Eur Arch Otorhinolaryngol
DOI 10.1007/s00405-013-2613-y

OTOLOGY

Identification of D179H, a novel missense *GJB2* mutation in a Western Sicily family

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Received: 21 April 2013 / Accepted: 19 June 2013

Abstract The main purpose of this study was to describe a novel missense mutation (p.D179H) found in a Western Sicily family and to examine the genetic and audiologic profiles of all family members by performing a *GJB2* and *GJB6* mutations analysis and a complete audiologic assessment. The proband was a 3-month-old infant with a congenital profound sensorineural hearing loss; direct sequencing of the *GJB2* revealed the presence of a c.35delG mutation in the heterozygous state and a heterozygous G>C transition at nucleotide 535 in trans; this novel mutation, called p.D179H, resulted in an aspartic acid to histidine change at codon 179. It was also evidenced in the heterozygous state in two members of this family, both with normal hearing. No *GJB6* mutations were evidenced in all subjects studied. Considering the genotypic and phenotypic analysis of all family members, we suggest, differently from the p.D179 N mutation previously reported, a recessive mode of inheritance. Functional studies on p.D179H have to be performed to confirm our hypothesis.

Keywords Novel mutation · Connexin mutation · *GJB2* · Sensorineural hearing loss · Congenital hearing loss

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Introduction

Mutations in *GJB2*, a gene encoding Connexin 26 (Cx26) protein on chromosome 13q11, are involved in the pathogenesis of more than 50 % of congenital, autosomal recessive, non-syndromic hearing loss (ARNSHL) [1].

Cx26 is one member of a family of gap-junction proteins which oligomerize in groups of six subunits making up hemichannels called "connexons". Each connexon can bind with another from adjacent cells, forming intercellular channels. They allow ions and small metabolites to pass between cells for synchronized function and homeostasis of tissues and organs. In the cochlea, Cx26 is expressed in the supporting cells of the organ of Corti, the fibrocytes of the spiral ligament and the basal cells of stria vascularis [2] play a crucial role in K^+ recycling [3]. This cyclic ionic flux is essential to maintain K^+ homeostasis by removing K^+ from the hair cells during auditory transduction. In addition, the permeability to Ca^{2+}-mobilizing messenger inositol triphosphate (IP_3), regulated by Cx26, was demonstrated to be necessary to generate an endocochlear potential [4].

Over 90 mutations of *GJB2* were identified (http://davinci.crg.es/deafness), inherited in recessive or dominant mode and influencing the audiological phenotype differently.

Non-syndromic hearing loss can be linked also with mutations of *GJB6*, a gene encoding Connexin 30 (Cx30)

Published online: 28 June 2013

protein and coexpressed with *GJB2* in the inner ear. Two large deletions in *GJB6* have been described and could often occur with single recessive mutations in trans of the *GJB2*, suggesting a possible ARNSHL digenic inheritance [5, 6]. It has been also hypothesized that *GJB6* deletions are responsible for deafness in patients who also carry one *GJB2* recessive mutation in trans, as a result of the abolition of an as-yet-unidentified cis-regulatory element responsible for the expression of the *GJB2* gene in the inner ear [7]. Furthermore, a deletion distant from the transcriptional sites of both *GJB2* and *GJB6* and cosegregating as a pathologic allele with a *GJB2* mutation has been linked with a reduced expression of Cx26 and Cx30 [8].

In 2003, Primignani et al. [9] discovered a novel dominant *GJB2* mutation (p.D179 N) identified in four deaf members of a family from Southern Italy; they were all heterozygous for a c.535 G>A mutation (resulting in an aspartic acid to asparagine change at codon 179) and presented a mild to moderate post-lingual sensorineural hearing loss (SNHL).

In this paper, we report a novel missense mutation of the *GJB2* (p.D179H), found in the heterozygous form in three members of a Western Siciliy family; only one member, who carried also a c.35delG mutation in trans, presented a pre-lingual non-syndromic SNHL whereas the others were normal hearing. Nevertheless, p.D179H causes a nucleotide substitution at the same codon of p.D179 N, genetic and audiological evaluation suggested a recessive mode of inheritance.

