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# Functional characterization of a novel Cx26 (T55N) mutation associated to non-syndromic hearing loss

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**Abstract**

Mutations of the *GJB2* gene, encoding connexin 26, are the most common cause of hereditary congenital hearing loss in many countries and account for up to 50% of cases of autosomal-recessive non-syndromic deafness. By contrast, only a few *GJB2* mutations have been reported to cause an autosomal-dominant form of non-syndromic deafness. Here, we report a family from Southern Italy affected by non-syndromic autosomal dominant post-lingual hearing loss, due to a novel missense mutation in the *GJB2* gene, a threonine to asparagine amino acid substitution at codon 55 (T55N). Functional studies indicated that the mutation T55N produces a protein that, although expressed to levels similar to those of the wt counterpart, is deeply impaired in its intracellular trafficking and fails to reach the plasma membrane. The mutation T55N is located at the apex of the first extracellular loop of the protein, a region suggested to play a role in protein targeting and a site for other two mutations, G59A and D66H, causing dominant forms of deafness.


*Keywords:* Cx26; Deafness; Mutation; Intercellular communication

Genetic deafness is one of the most prevalent inherited sensory disorders, affecting about 1 in 2000 children in developed countries [17]. Sensorineural hearing loss comprises both syndromic and non-syndromic deafness. Mutations in Cx26, Cx30, and Cx31 (GJB2, GJB6, and GJB3 genes), which are all found in the organ of Corti [12], have been linked to sensorineural hearing loss [11,28,10]. These three Cx genes are associated to different forms of dominant (NSAD) and recessive non-syndromic deafness (NSRD).

Oligomerization of connexins in intercellular channels, clustered in gap junctions, allows the selective diffusion of ions and small water soluble molecules among adjacent cells and are structurally more complex than ion channels. They span two plasma membranes, each cell contributing half a channel, or connexon, which interacts and aligns with another connexon from the adjacent cell. Mutations in the GJB2 gene, encoding the gap junction channel protein connexin 26, account for the majority of recessive forms and some of the dominant cases of deafness. The most frequent recessive Cx26 mutation is a single base deletion (35delG) that results in a frame-shift at position 12 in the coding sequence and premature termination of the protein [11,24,7,25,9]. All the other mutations are located in the coding region of the gene (exon 2), with the exception of a splice-site mutation located at the end of the non-coding exon 1 [18]. Dominant mutations described in the same

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gene encode full length products containing non-conservative amino acid substitutions and exert a noxious dominant-negative effect on wild type Cx26 channel function [19,2]. Members of the Cx family have highly conserved sequences with four transmembrane domains separating two extracellular and one cytoplasmic loop, with cytoplasmic carboxy- and amino-terminal ends [3].

In this report, we describe a novel missense mutation in the coding region of connexin 26, associated to autosomal dominant non-syndromic hearing loss. This mutation has been found in a family from the Southern Italy and shows a clear dominant inheritance. When transiently expressed in HeLa cells, the mutation T55N produces a protein that, although expressed to levels similar to those of the wt counterpart, is deeply impaired in its intracellular trafficking and fails to reach the plasma membrane. However, the T55N mutant failed to act as a dominant inhibitor of both wild type Cx26 and wild type Cx30 channel activity when the two proteins were co-expressed in a manner mimicking a heterozygous Cx26 genotype.

## Materials and methods

*Patients.* Three patients (Fig. 1A; II-1, II-2, and II-3) were studied as a part of the routine genetic deafness diagnostic test allowed by our Institute. Three other affected people (Fig. 1A; II-4, III-1, and III-2) from the same family were collected successively. All patients underwent ear, nose, and throat (ENT) and audiological examinations: pure tone audiometry, tympanometry with stapedius reflex, otoacoustic emissions, auditory evoked potential (ABR, ASSR). Also, vestibular function were studied: positional spontaneous nistagmus in the primary positions with and without visual fixation; evoked nistagmus; head shaking test; nistagmus evoked by caloric stimulation; Hallpike's manoeuvre; vestibular potential (VEMPs).

DNA was extracted from peripheral blood according to standard protocols after obtaining informed consent.

*Cx26 gene analysis.* The whole CX26 gene coding region was amplified by polymerase chain reaction (PCR) using two oligos: CX26ORF-F (TGCTTACCCAGACTCAGAGAA) and CX26ORF-R (GACTGAGCCTTGACAGCTGAG). PCR was performed in 50 μl reaction volumes containing 15 pmol each primer, 100 μM dNTP, 5 μl of 10× reaction buffer (100 mM Tris, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% gelatin), 2.5 U Ampli*Taq* Gold Polymerase (Perkin-Elmer, Foster City, CA) and 100 ng DNA. The reaction was performed in an automated Thermal Cycler 9700 (Perkin-Elmer) with the following cycling profile: an initial denaturation step at 94 °C for 12 min followed by 35 cycles each one characterized by a denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 80 s, and a final extension step at 72 °C for 10 min.

