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MUTATION ANALYSIS OF THE COMMON DEAFNESS GENES IN PATIENTS WITH NONSYNDROMIC HEARING LOSS IN REPUBLIC OF MACEDONIA

МОЛЕКУЛАРНА КАРАКТЕРИЗАЦИЈА НА НАСЛЕДНАТА ГЛУВОСТ КАЈ ПАЦИЕНТИ СО НЕСИНДРОМСКО ОШТЕТУВАЊЕ НА СЛУХОТ ВО РЕПУБЛИКА МАКЕДОНИЈА

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Abstract

Hearing impairment is the most common sensory disorder, which occurs in 1 of 1000 newborns. It is caused by heterogeneous conditions with more than a half due to genetic etiology. Although hundreds of genes are implicated in hearing process and have been found to be associated with nonsyndromic hearing loss, pathogenic variants in *GJB2* gene have been considered as the main cause of deafness among nonsyndromic hearing loss (NSHL) population worldwide. Pathogenic variants in *MT-RNR1* or *mtDNA12SrRNA* gene were also implicated predominantly in postlingual progresive deafness.

The aim of this study was to analyze the implication of *GJB2* and *MT-RNR1* genes in the molecular etiology of deafness among 130 NSHL patients in the Republic of Macedonia. The presence of the del (*GJB6-D13S1830*) was also analysed. We performed SSCP and/or sequence analysis of *GJB2* and identified sequence variants in 62 out of 130 patients (47.7%); (51 homozygous or compound heterozygous and 11 with only one variant allele). We found 8 different allelic variants, the most prevalent being c.35delG (65.49%), and p.W24*(23.01%), followed by other less frequent alleles (p.V27I, p.V37I, p. P175T and cd. delE120 or delGAG at 360). In addition, two polymorphic substitutions in the *GJB2* gene with no clinical significance (p.V153I and p.R127H) were detected. No del(*GJB6-D13S1830*) was found.

SNaPshot analysis was used to screen for the five most frequent allelic variants in the MT-RNR1 gene. Two MT-RNR1 mutations (A827G and T961G) were detected in three patients where only one GJB2 pathogenic variant was found. A new MT-RNR1 gene variant G1303A was also detected.

In conclusion, MT-RNR1 mutations were not a significant contributor to the etiology of deafness in Macedonia, although could be considered as a modifier gene affecting the expression of deafness in patients carrying one GJB2 variant. On the other hand, the high percenttage of *GJB2* pathogenic variants identified among NSHL cases indicates the necessity of molecular newborn screening for the two most common *GJB2* variants (c.35delG and p.W24*) in the Republic of Macedonia.

Keywords: Nonsyndromic hearing loss, *GJB2* gene, pathogenic variants

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Апстракт

Губењето на слухот е најчесто сензорно пореметување, кое се јавува со инциденца од 1 на 1000 новородени деца. Постојат бројни причини за глувоста, но наследните фактори се повеќе го заземаат приматот во нејзината етиологија. Во остварувањето на слушниот процес се инволвирани бројни гени и промени во овие гени, кои водат кон оштетување на слухот. Но, и покрај извонредно хетерогената генетска основа, патолошките варијанти во *GJB2* генот, во светски рамки, се сметаат за главна причина за појава на несиндромска наследна глувост (НСНГ). Патолошки варијанти во *MT-RNR1* генот, исто така, се имплицирани, главно, во постлингвалната прогресивна глувост.

