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Identification of a novel homozygous mutation in transmembrane channel like 1 (*TMC1*) gene, one of the second-tier hearing loss genes after *GJB2* in India

Pawan Kumar Singh[1], Manju Ghosh[1], Shipra Sharma[1], Shivaram Shastri[1], Neerja Gupta[1], Madhumita Roy Chowdhury[1], Anuranjan Anand[2] & Madhulika Kabra[1]

[1]Division of Genetics, Department of Pediatrics, All India Institute of Medical Sciences, New Delhi & [2]Molecular Biology & Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India

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***Background & objectives*: Hearing impairment is a common and heterogeneous sensory disorder in humans. Among about 90 genes, which are known to be associated with hearing impairment, mutations in the *GJB2* (gap junction protein beta 2) gene are the most prevalent in individuals with hereditary hearing loss. Contribution of the other deafness-causing genes is relatively poorly understood. Here, we present our findings on two families with transmembrane channel like 1 (*TMC1*) gene variants of the 47 families with nonsyndromic hearing loss (NSHL) studied.**

***Methods*: Forty seven families including 26 consanguineous families with at least two hearing impaired children and one normal hearing child and 21 non-consanguineous families having at least three hearing impaired children and one normal hearing child were enrolled for this study. Genetic linkage studies were carried out in 41 families that were *GJB2* (Connexin 26) negative. Seven polymorphic short tandem repeat markers at the DFNB7/11 locus were studied employing fluorescently labelled markers.**

***Results*: A novel homozygous missense mutation c.1283C>A (p.Ala428Asp) was identified co-segregating with hearing loss. This change results in substitution of a highly conserved polar alanine to a charged aspartic acid and is predicted to be deleterious. In addition, a previously reported nonsense mutation, p.R34X in *TMC1*, was found.**

***Interpretation & conclusions*: While mutations in *TMC1* are not as common a cause of NSHL as those in *GJB2*, *TMC1* should be considered for diagnostic investigations in cases of NSHL in *GJB2*-negative families.**

Key words DFNB7/11 locus - DNA sequencing - hearing impairment - India - linkage study - short tandem repeat markers - transmembrane channel like 1 gene

Hearing impairment is a common and genetically heterogeneous sensory disorder in humans. The frequency of congenital hearing loss is estimated to be 155 per 100,000 live births or around 1 in 650 newborns[1]. In 15-30 per cent of cases, hearing impairment is syndromic in its manifestation, and

among the remaining, non-syndromic. It is estimated that more than 200 genes may be associated with hearing impairment in humans. To date, more than 90 genes have been identified[2]. The gap junction protein beta 2 (*GJB2*) gene is the most common gene responsible for congenital nonsyndromic hearing impairment across different ethnic populations of the world including India[3-10]. Other frequently involved genes include *SLC26A4*, *MYO15A*, *TMC1*, *CDH23* and *TRIOBP*. Mutations in the *MT-RNR1* gene increase the risk of developing nonsyndromic hearing impairment due to antibiotic exposure[11-13]. Transmembrane channel like 1 or transmembrane cochlear-expressed gene 1 (*TMC1*) is one of the hearing loss genes which is implicated in both autosomal recessive (DFNB7/11, MIM: 606705) and dominant (DFNA36, MIM: 600974) hearing impairment. *TMC1*-linked dominant hearing loss is not very common, and only three different missense mutations, p.D572N[14], p.D572H[15] from North American families and p.M418K[16] in a Chinese family have been reported. However, recessive mutations in *TMC1* have been identified in many ethnic populations of the world including Sudan[17], Iran[18], Pakistan[15,19], Turkey[20-22] and Tunisia[23]. Two previous reports from the Indian subcontinent have also described mutations in the *TMC1* gene[14,24]. The objective of the study was to uncover the contribution of mutations in genes other than *GJB2* for nonsyndromic hearing loss (NSHL) in India.

Material & Methods

Family enrolment and clinical evaluation: This study was conducted between February 2008 and February 2014 at the department of Pediatrics, All India Institute of Medical Sciences, New Delhi, and Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India. The study was approved by the Institute's Ethics Committee. Written informed consent was obtained from all families.

