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ORIGINAL ARTICLE

GJB2 MUTATIONS IN NON SYNDROMIC HEARING LOSS IN THE REPUBLIC OF MACEDONIA

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ABSTRACT

Hearing impairment is a common sensori-neural disorder with the incidence of profound deafness in one per 1,000 births. Non syndromic recessive deafness (NSHL), accounts for approximately 80% of cases of hereditary deafness. It is extremely heterogeneous genetically with over 130 gene loci. Mutations in the *GJB2* and *GJB6* genes for DFNB1 locus (13q12) are responsible for about half of all cases of autosomal recessive prelingual hearing loss.

We determined the prevalence and mutations in the *GJB2* gene, and the presence of delD13S1830 in the *GJB6* gene in DNA samples from 33 unrelated Macedonian families with recesive NSHL and 200 normal hearing individuals using single-strand conformation polymorphism (SSCP) analysis followed by direct sequencing and specific polymerase chain reaction (PCR).

We found mutations in the *GJB2* gene in 12 patients, but no delD13S1830 in the *GJB6* gene. In 22 mutated chromosomes, 15 (68.2%) had the 35delG mutation, four (6.1%) W24X, two (3.0%) V37I and one (1.5%) R127H.

Because of the high mutation rate (36.4%) in the *GJB2* gene in NSHL patients, testing should be performed in all cases with prelingual deafness.

Key words: Deafness, Non syndromic hearing loss (NSHL), GJB2 Gene, Connexin 26, Mutations, 35delG

INTRODUCTION

Hearing impairment is the most common sensori-neural disorder in humans, about 1 in 1000 newborns being affected by severe to profound deafness at birth or during early childhood. In infants, it may have dramatic effects on language acquisition and on social and working lives. Genetic causes account for about half of these cases, the remainder being due to environmental factors [1]. Non syndromic hereditary hearing impairment is extremely heterogeneous genetically. More than 130 different gene loci have been identified [2,3]. These include 54 loci associated with autosomal dominant inheritance (DFNA), 67 loci with autosomal recessive inheritance (DFNB), five loci that are X-chromosome linked and four that occur in mitochondrial DNA. Within these loci, approximately 46 genes have been found to be responsible for hearing loss. They encode a wide variety of protein classes including transcription factors, cytoskeletal and extracellular matrix components, and ion channels.