Целта на оваа студија е утврдување на инциденцата и типот на патолошки промени во *GJB2* и *MT-RNR1* гените во молекуларната етиологија на наследна глувост кај 130 лица со НСНГ во Република Македонија. Исто така, е испитувано и присуство на делецијата-del (*GJB6-D13S1830*). Спроведени се анализи на едноверижен конформациски полиморфизам (SSCP) и/или директно секвенционирање на

егзонските секвенци од *GJB2* генот. Утврдени се варијанти во секвенцата на *GJB2* генот кај 62 од испитувани 130 лица или 47.7 проценти. Кај 51 лице се утврдени промени во хомозиготна форма или комбинирана хетерозиготна форма, додека кај 11 пациенти е утврдена промена во *GJB2* генот само на еден алел. Идентификувани се осум различни генски варијанти, од кои најчеста е c.35delG, со застапеност од 65.49%, како и p.W24* со застапеност од 23.01%. Други помалку чести варијанти, утврдени во текот на студијата се: p.V27I, p.V37I, p.P175T и cd.119/120delGAG. Дополнително се утврдени и две полиморфни замени во *GJB2* генот, кои немаат клиничка сигнификантност: p.V153I и p.R127H. Кај ниту еден пациент не е утврдено присуство на делецијата del (*GJB6-D13S1830*).
Воведена е и SNaPshot анализа за утврдување на петте најчести промени во *MT-RNR1* генот. Утврдено е присуство на две *MT-RNR1* патолошки варијанти (A827G and T961G) кај тројца пациенти, каде е откриена само по една патолошка варијанта во *GJB2* генот. Исто така, е утврдено присуство и на нова варијанта G1303A во *MT-RNR1* генот.
Како заклучок-*MT-RNR1* мутациите немаат значителен продонес кон молекуларната етиологија на глувоста во Република Македонија, иако нивното присуство може да се смета за модификатор, кој ја афектира тежината на оштетувањето на слухот кај лицата носители, само на една *GJB2* патолошка варијанта. Од друга страна, високиот процент на застапеност на патолошките варијанти c.35delG и p.W24* во *GJB2* генот кај лицата со НСНГ во нашата земја, укажува на потреба од скринирање за присуство на патолошки варијанти во *GJB2* генот кај сите лица со несиндромско оштетување на слухот, а воедно и воведување неонатална скрининг програма за детекцијата на овие две варијанти.

Клучни зборови: несиндромска наследна глувост, ГЈБ2 ген, патолошки варијанти.

Introduction

Hearing loss is the most prevalent sensory defect. According to WHO 5% of the population or 360 million people are affected worldwide. Both genetic and environmental factors are associated with deafness but the inherited causes are exposed as the most prominent etiological factor in developed countries. Deafness has dramatic effects on language acquisition seriously compromising the quality of life and leads to social isolation [1].
The genetic basis of hearing loss is complex. To date, more than 70 genes (http://hereditaryhearingloss.org) have been associated with hearing loss. At least 70% of all cases are classified as nonsyndromic hearing loss (NSHL) manifested with isolated hearing loss without other associated clinical features. Of all NSHL, 75%-80% are autosomal recessive disorders, 15%-20% are autosomal dominant, 5% are X-linked, and 1% is inherited by mitochondrial genes. [2].
Despite the enormous genetic heterogeneity, pathogenic variants in only one gene, *GJB2*, located at the DFNB1 locus (13q12) are responsible for approximately half of all cases with NSHL [3]. The locus contains three genes, *GJB2*, *GJB6* and *GJA1*, encoding for the transmembrane gap junction proteins connexin 26, connexin 30 and connexin 31, respectively, responsible for creating hexameric hemichannels (connexions) implicated in the maintenance of K^+ homeostasis in the inner ear [4,5]. Other connexin genes *GJB6* and *GJA1* have also been associated with deafness, but more rarely.
Pathogenic variants in *GJB2* gene (OMIM 121011) are the most common causes of sporadic and recessive NSHL, in many populations worldwide. More than 200 different pathogenic variants in this gene (davinci.crg.es/deafness/) have been described with specific prevalence in differrent ethnic groups and geographic regions. c.35delG predominates in Caucasians [6], 176delT in Ashkenazi Jews, 235delC in Japanese, while p.W24X in Indian and p.R142W in African population [7,8].
Due to the high influence of *GJB2* in deafness, molecular testing for *GJB2* pathogenic variants has rapidly become the standard of care for the diagnosis and counselling of patients with nonsyndromic hearing impairment of unknown cause.
Interestingly, in many studies 10% of patients with prelingual nonsyndromic deafness were found to carry a single heterozygous recessive mutation in the *GJB2* gene. Presence of the deletion in DFNB1 locus affecting *GJB6* and the promoter region of *GJB2*, a del(*GJB6-D13S1830*), as a digenic effect provided an explanation for the deafness in as many as 30% of affected *GJB2* heterozygotes in some populations [9]. But, the molecular etiology of nonsyndromic sensorineural hearing loss (SNHL) in subjects with only one detectable autosomal recessive GJB2 pathogenic variant is still unclear. Various studies searching for other modifier genes within DFNB1 or elsewhere have been performed in order to answer this question [10].
Nonsyndromic deafness can be caused by mutations in mitochondrial genes as well. *MT-RNR1* (Mitochondrially Encoded 12S RNA) is an RNA Gene, and is affiliated with the non-coding RNA class. Several mutations in the mitochondrial *MT-RNR1* gene have been found to be responsible for both aminoglycoside-induced and nonsyndromic hearing loss. The most common mutations in 12S rRNA (*MT-RNR1*) gene causing nonsyndromic hearing impairment are: A1555G, T961deT/ insC, T961G, T1005C, T1095C, A1116G, C1494T and A827G. These mutations make the

