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A mutation of *MET*, encoding hepatocyte growth factor receptor, is associated with human *DFNB97* hearing loss

Ghulam Mujtaba[1], **Julie M Schultz**[2], **Ayesha Imtiaz**[1], **Robert J Morell**[2], **Thomas B Friedman**[2], and **Sadaf Naz**[1,*]

[1]School of Biological Sciences, University of the Punjab, Lahore, Pakistan

[2]Laboratory of Molecular Genetics, National Institutes on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, MD, USA

Abstract

Background—Hearing loss is a heterogeneous neurosensory disorder. Mutations of 56 genes are reported to cause recessively inherited nonsyndromic deafness.

Objective—We sought to identify the genetic lesion causing hearing loss segregating in a large consanguineous Pakistani family.

Methods and Results—Mutations of *GJB2* and all other genes reported to underlie recessive deafness were ruled out as the cause of the phenotype in the affected members of the participating family. Homozygosity mapping with a dense array of one million SNP markers allowed us to map the gene for recessively inherited severe hearing loss to chromosome 7q31.2, defining a new deafness locus designated *DFNB97* (maximum LOD score of 4.8). Whole-exome sequencing

[*]**Corresponding author** Sadaf Naz, School of Biological Sciences, University of the Punjab, Lahore 54590, Pakistan, Phone: 92-42-99231819, naz.sbs@pu.edu.pk.
JMS Present address GeneDx, Gaithersburg, MD, USA

WEB RESOURCES 1000 Genomes Project, http://www.1000genomes.org
Clinical Variation, http://www.ncbi.nlm.nih.gov/clinvar/
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CUPSAT, http://cupsat.tu-bs.de/
DNAnexus, https://www.dnanexus.com/
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I-Mutant 2.0, http://folding.biofold.org/i-mutant/i-mutant2.0.html
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Protein Variation Effect Analyzer, PROVEAN, http://provean.jcvi.org/
Rutgers combined linkage-physical map, http://compgen.rutgers.edu/rutgers_maps.shtml
Shared Harvard inner-ear laboratory database, SHIELD, https://shield.hms.harvard.edu/
Sorting Intolerant from Tolerant, SIFT, http://sift.bii.a-star.edu.sg/
UCSC Genome Browser Feb. 2009 (GRCh37/hg19), https://genome.ucsc.edu/cgibin/hgGateway

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COMPETING INTERESTS None

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revealed a novel missense mutation c.2521T>G (p.F841V) in *MET*, which encodes the receptor for hepatocyte growth factor. The mutation co-segregated with the hearing loss phenotype in the family and was absent from 800 chromosomes of ethnically matched control individuals as well as from 136,602 chromosomes in public databases of nucleotide variants. Analyses by multiple prediction programs indicated that p.F841V is likely damaging to MET function.

Conclusion—We identified a missense mutation of *MET*, encoding the hepatocyte growth factor receptor, as a likely cause of hearing loss in humans.

Keywords

Hearing Loss; Deafness; Hepatocyte Growth Factor Receptor (HGFR); MET; Pakistan

Nonsyndromic recessively inherited hearing loss contributes to an estimated 77% to 93% of hereditary deafness in humans.[1] The majority of the reported mutations in the 56 genes identified to date cause an increase in hearing level (HL) threshold of greater than 91 dB, referred to as profound deafness. Alleles of some of these same genes can also cause progressive hearing loss or a stable loss ranging in severity from moderate (41–55 dB HL), to severe (71–90 dB HL).[2] The genes with an essential role in hearing encode proteins with a variety of functions,[3] including gap junctions, unconventional myosins, and several proteins that are necessary to form and maintain the stereociliar cytoskeleton. Mutations of two genes encoding receptors, ESRRB (estrogen-related receptor beta, OMIM 60627) and ILDR1 (immunoglobulin-like domain containing receptor 1, OMIM 609739) are associated with deafness in humans. *HGF* encodes a growth factor (hepatocyte growth factor/scatter factor) and noncoding mutations of *HGF* segregating in numerous Pakistani families cause nonsyndromic severe to profound deafness[4] (OMIM 142409).