Thus the identification of a new recessive *GJB2* mutation might help in understanding the relationship between this gene and deafness phenotypes; moreover, it provides additional evidences about the role of single *GJB2* point mutations in the pathogenesis of hearing defects and their inheritance mode.

Materials and methods

We investigated a family from Western Sicily composed of five members over three generations (Fig. 1). The proband (III1) was a 3-month-old infant who was admitted to the Audiology Section of the University of Palermo after failing two neonatal hearing screenings (performed with TEOAEs) in both ears; because the Audiology Section of Palermo University represents the main speech and hearing third level centre in Western Sicily, the infant was evaluated with a global audiological assessment; environmental risk factors for SNHL (JCIH 2007) [10] were investigated through the compilation of a specific questionnaire by the parents. An experienced audiologist and otorhinolaryngologist examined the condition of the external auditory canal and tympanic membrane with otoscopy, and nose, throat, head and face in search of ear anomalies and syndromic features related to hearing impairment [11–14].

The audiological assessment was performed by the same qualified biomedical staff and consisted of performing tympanometry measurement and recording auditory brainstem responses (ABR) and TEOAEs.

After a clinical evaluation including micro-otoscopy, the proband's parents (II1,II2) and grandparents (I1,I2) were evaluated with audiometric test (calculating air conduction pure tone average thresholds at frequencies 0.5–1–2–4–8 kHz for each ear) and tympanometry with stapedius reflex testing.

All family members were tested for the molecular analysis of the entire *GJB2* and the *GJB6* genes after extracting DNA samples from peripheral blood. Blood samples were taken from family members after obtaining informed consent from each member and from the parents of patient III1.

Genomic DNA was extracted classically from peripheral blood samples by the salting-out method. Molecular analysis of the entire *GJB2* gene was performed with PCR and direct sequencing. Exon 1 was amplified with the primers Cx1 forward (5′-TCAAAGGAACTAGGAGATCGG-3′) and Cx1 reverse (5′-CAAGGACGTGTGTGGTCCAG-3′). Exon 2 was amplified with the primers GAP1 forward (5′-CCTATGACAAACTAAGTTGGTTC-3′) and CONN reverse (5′- GACAGCTGAGCACGGGTGCCTC-3′).

USB® ExoSAP-IT® PCR Product Cleanup protocol (37 °C for 30 min and then 95 °C for 5 min) was performed to remove leftover primers and unincorporated dNTPs (Affymetrix, Inc.).

Cleaned up PCR products were sequenced from both ends, using the same primers as used for PCR reactions, with Applied Biosystem (ABI) PRISM® BigDye®

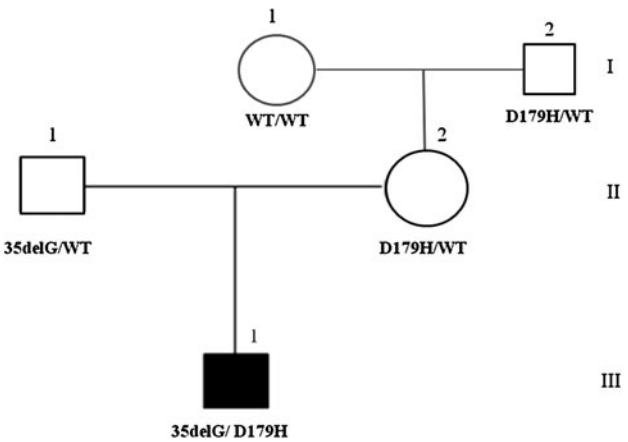

Fig. 1 Pedigree and GJB2 genotypes for the study family segregating non-syndromic recessive deafness

Terminator v3.1 Cycle Sequencing Kits and analyzed with ABI PRISM 3100 DNA automatic sequencer.

Furthermore, following PCR protocol and using primers described by Del Castillo [5], we also checked the *GJB6* gene to rule out the presence of deletions (del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854).