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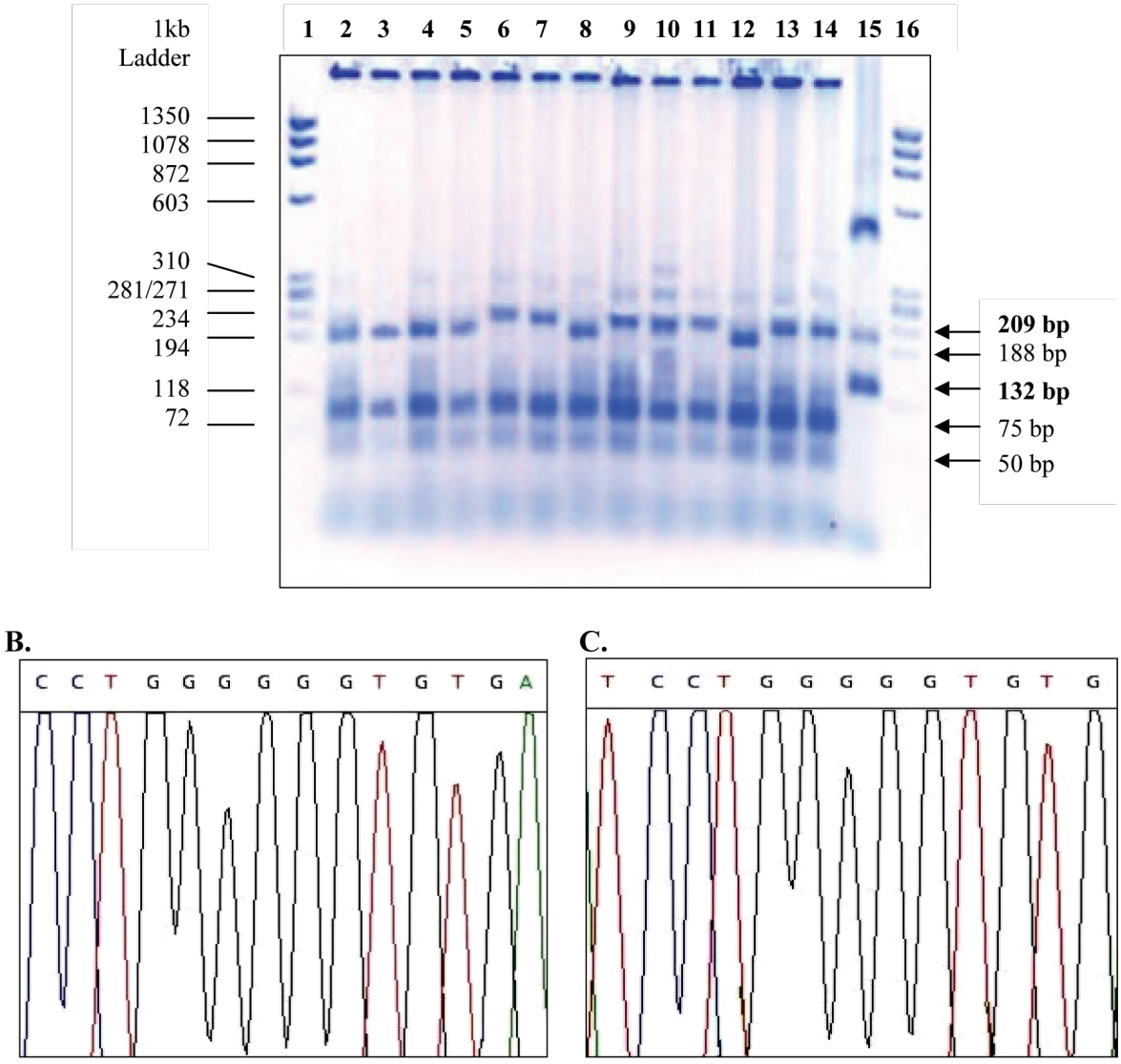

Figure 1. A) BsiYI digestion of a 209 bp PCR fragment containing the region within a stretch of six Gs, and control PCR fragment of 132 bp containing the BsiYI restriction site run on a 2% agarose gel. Lanes 1 and 16: 1 kb ladder; lanes 2, 3, 4, 5, 8 and 12: homozygotes for the 35delG mutation in the GJB2 gene; lanes 6, 7, 9, 10, 11, 13 and 14: patients without the 35delG mutation, lane 15 undigested PCR fragments of 209 and 132 bp. B) Normal; and C) the 35delG sequence of the GJB2 gene.

The gene with most significant impact on the population genetics and genetic counseling is the *GJB2* gene located on chromosome 13q12, mutations in which account for about 50% of all congenital cases of hearing impairment. This gene encodes gap junction protein connexin 26 which is implicated in intracellular communication. A frequent point mutation is 35delG, deletion of the sixth G within a stretch of Gs at position 30-35 [4]. This mutation is frequent in Mediterranean countries, with a carrier frequency of 2.3-4.0%. Over 100 other mutations have been found in the *GJB2* gene, of which 167delT, 235delC and R143W are most common in Ashkenazi Jews, and Japanese and Ghanaian populations [5].

Many patients with autosomal recessive hearing impairment carry only one mutant *GJB2* allele. Some families have clear evidence of linkage to the DFNB1 locus, which contains the *GJB2* and *GJB6* genes, which encode connexin 30 [6]. A 342 kb deletion involving the *GJB6* gene, delD 13S1830, has been found to be very common in non syndromic hearing-impaired patients from Spain, France, Israel, the United Kingdom and Brazil, which also suggests a possible *GJB2/GJB6* digenic pattern of inheritance of deafness [7].