We recruited a large family HLGM17 (figure 1A) and proceeded to map and identify the gene responsible for hearing loss segregating in the affected members. Institutional Review Board approval was obtained from the School of Biological Sciences, University of the Punjab, Lahore, Pakistan and the National Institutes of Health, USA. Written informed consent was obtained for all participants. The family includes 9 individuals (age range = 5–60 years old) with hearing loss at or before 2 years of age, noticeable due to delay in development of speech. Audiometry in ambient noise conditions revealed a severe degree of sensorineural hearing loss (pure tone average, $PTA_{500\ Hz\text{-}4000\ Hz}$, 74–89 dB HL) with intra-familial variations in thresholds, (figure 1B). The participants were reported to independently ambulate by 12–13 months of age. The results of tandem gait and Romberg tests were normal, suggesting intact, or at least residual, peripheral vestibular function. Medical conditions including those related to liver, kidney and heart were not reported and there was no history of cancers in the family. Results of clinical evaluations, including complete blood counts, serum chemistries, urinalysis, liver function tests and funduscopy, were normal for two affected individuals (12 and 15 years old).

Mutations of *GJB2* and all other genes reported to underlie recessive deafness were ruled out in our study family by sequencing or linkage analyses with microsatellite markers tightly linked to the respective loci. Samples from four individuals were selected for genome-wide homozygosity mapping: the unaffected mother (IV:2), her two affected offspring (V:1, V:2)

and her nephew with hearing loss (V:3). SNP genotyping was performed (Atlas Biolabs, Germany) with the Affymetrix SNP 6 array of one million SNP markers across the genome. KinSNP analyses[5] revealed three regions of homozygosity on chromosomes 2, 6 and 7 (table S1). Genotyping with microsatellite markers across samples from other members of the family confirmed linkage to a 4.06 cM region on chromosome 7 (figure 1A, table S2). A maximum LOD score of 4.8 at $\theta = 0$ was obtained with markers *D7S486* and *D7S2460*. This locus was designated *DFNB97* by the HUGO Gene Nomenclature Committee (HGNC). *DFNB97* overlaps with the originally reported interval for the *DFNB17*[6] locus (OMIM 603010). However, the *DFNB17* interval was subsequently refined[7] to a non-overlapping 5.5-Mb region centromeric of *DFNB97*. In order to find the contribution of the newly mapped locus to deafness, we screened 100 families in which the moderate to profound hearing loss was not attributable to a known deafness gene variant. No other families were identified, which suggests that *DFNB97* is a rare cause of hearing loss in Pakistan.

Whole-exome sequencing (WES) was performed on a DNA sample from individual V:1 (Otogenetics, USA). The exome was enriched using a NimbleGen V2 kit and sequenced on a HiSeq2000 using a paired-end (2×100 bp) protocol. FASTQ reads were mapped to the human genome (GRCh37/ hg19) by DNAnexus software with default settings on a cloud platform (table S3). We analyzed the exome data and generated a list of all variants in the genes known to cause hearing loss. Consistent with the genetic linkage analysis results, none of these genes had a pathogenic mutation. Next, the exome data was filtered and the analysis confined to variants located in the *DFNB97* interval (chromosome 7:115181357 -120965265). We inspected the data alignment in this region to the reference genome at base pair resolution for each exon and the surrounding introns (DNAnexus and UCSC genome browsers). In the *DFNB97* interval, all exons except one were fully captured for exome analysis (WES) and were sequenced with at least 50 bp of flanking intronic boundaries and a minimum of 10 reads. A GC-rich region of an exon was partially sequenced and probes for alternative exons of 6 genes were absent from the NimbleGen array (table S4). These exons not covered or captured by WES were analysed by Sanger sequencing of PCR amplification products, but no mutations were identified. A homozygous non-synonymous variant located in *MET* (Mesenchymal Epithelial Transition factor, NM_000245.2), c.2521T>G (p.F841V), hg19, chr7:116403260T>G, was identified in the exome data, ClinVar# SCV000211990 (figure 1C, table S5). The variant co-segregated with the phenotype, being homozygous in all 9 affected individuals, and was absent from DNA of 400 ethnically matched individuals (800 chromosomes). The mutation was also absent from the Exome Variant Server (6,503 individuals), Exome Aggregation Consortium dataset (60,706 individuals) and the 1000 genomes project database (1,092 individuals). These results support the hypothesis that this mutation in *MET* is the cause of *DFNB97* hearing loss in our study family. However, since we only analyzed exonic regions, it is possible that a mutation in a regulatory region of *MET* or in a cis-acting element of any of the other 16 genes in the critical *DFNB97* interval may be responsible for the hearing loss in family HLGM17 and the p.F841V variant is in linkage disequilibrium with the pathogenic allele.