Results

The audiological assessment of the whole family revealed a profound, bilateral and symmetric SNHL only in the 3-month-old infant (III1), evidenced by absent TEOAEs and not recordable ABR (evocated by click stimuli at a maximum level of 100 dB HL, Fig. 2); the other family members instead presented a bilateral normal audiometric threshold (<20 dB for the frequencies 0.5–1–2–4–8 kHz).

From the questionnaire answered by the parents it was possible to exclude the main risk factors for SNHL, such as family history of hearing impairment, consanguinity, pregnant maternal infection (TORCH), ototoxic drugs administration (furosemide, dexamethason, vancomycin, gentamycin and tobramycin), prematurity, very low birth weight (<1500 g), respiratory distress, intensive care in excess of 5 days, hyperbilirubinemia requiring exchange transfusion, perinatal infections and others conditions, as recommended by JCIH 2007; no ear anomalies and syndromic features related to hearing impairment were identified.

All family members were tested for the molecular analysis of *GJB2* and *GJB6* genes. Sequencing of *GJB2* revealed that the proband (III1) presented a heterozygous c.35delG mutation associated to a heterozygous c.535G>C mutation resulting in an aspartic acid to histidine change at codon 179 (p.D179H); individuals I2 and II2, who had normal hearing, carried p.D179H in the heterozygous state whereas subject II1 was only heterozygous for the c.35delG mutation (Fig. 1).

Sequencing and deletional analysis of *GJB6* were performed in all family members to rule out, as stated previously, the hypothesis of a co-segregating pathological allele; no mutations or deletions in the *GJB6* gene were found in any of the subjects tested.

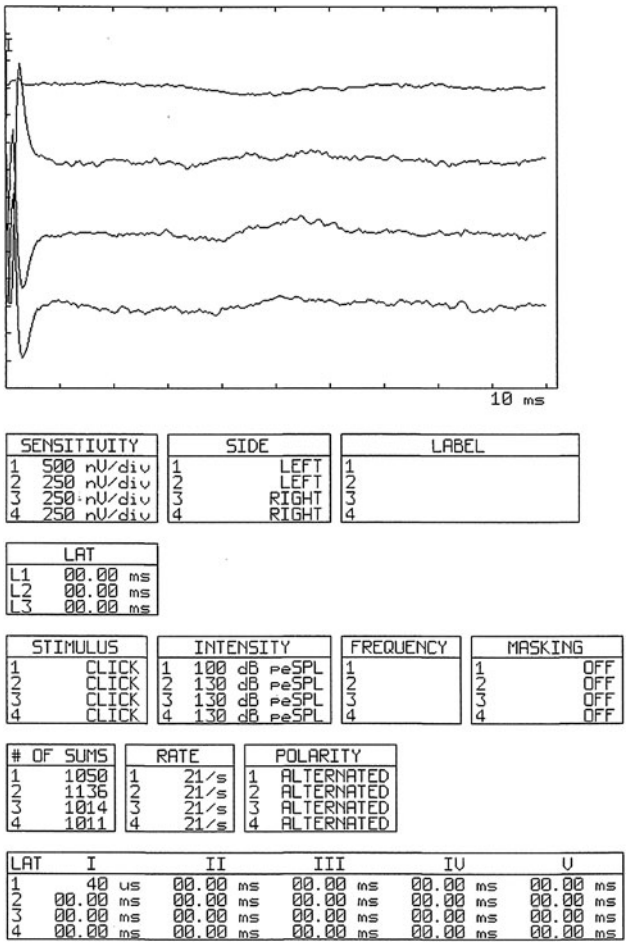

Fig. 2 Auditory Brainstem Responses of subject III1

Discussion

GJB2 mutations are the most frequent genetic cause of congenital hearing loss, identified in over 50 % of AR-NSHL. More than 90 different mutations of this gene were recognized, characterized by a wide spectrum of phenotypes [15], different modes of inheritance and deafness' time of onset. *GJB2* encodes Cx26, a member of a large family of highly conserved proteins involved in the formation of gap junctions which allows the direct transfer of small molecules and ions between neighbouring cells. Each member of this gap-junction family is constituted by four transmembrane helices (TM1-4), N- and C-cytoplasmatic termini, two extracellular loops (EC1-2) and one intracellular loop. The N-terminal domain determines the localization of the nascent polypeptide chain in the endoplasmic reticulum and the voltage gating with the first TM domain. Similarly the C-terminal domain and the intracellular loop regulate the pH gating. The EC loops are involved in the connexon–connexon interactions forming a complete channel. Because different amino acids of the EC domains are conserved among either species and connexins, mutations involving these regions are often responsible for a Cx26 loss of function.