MATERIALS AND METHODS

There are approximately 6,000 persons with hearing loss or hearing impairment in Macedonia. Patients with hearing impairment have been ascertained through two regional otorhinolaryngology centers: Audiology Center, Clinic for Othorinolaryngology, Medical faculty, Skopje and Association of Deaf and Hard of Hearing, Skopje, R. Macedonia. We performed molecular analyses of the *GJB2* gene on DNA samples from 80 individuals with non syndromic hearing loss (NSHL) belonging to 33 affected, unrelated families. Twenty-three families were of Macedonian, six of Albanian, one Turkish and three of Gypsy origin. After informed consent was obtained and examination of complete medical histories was made, the family inheritance and audiological status were ascertained. DNA was isolated from peripheral white blood cells using a phenol/chloroform extraction, ethanol precipitation method [8].

The 35delG mutation was identified by the method described by Storm *et al.* [9], in which polymerase chain reaction (PCR) amplifications were performed with two sets of primers: GJB1: 5'-GTG AGG TTG TGT AAG AGT TG-3'; GJB2: 5'-CTG GTG GAG TGT TTG TTC CCA-3'; and as control sequence: GJB7 5'-CCA GGC TGC AAG AAC GTG TGC-3' and GJB9: 5'-CTC ATG TCT CCG GTA GGC CAC-3' under standard PCR conditions. The PCR products were digested with *Bsi*YI (Boehringer, Mannheim, Germany) and analyzed by 2% agarose gel electrophoresis (Figure 1A).

For other aberrations in the *GJB2* gene, the single strand conformation polymorphism (SSCP) method was used. Amplification of a 286 bp fragment belonging to the 5' end of the exon 2 coding sequence was performed using the following primers: GJB5 5'-TCT TTC CAG AGC AAA CCG C-3' and GJB8 5'-GAC ACG AAG ATC AGC TGC AGG-3', while a 270 bp fragment belonging to the 3' end of the GJB2 gene, was amplified using primers: GJB10 5'-GCA GCA TCT TCT TCC GGG T-3' and GJB6 5'-GGG CAA TGC GTT AAA CTG GC-3'. The SSCP was performed on the Bio-Rad DeCode System (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were loaded onto a non denaturing 12% acrylamide/Bisacrylamide (39:1) gel. After electrophoresis, the PCR fragments were visualized with silver staining of the gel.

To identify the nucleotide substitutions responsible for altered the electrophoretic mobility detected by SSCP analysis, the PCR fragments were sequenced by BigDye sequencing kit v3.1 (PE Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions and separated on an Applied Biosystems 310 Genetic analyzer.

The presence of the deletion delD13S1830 encompassing part of the *GJB6* gene was analyzed by use of three primers to simultaneously amplify the breakpoint-containing fragment and the normal *GJB2* allele [10]. The primers used were: GJB6-1F 5'-AGT GAT CCA TCT GCC TCA GC-3'; GJB6-2RN 5'-GTC TGT GCT CTC TCT TTG ATC TC-3' and GJB6-3RD 5'-GGA AGG TGT GGA TCA CAG TC-3', under cycling conditions of initial denaturation of 10 min. at 95°C, followed by 30 cycles of 1 min. at 94°C, 1 min. at 60°C and 1 min. at 72°C in the Applied Biosystems 2720 thermalcycler. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis. The presence of a 478 bp fragment indicates the presence of the 342 kb deletion (Primers GJB6-1F and GJB6-3RD), while the presence of a 650 bp fragment indicates the normal allele (primers GJB6-1F and GJB6-2RN).

RESULTS AND DISCUSSION

Mutation analysis revealed mutations in the *GJB2* gene in 12 out of 33 (33.4%) of the studied unrelated families (Table 1, Figure 1). Of the 22 mutated chromosomes, 15 (68.2%) carried the 35delG mutation, with homozygosity for 35delG in seven unrelated patients (five Macedonian, one Albanian

Table 1. Mutations in *GJB2* gene found in non syndromic hearing loss patients from R. Macedonia.