human mitochondrial ribosome more bacteria-like and alter binding sites for aminoglycosides [11].
Aminoglycoside antibiotics such as gentamycin, streptomycin, kanamycin and tobramycin, are commonly used in the treatment of patients with aerobic Gram-negative bacterial infections. These drugs have well-documented adverse reactions such as ototoxicity and nephrotoxicity. The nephrotoxicity is usually reversible but the ototoxicity, which is most likely due to damage of the sensory hair cells and the stria vascularis in the cochlea, is permanent [12].
In order to determine the molecular etiology of deafness in our country, we have analyzed the type and frequentcy of *GJB2* pathogenic variants among NSHL patients. The prevalence of mitochondrial *MT-RNR1* pathogenic variants was also analyzed using SNaPshot method for the five most common mutations. In addition, complete 12S rRNA sequencing in patients with only one detected *GJB2* mutation was performed in order to examine the modifier effect of the mtDNA pathogenic variants on severity of hearing loss. The presence of del (*GJB6*-D13S1830) was also analyzed.

Materials and methods

This study was conducted on 130 unrelated cases with NSHL of different ethnic origin [Macedonians (75), Albanians (20), Gypsies (31) and Turks (4)]. They were referred to our laboratory by the Audiology Center, University Clinic of Otorhinolaryngology, University Pediatric Clinic or the specialized units for speech rehabilitation in our country where audiologic examination and detailed family history analyses were performed. A total of 120 patients had a moderate-to-profound sensorineural hearing loss, while ten patients progressively lost their ability to hear during early childhood. No other clinical features were detected in the analyzed group.
After obtaining an informed consent from all participants and/or members of their families, peripheral blood was taken and total DNA was extracted using standard phenol-chlorophorm extraction, ethanol precipitation method [13].
Molecular studies included screening for: mutations in *GJB2* gene using Single Strand Conformation Polymorphism analysis (SSCP) and/or direct sequencing, large deletions in chromosome 13p region by Multiplex Ligation Probe Amplification (MLPA) method for and del (*GJB6*-D13S1830) mutation by specific PCR analysis. Single base extension or SNaPShot method was introduced for analysis of five common mutations in mithochondrial DNA connected with inherited deafness.
Amplification of non-coding (exon 1), coding (exon 2) and flanking intronic regions of the *GJB2* gene was conducted by PCR on an ABI2720 thermalcycler (Life Technologies). Oligonucleotide primers 5'- CCGGGAAG-CTCTGAGGAC-3' and 5'-GCAACCGCTCTGGGTCTC-3' were used for amplification of exon 1 [14] while exon 2 was amplified in two different PCR reactions, one for amplification of 286 bp PCR fragment of the 5' end of exon 2 using the GJB5- TCT TTC CAG AGC AAA CCG C and GJB8-GAC ACG AAG ATC AGC TGC AGG primers, and the other for amplification of a 270 bp fragment belonging to the 3' end of the GJB2 gene using GJB10 5'-GCA GCA TCT TCT TCC GGG T-3' and GJB6 5'-GGG CAA TGC GTT AAA CTG GC-3' primers.