GJB2 allele variants have been classified as truncating and non-truncating mutations [16]; truncating mutations, such as the small deletions c.35delG, c.167delT, c.235delC and the splice mutation IVS1+1G>A, included nonsense mutations, deletions, insertions and duplications that

introduce an anticipated stop codon; non-truncating mutations, such as the p.M34T, the p.V95 M and the p.L90P, contained amino acids substitutions and one inframe deletion. Truncating mutations are often associated with a greater degree of hearing loss than non-truncating mutations; however, the presence in most patients of combined mutations in the heterozygous state, makes difficult to predict, on the basis of this classification, the severity of the phenotype and the responsibility of each mutations in its pathogenesis. In addition, functional studies of the Cx26 reported many times in literature, which demonstrated complete loss of channel activity for mutations like c.35delG and c.IVS1+1G>A [17, 18], resulted often discrepant with the phenotype expected.

In the 2003 Primignani et al. described a novel non-truncating *GJB2* mutation found in three members of a Southern Italy family suffering from post-lingual bilateral SNHL limited to high frequencies; the direct sequencing of the *GJB2* gene evidenced a heterozygous G>A transition at nucleotide 535, resulting in an aspartic acid to asparagine substitution at codon 179 (p.D179 N). No mutations or deletions in the *GJB6* gene were found in any of the three family members analyzed. The authors suggested a dominant inheritance because of the presence in all hearing impaired subjects of p.D179 N mutation in the heterozygous state and the absence of a co-segregating pathological allele of *GJB6*; the delayed onset of deafness in all family members, frequent feature of dominant genetic hearing loss, supported the hypothesis [9]. Furthermore, functional studies performed on HeLa cells stably expressing the previously described p.D179 N mutation, revealed how this mutation only partially inhibited dye transfer of Cx26 and did not affect dye transfer of Cx30 [19, 20].

The novel non-truncating p.D179H mutation, which was found in a Western Sicily family, occurs in the highly conserved second EC loop of Cx26. The aspartic acid at position 179, contained in this Cx26 domain, is conserved in five different species (human, cow, sheep, rat and house mouse) and in several human connexins, so it is crucial for the activity of the gap junction.

Particularly p.D179H mutation, with a G>C transition at nucleotide 535, replaces a negatively charged amino acid (aspartic acid) with a positively charged amino acid (histidine), what is predicted to cause a modification in local conformation of EC2 and a defect in connexon–connexon interaction.

Even if p.D179H mutation is localized in the same place of p.D179 N, we think it could be possible to exclude, on the basis of clinical and genetical findings, a dominant pattern of inheritance for p.D179H; in fact individuals I1 and II1, who had normal hearing, carried this mutation in the heterozygous state, while subject III1, who suffered from SNHL, was found to be compound heterozygous for Cx26 mutations (c.35delG/p.D179H) (Fig. 2); no mutations or deletions in the *GJB6* gene were found in all individuals studied.

The identification of this novel *GJB2* mutation highlights some critical data: the severity of the phenotype associated with p.D179H is in contrast with Snoeckx et al.'s classification, according to which non-truncating mutations are associated with lower degree of hearing loss. However, our case is a compound truncating (c.35delG)/non-truncating (p.D179H) heterozygous, so we cannot determine precisely the role of p.D179H in the pathogenesis of the phenotype. Moreover, considering the complex structure and function of Cx26 gap junctions, it is really difficult to predict the pathogenicity of this missense mutation and to understand the different mode of inheritance only on the basis of the amino acid substitution. Finally, functional studies have to be performed to study the effects of p.D179H on Cx26 channel activity and further support would be provided not only by the finding of a homozygous affected subject, but also by the other affected compound heterozygotes in other families.

Conflict of interest The authors declare that they have no conflict of interest.

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