Mutation	*n*	Ethnicity
35delG/35delG	7	Macedonian (5); Albanian (1); Turk (1)
35delG/V37I	1	Macedonian
V37I/N	1	Albanian
W24X/W24X	2	Gypsy
R127H/N	1	Gypsy
TOTAL	12	

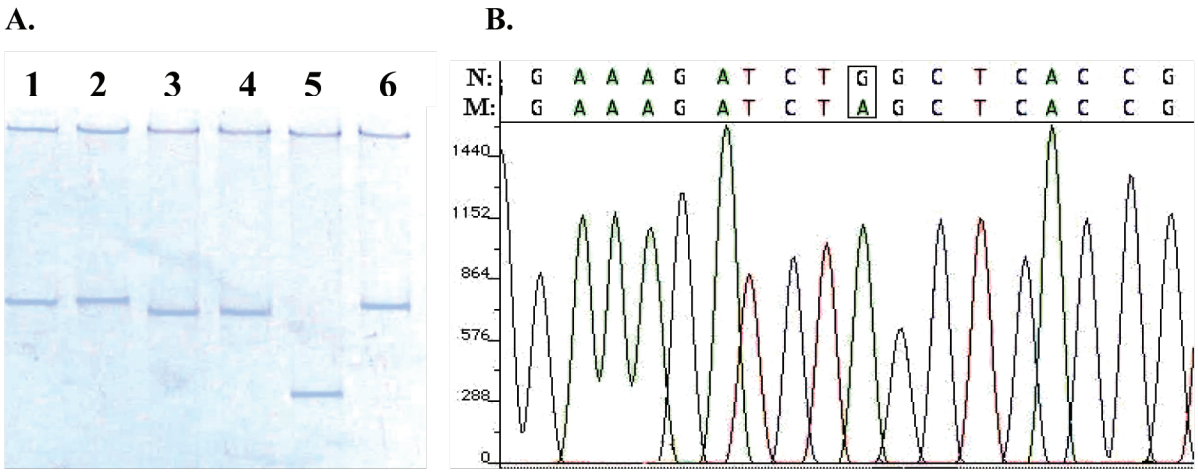

Figure 2. A) The SSCP analysis identifying the 35delG (lanes 3 and 4) and W24X mutation (lane 5) in the GJB2 gene. Lanes 1 and 2 are other hearing loss individuals without mutation in GJB2; lane 6: normal control. B) The sequence analysis determining a homozygosity of TGG> TAG (Trp→Stop) nucleotide substitution at codon 24 of the GJB2 gene in a Gypsy family with NSHL is shown.

