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Letter to the Editor

Detection of the GJB2 gene mutations in two children with hearing impairment

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To the Editor:

Hearing loss is the most common sensory disorder worldwide. One in 500-650 newborns has permanent bilateral deafness (1). Epidemiological studies have shown that 50-60% of deafness cases have genetic causes and the mutations at the nonsyndromic hearing loss and deafness locus 1 (DFNB1) account for over half of autosomal recessive nonsyndromic hearing loss (ARNSHL) cases (2). DFNB1 locus is mapped to chromosome 13q11-q12 and contains two genes - Gap Junction Protein, Beta 2, 26kDa (GJB2) and Gap Junction Protein, Beta 6, 30kDa (GJB6) (3).

The 35delG mutation in the GJB2 gene, which encodes connexin 26, is considered to be the most common genetic deficiency in Caucasians. This mutation is estimated to be about 14,000 years old, with a Greek origin (4), age that explains the spread of the mutation in Europe from the Mediterranean coast to Italy and Spain, and then to the Danube and Rhine valleys to northern Europe (5).

It is estimated that 10-42% of persons with GJB2 mutations have one mutant allele, with a second mutation in the GJB6 gene, which encodes the connexin 30. Two deletions in the GJB6 gene, del(GJB6-D13S1830) – encompassing 309 kb and del(GJB6-D13S1854) – spanning 232 kb have been shown to be a common cause of hearing loss. Haplotype analysis showed a common founder for the del(GJB6-D13S1854) mutation in Spain, Italy, and United Kingdom, while for the mutation del(GJB6-D13S1830) it indicated a founder effect in Ashkenazy Jews (6). Findings in our previous works indicate no detection of deletions in the GJB6 gene and suggested that these deletions are not significant for Romanian population.

Considering all these aspects and based on our previously published study (7), where we found a carrier rate for 35delG GJB2 of ~3.14% in Romanian population, we investigated two children with prelingual nonsyndromic hearing loss for GJB2 gene mutations, to elucidate the respective causative genetic mechanism.

We investigated two pediatric patients - a male aged 3 and a female aged 4 years old - clinically diagnosed with hearing loss, referred to our laboratory for genetic testing. Both children presented congenital sensorineural prelingual, stable, bilateral, and profound nonsyndromic deafness. Congenital hearing loss caused by infections was excluded. Their parents had normal hearing and no familial history of deafness was identified. All parents signed an informed consent. Genetic counseling was offered to the parents.

Blood specimens were collected on EDTA vacutainers. DNA extraction from peripheral blood cells was performed using the QIAamp DNA Blood Mini Kit (Qiagen).

To detect the c.35delG GJB2 gene mutation, we performed Amplification-Refractory Mutation System (ARMS) analysis using the primers described by Scott et al (8). β-actin was introduced as an internal control of PCR (Polymerase Chain Reaction) reactions. 200 ng genomic DNA was amplified following the PCR conditions described in our previously study (7).

The coding exon 2 of the GJB2 gene was amplified using the three primer sets and PCR conditions described by Mkaour et al. (9). The expected size of the amplification products were

286 bp, 279 bp and respectively 279 bp. The PCR reaction was performed in a final volume of 50 µl, containing 200 ng genomic DNA, 10 pmol each primer, 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTP and 3 U Taq DNA polymerase (Promega).

The non-coding exon 1 was amplified using the primer set described by Ferraris et al. (10). The amplicon includes the region where the splice site mutation c.-23+1 G>A is located. The reaction was performed in 50 µl reactions, containing 200 ng genomic DNA, 10 pmol each primer, 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTP, 3 U Taq DNA polymerase (Promega). The PCR program consisted in an initial denaturation at 95°C for 5 min, five touchdown cycles of denaturation at 94°C for 1 min, annealing for 50 sec at 63°C for the first cycle and 1°C reduction per cycle, extension 1 min at 72°C, and then 40 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 50 sec, and extension at 72°C for 1 min. Final extension was at 72°C for 7 min.

The PCR reactions were performed on the GeneAmp PCR System 9700 thermal cycler (Applied Biosystem). After the PCR products purification (Wizard SV Gel and PCR Clean-up System, Promega), bi-directional DNA sequencing with the PCR primers on CEQ8800 Genetic Analyzer (Beckman Coulter) was performed.

The UCSC Genome Bioinformatics Website was used for the blast homology searches.

Based on the clinical data and patient history, we established that both children were sporadic cases and expressed DFNB1 phenotype. Therefore, we investigated both children for 35delG GJB2 mutation.