Human MET exhibits extensive amino acid sequence identity with its orthologues, ranging from 98% for the chimpanzee to 50.64% for the zebrafish. CLUSTALW analysis revealed

that the phenylalanine residue substituted by the mutation in family HLGM17 is conserved among diverse vertebrate species (figure 1D), indicating that p.F841 is important for MET function. Although SIFT predicts that p.F841V is “tolerated”, PROVEAN, PolyPhen-2, Human splicing finder and Mutation Taster predicted it to be “damaging” to MET function.

Alternative splicing of *MET* results in multiple mRNA transcripts. The two longest isoforms encode proteins of 1,408, or 1,390 amino acids (figure 2A, 2B) with the mutation located in exon 11 of these two isoforms. Four of the other mRNA transcripts also encompass the exon that has the mutation (figure 2C, 2D, 2E, 2F). A predicted gain of an internal donor splice site by mutation taster (score 0.99), within exon 11 of *MET* due to the c.2521T>G, mutation was investigated by an exon trap assay.[4] Results of the experiments revealed that all but 2 of the total 65 clones derived from the transfection of mutant construct were identical to those obtained after transfection of the wild-type construct. However, one transcript obtained after transfection of the mutant construct, retained part of intron 10 with exon 11 while in the second transcript, part of intron 11 was retained with exon 11. This indicates that the c. 2521T>G mutation may have a limited effect on splicing or alternatively, the mutation does not affect splicing and the two aberrant splicing products are experimental artifacts.

MET is synthesized as a precursor protein and then proteolytically cleaved by furin to form alpha and beta subunits, which are covalently linked through disulphide bonds. The alpha subunit and part of the beta subunit of MET together form a low affinity binding site for HGF. The beta subunit has a cysteine-rich region termed PSI (Plexins, Semaphorins and Integrins) and four IPT (Immunoglobulin-like, Plexins, Transcription factors) domains, which are important structural components of the MET extracellular region (figure 2G). The beta subunit also traverses the plasma membrane and provides a cytoplasmic domain (figure 2G). The cytoplasmic portion of MET contains the tyrosine kinase domain which autophosphorylates the receptor at specific tyrosine residues upon HGF binding or upon activation by other cellular co-receptors.[8] Conserved residues at the C-terminus also serve as an interface for docking of MET with its partner proteins.[8] The activation of MET leads to the binding and phosphorylation within the cell of adapter proteins, such as GAB1 and GRB2, which in turn activate signalling transducers including PIK3, PTPN11, PTK2, and STATs.[9]

The extracellular domains of MET are involved in ligand-dependent receptor homodimerization and ligand-independent interactions with other cellular co-receptors such as CD44, EGFR and integrin α6β4.[8] The mutation p.F841V is located in the extracellular IPT4 domain (figure 2G). Modelling the p.F841V mutation with CUPSAT and I-Mutant 2.0 using the crystal structure of IPT2-IPT4[10] (PDB ID, 2CEW) and the amino acid sequence of MET (NP_000236.2) predicted a decrease in the overall stability of the resulting protein. The IPT3 and IPT4 domains together form a high-affinity binding site for HGF.[11] In the *donut* mutant zebrafish, a missense mutation in *met* affects the IPT3 domain (p.L775R equivalent to human p.I777R).[12] The maturation of Met, its transport to the plasma membrane and the rate of activation of the kinase domain are all negatively affected by the mutation and result in failed outgrowth of the exocrine pancreas in *donut* mutants.[12] We speculate that the p.F841V mutation may similarly affect human MET function, but with phenotypic consequences limited to the inner ear. Other possibilities for the pathogenic

effect of the p.F841V mutation are that it reduces receptor stability and affinity for binding to HGF or impairs interaction with cellular coreceptors. Alternatively, the mutation may change expression level of some *MET* isoforms, similar to the effect seen for a missense variant that causes a dystonia-ataxia syndrome.[13]

In contrast to the phenotype in *donut* mutants, zebrafish *met* morphants have strikingly different defects including dysmorphology of the hypaxial muscles and impairment of the development of the lateral line. The zebrafish lateral line contains the neuromasts which resemble vertebrate inner ear sensory epithelia.[14] The *met* morphants have diminished deposition of the proneuromast cells, as well as reduced number of migrating neuromast-derived hair cells and the non-neural supporting cells.[15] It remains to be investigated if the sensory cells in the inner ear of the zebrafish morphants are similarly affected.