Families with consanguineous parents, two hearing impaired children and at least one normal hearing child, and non-consanguineous parents, three hearing impaired children and at least one normal hearing child, were enrolled for the study by home visit. Before enrolling the families, detailed medical history of all the affected individuals was obtained to exclude syndromic and environmental causes of hearing loss. Families were examined for abnormalities of skin and hair pigmentation and problems relating to balance, vision, night blindness, heart, kidney and thyroid. In addition, clinical histories of infectious diseases such as rubella, mumps, meningitis, typhoid, trauma and antibiotic/ototoxic drugs usage were also considered. Families were asked about the onset of hearing loss in each affected individual to confirm the hearing impairment to be congenital. Clinical information was collected in a proforma, and the hearing loss was evaluated by an audiologist. All affected individuals had severe-to-profound hearing impairment. Twenty six families were consanguineous and had two or more hearing impaired children. Twenty one families were non-consanguineous and had three or more hearing impaired children. History of hearing loss in the extended family was present in some families. A total of 47 families were enrolled for the study through deaf schools in Delhi, Uttar Pradesh, Madhya Pradesh, Gujarat and 50 healthy individuals having normal hearing with no family history of hearing problems were enrolled as controls. Peripheral blood sample (5 ml) was collected from all the affected children, normal children and parents by making home visits. Genomic DNA was extracted by the salting out method[25].

Genotyping: Two hearing impaired children from each family were screened for mutations in exon 2 of *GJB2* (Transcript: ENST00000382848), exon 3 and 309 kb deletion of *GJB6* gene (Transcript: ENST00000241124) by polymerase chain reaction (PCR) and sequencing. Six families harbouring mutations (p.W24X, p.W77X, p.Q124X, c.303-316del14, IVS1+1G>A) in *GJB2* were excluded from linkage study for *TMC1*. In the remaining 41 families negative for *GJB2* and *GJB6* mutations, linkage analysis was performed using seven fluorescently labelled polymorphic short tandem repeat markers for the DFNB7/11 locus. The seven microsatellite markers used were D9S301, D9S1822, D9S1124, D9S1837, D9S1876, D9S237 and D9S175 between 66.32 cM (D9S301) and 70.33 cM (D9S175) within the DFNB7/11 locus. PCR (ABI 2720 Thermal Cycler, Applied Biosystems, USA) amplification was carried out in a total 10 µl of reaction volume, containing 50 ng of genomic DNA, 1 µl 10X PCR buffer, 1 µl 2 mM dNTPs each (G Biosciences, Geno Technology, Inc., USA), 0.5U Taq polymerase (Merck Bioscience, India) and 3 pmol primers. Forward primer was labelled with 6-FAM (6-carboxyfluorescein), HEX (hexachlorofluorescein) and NED. PCR amplification included 5 min denaturing step at 95°C followed by 10 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C

for 30 sec followed by the second step of 25 cycles of 89°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec and a final extension at 72°C for 30 min. Post-PCR products were pooled and diluted. One microlitre of pooled PCR product was mixed with 8.7 µl of 100 per cent Hi Di formamide (Applied Biosystems) and 0.3 µl of the internal size standard (GeneScan™ 500 ROX™ Size Standard, Applied Biosystems), denatured at 95°C for 5 min and separated by capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems). The allele sizes were analyzed using Gene Mapper v.4.0 (Applied Biosystems, USA).

Sequence analysis: PCR primers for *TMC1* were designed using Primer3 software (*http://bioinfo.ut.ee/primer3-0.4.0/*). Direct sequencing of *TMC1* was performed by amplifying its coding regions including the exon-intron boundaries. PCR-amplified products were incubated with Exonuclease I and shrimp alkaline phosphatase (Fermentas, Thermo Fisher Scientific, USA) to remove unincorporated primers and nucleotides. Cycle sequencing was done using ABI prism BigDye Terminator cycle sequencing Ready Reaction Kit v3.1 (Applied Biosystems) and was followed by ethanol/EDTA/sodium acetate precipitation and resuspension in 10 µl Hi Di formamide, denaturation and capillary electrophoresis using an ABI 3130 Genetic Analyzer. Sequence analysis was performed using Chromas Pro software (*http://technelysium.com.au*) and compared with reference sequence of *TMC1* in the NCBI database (*http://www.ncbi.nlm.nih.gov/*). The 1000 genomes database (*http://www.1000genomes.org/*), dbSNP database (*http://www.ncbi.nlm.nih.gov/snp/*) and Human gene mutation database (HGMD, *http://www.hgmd.cf.ac.uk/ac/index.php*) were used to check the pathogenicity and novelty of the variations identified in *TMC1*.

In silico analysis: PolyPhen2 (*http://genetics.bwh.harvard.edu/pph2*) and Sorting Intolerant from Tolerant (SIFT) (*http://sift.jcvi.org*) programs were used to predict the qualitative effect of missense change on the protein function. Multiple sequence alignments were performed to check the conservation of amino acid across species.