The ARMS-PCR detected the presence of one normal and one mutant allele in the male child (heterozygosity for 35delG mutation) and two normal alleles in the female child (Figure 1 - A, B). Since c.35delG mutation is recessive and the boy was heterozygous, and since the girl did not present this mutation, GJB2 gene was sequenced in both children.

DNA sequencing revealed the presence of the c.71G>A (p.W24X) heterozygous mutation in the male child (Figure 1C). We concluded that the child was compound heterozygous for the c.35delG/c.71G>A mutations at DFNB1 locus. GJB2 gene sequencing in his parents established that his mother was healthy carrier for c.35delG mutation and his father was healthy carrier for c.71G>A mutation.

GJB2 gene sequencing in the female child showed the presence of the recessive mutation c.-23+1G>A (originally named IVS1+1G>A) in a homozygous form. This mutation is also located at DFNB1 locus and consists in a G-to-A transition (Figure 1D). Her parents were therefore healthy carriers for the c.-23+1G>A mutation.

Genetic counseling was offered to their parents regarding the appropriate intervention for language development (auditory prosthesis, cochlear implant, speech therapy practice), thus increasing the chances of recovery of hearing in their children. Also, the parents were counseled regarding the recurrence risk in future pregnancies.

Our study revealed that the hearing impairment of both children was caused by mutations in the GJB2 gene. One of children was compound heterozygous for the c.35delG/c.71G>A mutations and the other was homozygous for the c.-23+1G>A mutation.

The c.35delG and c.71G>A (p.W24X) GJB2 recessive mutations mostly lead to profound bilateral hearing loss. c.35delG and c.71G>A are known as truncating mutations, with no functional monomers of connexin 26 present in cells. In the 35delG mutation, one residue from a sequence of six guanines between +30 and +35 positions is deleted, resulting in a frameshift and premature termination of the connexin 26 synthesis at the twelfth amino acid (11, 12). c.71G>A is a nonsense mutation which consists