The exon I and flanking splicing sites were amplified using the oligos EX1CX26F: tcaaaggaactaggagatcgg and EX1CX26R: aaggacgtgtgttggtccag.

The deletion of the GJB6 gene was detected amplifying the Δ(GJB6-D13S1830) deletion by PCR as suggested in De Castillo et al. All the amplicons were sequenced directly using the PCR oligos and the ABI-PRISM big-dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Foster City, CA). An aliquot of the sequence reaction was loaded and analysed on an automated sequencer (ABI 3100; Perkin-Elmer, Foster City, CA).

*Molecular cloning.* The coding region of either wild-type human Cx26 (hCx26) or wild-type hCx30 was cloned into the *Eco*RI and *Bam*HI sites of pEYFP-N1 vector (Clontech, Palo Alto, CA) [1]. Point mutation T55N of hCx26 was generated using the QuickChange (Stratagene) site-directed

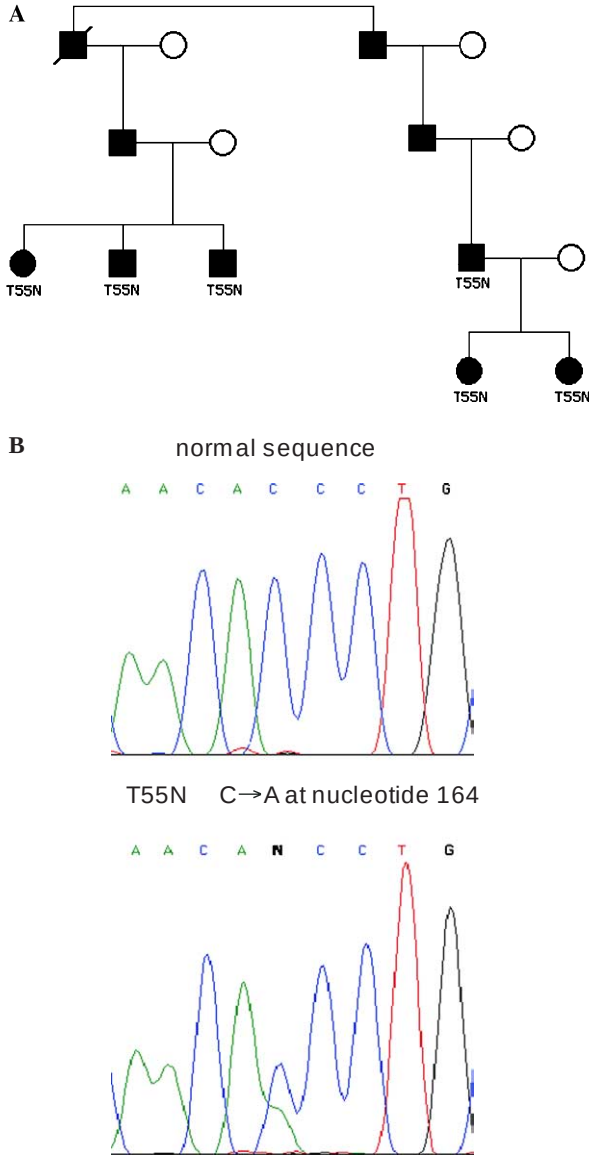


Fig. 1. (A) Pedigree of the family and (B) results of the sequences analysis of normal and mutant genes.

mutagenesis method. PCR was carried out with the following oligonucleotide primers:

Cx26 T55N for: 5′-GACTTTGTCTGCAACAACCTGCAGCCAGGCTGC-3′
Cx26 T55N rev: 5′-GCAGCCTGGCTGCAGGTTGTTGCAGACAAAGTC-3′

The mutated base was: C → A at nucleotide 164 resulting in Thr → Asn at codon 55.

The resulting PCR product was used to transform competent cells (*Escherichia coli* XL2-Blue). Miniplasmid preparation and restriction enzyme analysis were performed to identify positive clones. All constructs were sequenced using the Dye Terminator (Perkin-Elmer, Wellesley, MA), as recommended by the manufacturer, to verify that PCR amplification did not introduce unwanted mutations.

*Cell culture and transfection.* A clone of HeLa cells essentially devoid of connexins, as assessed by intercellular dye transfer [8], was kindly provided by Prof. Klaus Willeke (University of Bonn, Germany) and cultured according to standard procedures. Twenty-four hours after plating, cells