Results

As sequencing of the entire *TMC1* gene was expensive and time taking, linkage studies were done to screen the families. Seven microsatellite markers were analysed encompassing the DFNB7/11 locus in 41 families. Families were selected for *TMC1* gene sequencing on the basis of concordance of markers. In two unrelated families DDL4 and LKO5, concordance with all seven markers in all the affected children was observed. In the non-consanguineous family DDL4 with three affected siblings, all the seven markers co-segregated with hearing loss exhibiting homozygosity between D9S1124 and D9S237 markers which were at an interval of 0.54 cM (Fig. 1). In the

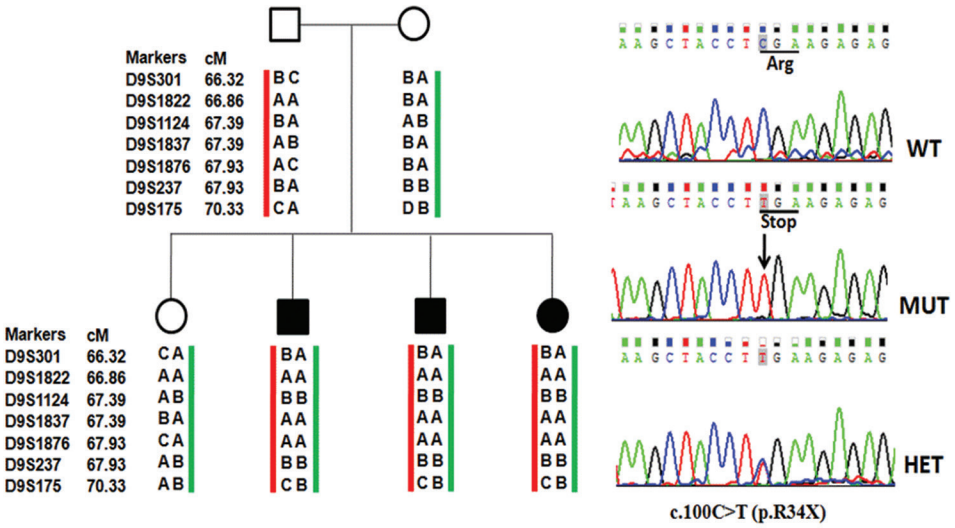

Fig. 1. Pedigree and haplotypes (family DDL4), co-segregating nonsyndromic autosomal recessive hearing loss (filled symbols) with short tandem repeat markers at the DFNB7/11 locus. Sequence chromatogram of transmembrane channel like 1 gene showing homozygous change c.100C>T (p.R34X) in hearing impaired children, parents and normal hearing child are heterozygous for c.100C>T.

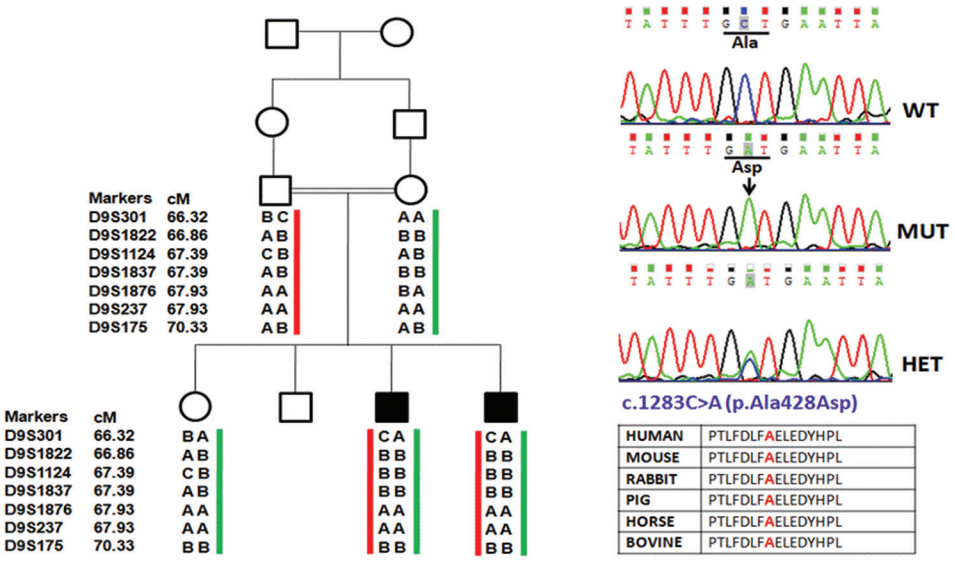

Fig. 2. Pedigree and haplotypes (family LKO5), co-segregating nonsyndromic autosomal recessive hearing loss (filled symbols) with short tandem repeat markers at the DFNB7/11 locus. Sequence chromatogram of *TMC1* gene show a novel homozygous change c.1283C>A (p.Ala428Asp) in hearing impaired children, parents and normal hearing child are heterozygous for c.1283C>A.

consanguineous family LKO5 with two affected siblings, a small homozygous region was found between D9S1822 and D9S1837 which were 0.54 cM apart (Fig. 2). DNA sequencing of *TMC1* revealed mutations in both the families. In DDL4 (Fig. 1), a homozygous mutation, c.100C>T (p.R34X), segregated with hearing loss. Both parents and normal hearing child were heterozygous for c.100C>T. In LKO5 (Fig. 2), a novel homozygous change c.1283C>A (p.Ala428Asp) was found in the affected children. Parents and normal hearing children were heterozygous for this change. *In silico* analysis of p.Ala428Asp showed that the change was damaging and probably damaging by SIFT and PolyPhen2, respectively.