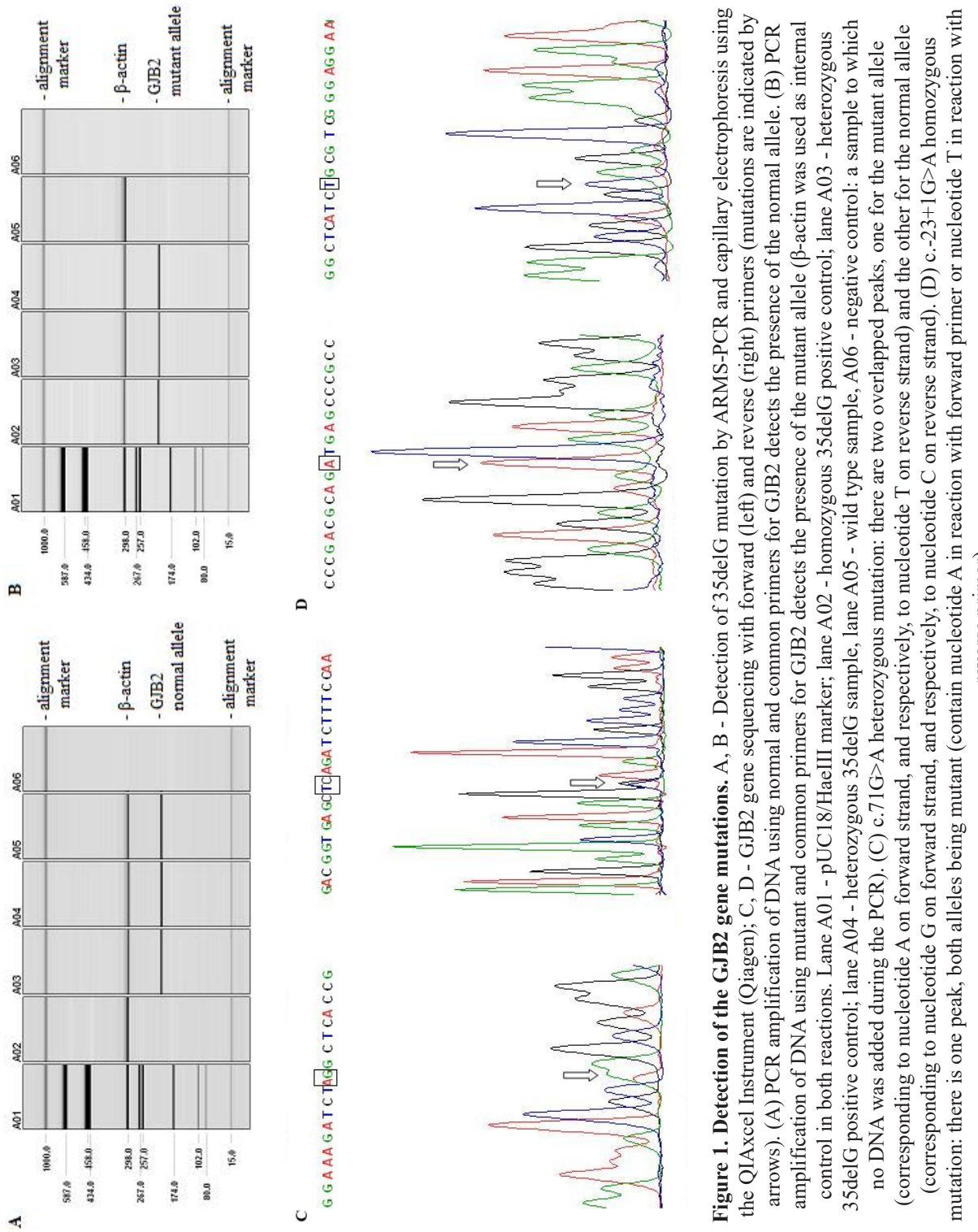

Figure 1. Detection of the GJB2 gene mutations. A, B - Detection of 35delG mutation by ARMS-PCR and capillary electrophoresis using the QIAxcel Instrument (Qiagen); C, D - GJB2 gene sequencing with forward (left) and reverse (right) primers (mutations are indicated by arrows). (A) PCR amplification of DNA using normal and common primers for GJB2 detects the presence of the normal allele. (B) PCR amplification of DNA using mutant and common primers for GJB2 detects the presence of the mutant allele (β-actin was used as internal control in both reactions. Lane A01 - pUC18/HaeIII marker; lane A02 - homozygous 35delG positive control; lane A03 - heterozygous 35delG positive control; lane A04 - heterozygous 35delG sample, lane A05 - wild type sample, A06 - negative control: a sample to which no DNA was added during the PCR). (C) c.71G>A heterozygous mutation: there are two overlapped peaks, one for the mutant allele (corresponding to nucleotide A on forward strand, and respectively, to nucleotide T on reverse strand) and the other for the normal allele (corresponding to nucleotide G on forward strand, and respectively, to nucleotide C on reverse strand). (D) c.-23+1G>A homozygous mutation: there is one peak, both alleles being mutant (contain nucleotide A in reaction with forward primer or nucleotide T in reaction with reverse primer).

in a G-to-A transition at position 71, resulting in a trp24-to-ter substitution (24 amino acids instead 226 amino acids) (12). c.-23+1G>A (originally named IVS1+1G>A) recessive mutation is located in the intron donor splice site and, is predicted to disrupt splicing, yielding no detectable mRNA (13). These aspects are consistent with the phenotypes of our patients.

Mutations in the GJB2 gene cause up to 50% of ARNSHL in Caucasian population and over 90 mutations have been identified. The c.35delG mutation is the most frequent pathogenic variant in Europeans and accounts for about 85% of all GJB2 gene mutations. The c.71G>A and c.-23+1G>A mutations also were found in Caucasian population but at a lower rate (14).

In summary, genetic prenatal and neonatal screening of GJB2 gene could lead to better chances of early diagnosis, taking into account that audiological testing is estimated to show a non-penetrance of 3.8% at birth. (15). In most cases, parents observe their child's disability too late for appropriate intervention (early management with hearing aids or cochlear implants, an early start on special education programs and use of assistive, adaptive, and rehabilitative devices), thereby reducing substantially the chances of recovery, including the development of language and, ultimately, of social integration. Molecular diagnosis could clarify the carrier status of the parents. Genetic counseling should be offered in case of marriages between individuals with GJB2 mutations in their families, as an opportunity for the identification of recurrence risk in future pregnancies.

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Abbreviations

ARMS - Amplification-Refractory Mutation System
ARNSHL - Autosomal Recessive Nonsyndromic Hearing Loss
DFNB1 - Nonsyndromic Hearing Loss and Deafness
GJB 2 - Gap Junction Protein, Beta 2, 26kDa
GJB 6 - Gap Junction Protein, Beta 6, 30kDa
PCR - Polymerase Chain Reaction

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