HGF is the only known ligand of MET and together the two proteins play a part in proliferation, migration and invasive growth.[8] MET also participates in epithelial development by interacting with the scaffolding protein, GAB1.[16] *Met* is expressed in the inner ears of rats [17] and mice (Shared Harvard Inner-Ear Laboratory Database). The role of HGF and MET interaction in the inner ear is not known. The involvement of HGF/MET signaling in healing of skin wounds,[18] suggests the possibility of a similar function in repair of cochlea after trauma due to chemicals or noise. This is supported by the observation that intrathecal injection of Haemagglutinating Virus of Japan Envelope (HVJ-E) containing *HGF* to cerebrospinal fluid in rats increases expression of endogenous *Hgf* and *Met* and was reported to prevent and ameliorate hearing impairment induced by kanamycin treatment.[17]

Mouse models have been investigated to determine the role of HGF/MET signalling. In mice, a null allele of *Met* is embryonic-lethal due to severe developmental defects.[19] Therefore, the inner ear pathophysiology due to loss of MET may be studied by using transgenic mice in which *Met* is deleted specifically in the inner ear using mice with a floxed *Met* [20] and *Cre* recombinase under the direction of an inner ear specific promoter. A second strategy would be to knock-in the equivalent human mutation of *MET* (c.2521T>G; p.F841V) in the mouse orthologue, *Met*.

MET and HGF are the first described receptor-ligand pair for which a mutation of either protein causes nonsyndromic recessive deafness in humans. We speculate that mutations in other genes encoding different proteins of the HGF/MET signalling pathway may also result in hearing loss. Continued research will yield insights into function of HGF/MET interaction in the auditory system and will also identify other associated proteins necessary for normal hearing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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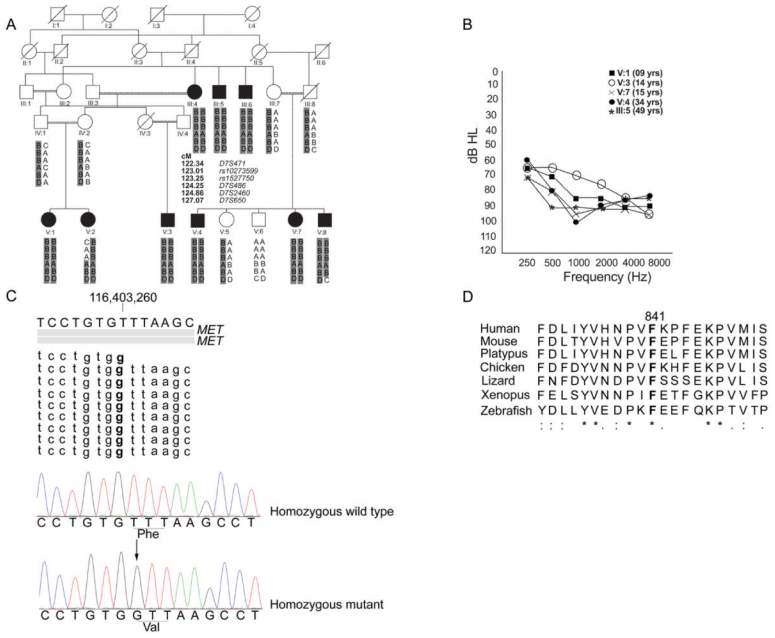

Figure 1. Family HLGM17, audiograms, mutation and conservation of p.F841

(A) Pedigree of Family HLGM17. Black symbols represent affected individuals. Double horizontal lines indicate consanguineous matings. The ancestral *DFNB97* haplotype with alleles of four STR markers and two SNP markers spanning 4.73 cM on chromosome 7 is shaded in gray. Samples from IV:2, V:1, V:2 and V:3 were used for SNP genotyping. Meiotic recombination breakpoints in individuals V:2 and V:8 define the proximal and distal boundary, respectively, of a linkage interval of 4.06 cM (chromosome 7:115181357 bp-120965265 bp,GRCh37/ hg19). Positions of markers are shown according to Rutgers Combined Linkage-Physical Map.

(B) Pure-tone audiometry results of selected affected individuals of family HLGM17. Variation in hearing loss thresholds was observed for most individuals.