The SSCP was performed on BioRad DeCode System (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were loaded onto a nondenaturing 12% acrylamide/Bisacrylamide (39:1) gel. Electrophoresis was performed at constant power of 25W, at 4ºC for about 20 hours. PCR fragments were visualized with silver staining of the gel. To identify the nucleotide substitutions responsible for altered electrophoretic mobility detected by SSCP analysis, the PCR fragments were sequenced by BigDye sequencing kit v.1 (Life Technologies) and separated on an Applied Biosystems 3500 Genetic Analyzer (Life Technologies).
MLPA analysis using SALSA MLPA kit P163-C1 (MRC Holland, The Netherlands) was performed in order to determine the presence of deletions/duplications in the 13q region. This analysis allows detection of the three common GJB2 mutations: c.35delG, splice site mutation IVS1+1G>A or NM_004004.5(GJB2):c.-23+1G>A (HGVS), and 313del14.
The 309-kb del(*GJB6*-D13S1830) or NC_000013.10: g. 20797176_21100550del (HGVS) was also studied by multiplex PCR as previously described [9]. Namely, a set of three primers was used for simultaneous amplification of the normal *GJB6* allele and the allele with the del (*GJB6*-D13S1830) (primer1F: 5' AGT GAT CCA TCT GCC TCA GC; primer 2RN: 5' GTC TGT GCT CTC TTT GAT CTC and primer 3RD 5' GGA AGG TGT GGA TCA CAG TC).
In order to screen our patients for the presence of the five most common mitochondrial mutations associated with deafness (A827G, 961delT+Cn, T1095C, C1494T and A1555G), a SNaPshot method was designed according to Bardien *et al.* [15]. A 1124bp fragment of the MT-RNR gene was amplified using the following primers: MT-RNR-For: CAA CCA AAC CCC AAA GAC AC и MT-RNR-Rev: GCT CAG AGC GGT CAA GTT AAG. The PCR fragment was cleaned up with 1 unit of Illustra ExoProStar 1-Step (GE Healthcare, Life Sciences). The SNaPshot PCR reaction was performed in multiplex format by adding specific primers for each variant in concentration of 1.8 μM each, except A1555G in concentration of 2.9 μM (Table 1). The reaction was performed on a thermal cycler using the following conditions: 25 cycles at 96ºC/10 seconds, 50ºC/30 seconds and 60ºC/30 seconds. The SNaPshot reaction was cleaned

up with one unit of Shrimp alkaline phosphatase for an hour at 37ºC, followed by capillary electrophoresis on 3130 Genetic analyzer (Life Technologies). The representative electrophoregram of the five *MT-RNR1* normal variants is given in Figure 1a.

Table 1. Specific oligonucleotides used for the SNaPshot analysis of the five most common MT-RNR1 deafness causing variants

Variant	Primer sequence 5'-3'	Orientation	Size	Label (N / M)
A1555G	TTG GCA TTT ATA TAG AGG AG	F	20bp	A / G
C1494T	CGT ACA CAC CGC CCG TCA	F	19bp	C / T
T1095C	CTG GGA TTA GAT ACC CCA CTA TGC T	F	25bp	T / C
961delT+C(n)	ACA GGT GAG TTT TAG CTT TAT TGG GG	R	26bp	A / G
A827G	GCT TAG TTA AAC TTT CGT TTG TTG CTA AAG G	R	31bp	T / C