Discussion

Homozygosity mapping is a useful method of linkage analysis for autosomal recessive inherited disorders leading to the identification of hundreds of genes responsible for autosomal recessive genetic diseases. The *TMC1* gene is one of the hearing loss genes which is implicated in both autosomal recessive (DFNB7/11) and dominant (DFNA36) hearing impairment. The chromosomal position of DFNB7/11 locus (OMIM 606706) is 9q13–q21. *TMC1* contains 24 exons (NCBI Reference Sequence: NM_138691.2, Transcript: ENST00000297784), encodes 760 amino acids, 87.8 kDa multipass transmembrane protein, with six transmembrane domains. However, the exact function of *TMC1* protein in the inner ear is unknown but is predicted to have a role in maintaining the electrochemical homeostasis, structure and function of neurosensory hair cells of the inner ear.

More than 53 mutations in *TMC1* (missense/nonsense, splicing, small and large deletions) have been reported in HGMD (*http://www.hgmd.cf.ac.uk/*) in different ethnic populations of the world, in which c.100C>T (p.R34X) is the most common recessive mutation in the gene and is most frequently reported in Pakistani and Tunisian populations and is attributed to founder effect[15,23]. Kurima *et al*[14] reported an incidence of *TMC1* mutations in India and Pakistan to be 5.4 per cent in the 230 families that were screened. c.1960A>G and c.295delA mutations were identified in the Indian families studied. In another study of 374 Indian families negative for the *GJB2* gene mutations, c.100C>T, c.237-6T>G, c.453+2T>C, c.628_630del, c.800G>A, c.1114G>A, c.1333C>T and c.1566+1G>A mutations were reported and the prevalence of *TMC1* gene mutations was 1.6 per cent[16].

We identified a novel homozygous mutation c.1283C>A (p.Ala428Asp) in a consanguineous family, LKO5, having change involving a transversion from cytosine to adenine at nucleotide position of 1283 (NCBI Reference Sequence: NM_138691.2, Transcript: ENST00000297784) which causes substitution of a highly conserved polar amino acid alanine, at codon 428, to a charged aspartic acid (p.Ala428Asp). This

change was predicted as damaging and probably damaging by SIFT and PolyPhen2, respectively. This novel mutation was absent in 50 healthy normal controls. c.1283C>A (p.Ala428Asp) mutation is located in the extracellular topological domain and amino acid sequence alignment using MEGA software 9 (*http://www.megasoftware.net/previousVersions.php*) of different vertebrates (mouse, rabbit, pig, horse and bovine) shows that the Ala428 residue is conserved across species.

In the DDL4 family, with three hearing impaired children with recessive, prelingual, severe-to-profound hearing loss and one normal hearing child, a nonsense mutation c.100C>T (p.R34X), previously reported in a Pakistani family[14], was identified. The parents and the normal hearing child were carriers for this mutation. Numerous studies indicate that p.R34X is a common mutation of *TMC1* reported in several hearing impaired individuals across many ethnic populations. It has been suggested that p.R34X arose from a common founder and the estimated age of mutation was calculated to be between 1075 and 1900 yr by setting the marker mutation[26] rate at 10^{-3} to 10^{-6}.

The genetic aetiology of NSHL is heterogeneous in India as in the rest of the world. Except *GJB2,* only a few genes have been reported from India; these are *TMIE*[24,27], *TMC1*[14,24], *USH1C*[24,28,29], *TMPRSS3*[24], *CDH23*[24], *MYO15A*[30] and *SLC26A4*[31] and can be considered to be second-tier genes. None of these have the frequency similar to *GJB2* mutations, hence it is difficult to specify the second most common causative gene.

In conclusion, the identification of a previously identified c.100C>T mutation, and a novel homozygous mutation, c.1283C>A in *TMC1,* in this study supports *TMC1* gene as one of the second-tier hearing loss genes, after *GJB2* in India. Testing for *TMC1* may be considered in all *GJB2*-negative NSHL cases.

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***Conflicts of Interest*:** None.

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Reprint requests: Dr Madhulika Kabra, Department of Pediatrics, Division of Genetics, All India Institute of Medical Sciences, New Delhi 110 029, India
e-mail: madhulikakabra@hotmail.com