

Immunohistochemistry in P2 whole-mount cochlea showed expression of TBC1D24 primarily in inner and outer hair cells although there was also expression in spiral ganglion (SG) neurons (Figure 2F). Rehman et al., in contrast, reported TBC1D24 expression exclusively in SG neurons, a difference that may reflect: 1) a different staining technique (they used cryosections while we used whole-mount cochlea, which facilitates assessment of hair cells); 2) changes in spatio-temporal expression of TBC1D24 during cochlea development (they used P30 and we used P2). Several databases confirm TBC1D24 expression in the cochlea. The Goodrich Auditory and Vestibular Gene Expression Database (<http://goodrich.med.harvard.edu/>) reports expression in mouse SG neurons from E12, when SG neurons first extend projections, to P15, after the onset of hearing. The mouse cochlea gene database (<http://research.meei.harvard.edu/Otopathology/tbimages/mouse.html>) also detected expression in 9-week-old mouse cochlea, but levels of expression in specific cell types were not specified. SHIELD (Shared Harvard Inner-Ear Laboratory Database) (<https://shield.hms.harvard.edu/>) reports moderate expression in hair cells and a strong expression in the SG from E16 to P16. Hertzano et al detected expression in various cell populations within the organ of Corti from P0-P1 mice (Hertzano, et al., 2011) (unpublished data).

In summary, we show that a novel variant in *TBC1D24* segregates with progressive ADNSHL, a finding that adds *TBC1D24* to the short list of genes that cause ADNSHL, ARNSHL and syndromic deafness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Pedigree and audiometric profiles

A: The pedigree of a four-generation family segregating ADNSHL. DNA samples were available for 10 affected and 11 unaffected individuals. Males are denoted in squares and females in circles. Black shading indicates ADNSHL while no shading represents normal hearing. G, W, and * denote genotyping, WES and OtoSCOPE® testing, respectively. Individuals marked as +/- are heterozygous for the *TBC1D24* p.Ser178Leu variant. Individuals marked as -/- do not carry the variant. **B:** Cross-sectional linear regression analysis of binaural mean air conduction threshold on age (years) for each frequency separately. The regression line (dashed) is included for each frequency. ATD, (in bold print) the regression coefficient (dB/year), is included in each panel; asterisk indicates significant progression. **C:** ARTA. Binaural mean air conduction threshold (dB HL) is presented for the ages 30, 40, 50, and 60 years. Hearing levels ranged from 20 to 70 dB, depending on age and frequency; the ATD was ~0.8 dB/year at 2-4 kHz, and ~1.6 dB/year at the other frequencies.

Figure 2. WES, segregation analysis and *TBC1D24* mutation and expression

A: WES results in affected individuals showing the final variant numbers for each exome and shared variants. 46 variants were shared amongst all three individuals. **B:** Identification of 3 regions on chr8, chr11 and chr16. SNP index is reported on the x-axis while the inheritance model score is indicated on the y-axis. **C:** Representative chromatograms from wild type and mutant sequences (red arrow indicates variant position). **D:** Alignment of *TBC1D24* orthologs shows the conservation of Ser178 (in red) across species. **E:** Localization of *TBC1D24* mutations associated with ADNSHL, ARNSHL, epilepsy phenotypes and DOORS syndrome. The variant p.Ser178Leu (in red) is located on the TBC domain. Phenotypes including deafness are shown above the diagram; epileptic phenotypes are shown below. **F:** *TBC1D24* expression in P2 mouse cochlea. **i:** Staining with actin antibody showing 3 rows of OHC and one row of IHC. **ii:** Staining with DAPI antibody. **iii:** *TBC1D24* staining in the cell body of OHC and IHC. **iv:** Merged pictures. **v, vi and vii:** SG staining with F-actin, DAPI and *TBC1D24* antibodies, respectively. Scale bar represents 50 μ m.