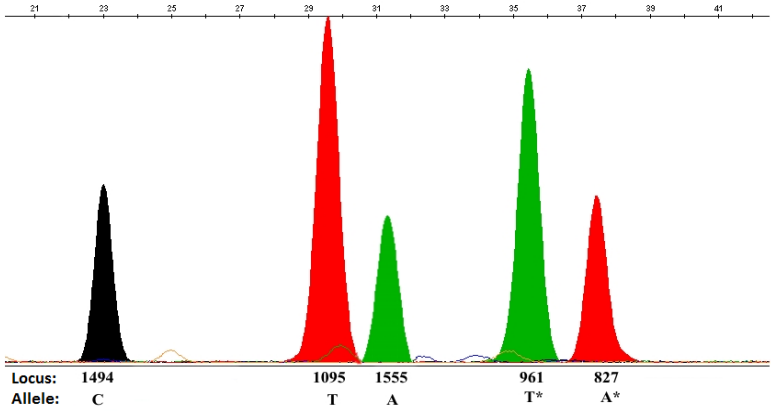

Fig. 1a. Representative electropherogram of the SNaPshot analysis for the five most common mitochondrial DNA mutations (C1494T, T1095C, A1555G, 961delT+C(n) and A827G) causing hearing loss. All shown fragments represent normal allele pattern

The sequencing data of mtDNA *12S rRNA* gene were compared with the Revised Cambridge Reference Sequence ("rCRS") (No. NC_012920) to identify mtDNA variants.

Results and Discussion

In this study we have analysed the type and frequency of *GJB2* variants among 130 unrelated NSHL cases from Macedonia, with different ethnic background. The presence of the five most common *MT-RNR1* pathogenic variants causing hearing loss was also analyzed.

GJB2 pathogenic variants were found in 62 patients (47.7%); 51 were homozygous or compound heterozygous while in 11 only one variant allele was detected (Table 2). We found 8 different allelic variants, the most prevalent being c.35delG (65.49%), found among Caucasians (Macedonian and Albanian patients), followed by p.W24* (23.01%), found only among Gypsy patients (Table 3). Other *GJB2* pathogenic variants: p.V27I, p.V37I, p.P175T and cd.119/120 delGAG were less frequent, found with allelic frequency of 0.88%, 1.77%, 0.88% and 0.88%, respectively. In addition, two polymorphic substitutions in the *GJB2* gene, which do not have clinical significance (p.V153I and p.R127H), were detected with a frequency of 1.77% and 5.31%, respectively. These findings confirm our earlier reported data on the prevalence of the *GJB2* pathogenic variants among the deaf population from the Republic of Macedonia [16]. The pathogenic variant c.-23+1G>A in exon 1, although frequent among Slavic population [17] was not detected in our group of patients.

We found a Trp24Stop (p.W24*) pathogenic variant exclusively among deaf patients of Roma ethnic origin. This is the most frequent *GJB2* pathogenic variant in India [18], and is common among Roma/ Gypsy patients in Spain [19] as well. This finding is indicative that this mutation was brought by Romani people to Europe from their Indian homeland, but this assumption should be confirmed by DNA polymorphic haplotype analysis.

We have identified a high percentage of patients (11 out of 130 or 8.5%) carrying only one *GJB2* pathogenic variant. Detection of only one pathogenic variant in the *GJB2* gene is a common finding. Seeman *et al.* also found that approximately 10% of the analyzed patients carry only one pathogenic variant [20**]**. This indicates the posiblilty of a digenic effect, influence of other modifier genes, or could be a result of an incidental finding of a *GJB2* variant in deafness due to other etiology [21].

Deletions/duplications in DFNB1 locus, in regions that regulate the expresion of the *GJB2* gene, could be also a possible explanation of the severity of hearing loss in GJB2 heterozygous patients. For determination of the possible digenic effect among 11 cases carryng only one *GJB2* pathogenic variant, analysis for the presence of the del (*GJB6*-D13S1830) and/or other deletions in the locus using SALSA MLPA probemix P163-D1 GJB-WFS1, were performed. None of the analyzed patients carried del (*GJB6-D13S1830*), or other deletion in the DFNB1 locus.