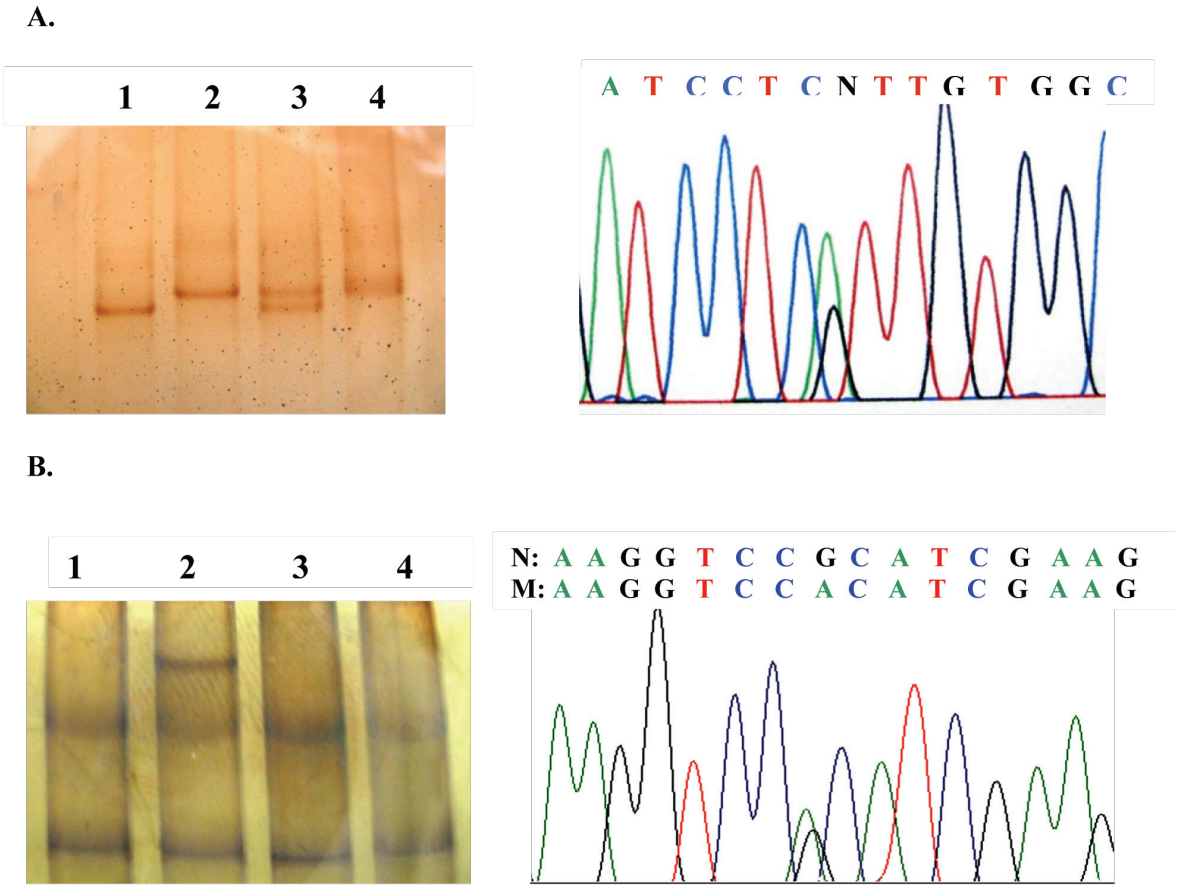

Figure 3. Representative photograph of SSCP analysis and sequencing reactions identifying GJB2 mutations in NSHL patients from R. Macedonia. A) The SSCP analysis identifying: homozygote for 35delG (lane 1); normal control (lane 2); heterozygote for 35delG (lane 3); and heterozygote for Val37Ile missense mutation with sequencing reaction identifying G>A substitution at codon 37 of the GJB2 gene. B) The SSCP analysis and sequencing reaction identifying G>A missense mutation at codon 127 leading to an Arg→His substitution in an NSHL patient of Gypsy origin

Table 2. Frequency of the mutations found in the GJB2 gene in non syndromic hearing loss patients from R. Macedonia.

Mutation	Chromosomes Analyzed	%
35delG	15/66	22.7
V37I	2/66	3.0
W24X	4/66	6.1
R127H	1/66	1.6
TOTAL	22/66	33.4

and one Turk). This indicate that the 35delG mutation is an important cause of NSHL in R. Macedonia. Early diagnosis by identification of the 35delG mutation would greatly improve genetic counseling, treatment and management of deafness in our country. However, we did not detect this deletion in the 200 normally hearing persons we studied, indicating that prevalence of this mutation in our population is lower than reported for other Mediterranean countries [5].

We found a Trp24Stop (W24X) homozygosity in two patients of Gypsy origin, to indicate an overall frequency of 6.1% (Figure 2). Since this nonsense mutation truncates connexin 26 protein, homozygotes have no functional protein in the cells. This mutation was most frequent in India and Pakistan [11], and is a common mutation in Roma/Gypsy patients in Slovakia [12] and Spain [13] as well. This finding is indicative that this mutation was brought by Romanies to Europe from their Indian homeland, but this assumption should be confirmed by DNA polymorphic haplotype analysis.

Mutations Val37Ile (V37I) and Arg127His (R127H), were found at a frequency of 3.0 and 1.6%, respectively (Table 2, Figure 3). Mutation V37I, generally considered as non syndromic causative (http://www.crg.es/deafness/) was found in a compound heterozygous state with 35delG. In the third Gypsy family only the R127H mutation was found. This mutation was the second most common mutation found in Roma/Gypsies from Slovakia [12]. They assume that R127H could be a polymorphism since patients carrying this mutation also had no other GJB2 mutation on the second chromosome. The delD13S1830 mutation in the *GJB6* gene was not found in our group of patients.

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