(C) DNAnexus analysis of the exome data from a region encompassing the c.2521T>G variant detected in *MET*. The gray bars depict two Refseq transcripts of *MET*. Nine of a total of 30 DNA sequence reads which cover this region are shown. The variation is visible in bold. Chromatograms generated through Sanger sequencing are also shown below this alignment, with the site of mutation underlined in both traces of affected and unaffected individuals. The “arrow” indicates the mutated base.

(D) ClustalW analysis of MET residues surrounding the site of the missense mutation showing conservation of p.F841 among diverse vertebrate species. The orthologous amino acid positions which are identical to p.F841 are shown in bold. Amino acid residues which are identical in all orthologoues are indicated by an asterisk below the alignment. The colons and periods indicate amino acid substitutions with highly conserved and less conserved residues, respectively.

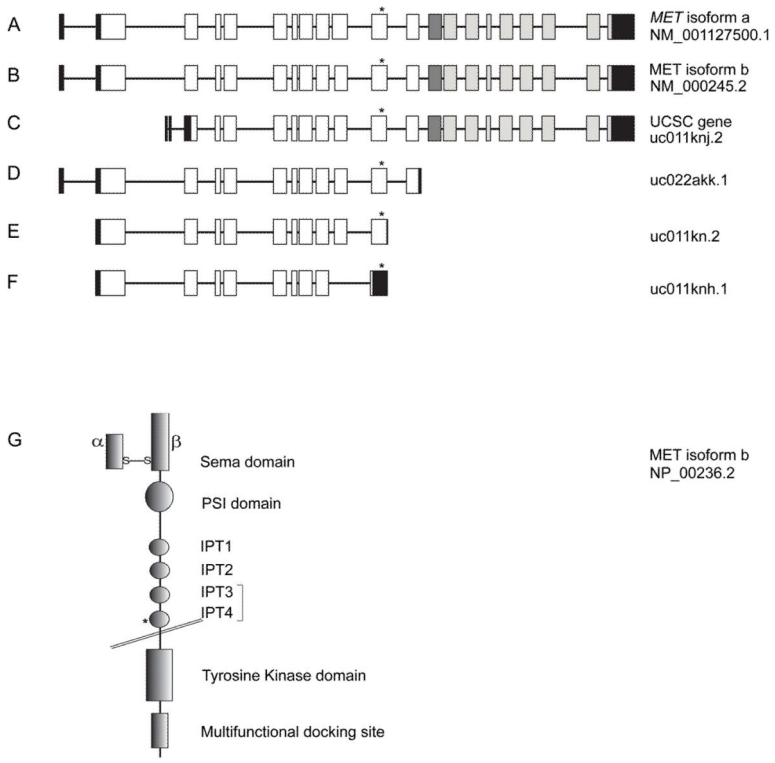

Figure 2. Schematic representations of *MET* isoforms and the processed receptor

(A) *MET* isoform *a* encodes the highest molecular-weight protein among all the isoforms. Black boxes denote non-coding exons. White boxes depict exons encoding the extracellular part of MET. The dark grey box represents exon 13 which encodes the transmembrane domain. Exons shown in light grey encode the cytoplasmic domain of MET. The asterisk marks the position of the mutation.

(B) *MET* isoform *b* is the highest transcribed isoform in all cell types examined to date and differs from isoform *a* by the absence of 54 nucleotides in exon 10. The asterisk shows the position of the c.2521T>G mutation in exon 11.

(C) A UCSC *MET* isoform lacks an exon which is known to encode most of the Sema domain.

(D) A predicted soluble MET isoform lacks sequences encoding the transmembrane anchoring amino acids.

(E) The *DFNB97* missense mutation in a *MET* transcript is located 113 nucleotides upstream of the stop codon within an exon which corresponds to exon 11 of isoforms *a* and *b*.

(F) A *MET* isoform is annotated in which a stop codon is introduced in the exon corresponding to exon 11 of isoforms a and b due to absence of an exon present in other isoforms. The *DFNB97* mutation is present in the 3′ UTR of this transcript.

(G) Schematic representation of the structure of MET isoform *b* showing the two processed subunits and different domains. The extracellular part of the receptor contains the ligand binding site and other domains. The Sema domain consists of the N-terminus part of the β-chain disulphide linked to the α-chain and binds to HGF. This is N-terminal to the PSI domain and four Ig or IPT domains. The bracket around IPT3 and IPT4 denotes a high-affinity binding site for HGF. The intracellular region of MET contains a kinase domain and

residues involved in protein interactions and downstream signalling. The position of p.F841V mutation within IPT4 is marked by an asterisk.