Table 2. GJB2 genotypes determined among 130 NSHL patients from different ethnic background in Macedonia

Genotype	Total No.	Ethnicity			
		Macedonian	**Albanian**	**Roma**	**Turks**
c.35delG / c.35delG	33	23	10	/	/
c.35delG / p.V37I	1	1	/	/	/
p.W24* / c.35delG	4	/	/	4	/
p.W24* / p.W24*	10	/	/	10	/
p.W24* / Cd120delGAG	1	/	/	1	/
p.R127H / p.R127H	1	/	/	1	/
p.R127H / p.V153I	1	/	/	1	/
c.35delG / N	3	2	1	/	/
p.W24* / N	1	/	/	1	/
p.V27I / N	1	1	/	/	/
p.V37I / N	1	/	/	/	1
p.P175T / N	1	1	/	/	/
p.R127H / N	3	/	/	3	/
p.V153I / N	1	1	/	/	/
Total	62	29	11	21	1

Table 3. Frequency of pathogenic variants in *GJB2* gene determined among NSHL patients from Macedonia and distribution according to their ethnicity

Variant	Chromosomes with *GJB2* variant		Frequency of GJB2 variants according to ethnical background			
	No.	**%**	**Macedonian**	**Albanian**	**Roma**	**Turks**
c.35delG	74	65.49	32.7%(49/150)	52.5%(21/40)	6.5%(4/62)	/
p.W24*	26	23.01	/	/	42.2%(27/62)	/
c.109G>A, p.V37I	2	1.77	0.7%(1/150)	/	/	12.5%(1/8)
p.R127H	6	5.31	/	/	9.7%(6/62)	/
p.V153I	2	1.77	0.7%(1/150)	/	1.6%(1/62)	/
Cd119/120delGAG	1	0.88	/	/	1.6%(1/62)	/
p.P175T	1	0.88	0.7%(1/150)	/	/	/
c.79G>A, p.V27I	1	0.88	0.7%(1/150)	/	/	/
Total	113	100	32.6%(45/138)	43.3%(13/30)	59.4%(38/64)	12.5%(1/8)

Molecular determination of *GJB2* gene variants is of high clinical significance. Biallelic pathological variants were found only in cases with profound deafness. None of the cases with progressive hearing loss carried pathological variants in *GJB2* gene.

Early diagnosis of deafness by identification of the 35delG or W24* variants would greatly improve genetic counseling, treatment and management of deafness in our country. Determination of a biallelic *GJB2* variant in a patient could allow an early decision for cochlear implantation and start of rehabilitation process earlier in the childhood when the success rate for speach development is higher [22]. Biallelic variants in *GJB2* are associated with variable clinical manifestation of hearing loss, mainly profound deafness, but moderete or mild forms could also be determined. Generally, phenotypic variability has been attributed to unknown modifier genes or environmental factors. On the other hand, the clinically most important point is that these cases always have normal development of vestibular apparatus and they are never accompanied with cochlear defects [23].

Pathogenic variants in mitochondrial DNA are associated potentially with nonsyndromic and aminoglycoside-induced hearing loss. Several nucleotide changes associated with hearing impairment were described, with variable distribution among different ethnic populations [15]. A SNaPshot method for determination of the five most common mitochondrial variants was performed in all 130 analyzed cases. Also, we have performed a systematic and extended sequencing of the mitochondrial *12S rRNA g*ene in 11 hearing loss patients where only one *GJB2* variant was detected. Mitochondrial DNA analysis revealed the presence of two deafness-associated variants, m.827A>G, and m.961T>G (Figure 1b). A sequence variant m.1303G>A was also found in one patient with only one *GJB2* mutation. According to UCSC Genome browser this G1303A variant is a highly

conserved variant in 100 species analyzed. Comparison with the reference sequence NC_ 012920.1 in MitoMap indicated a polymorphic variant. The frequency of this variant in full length sequence is 0.11%.

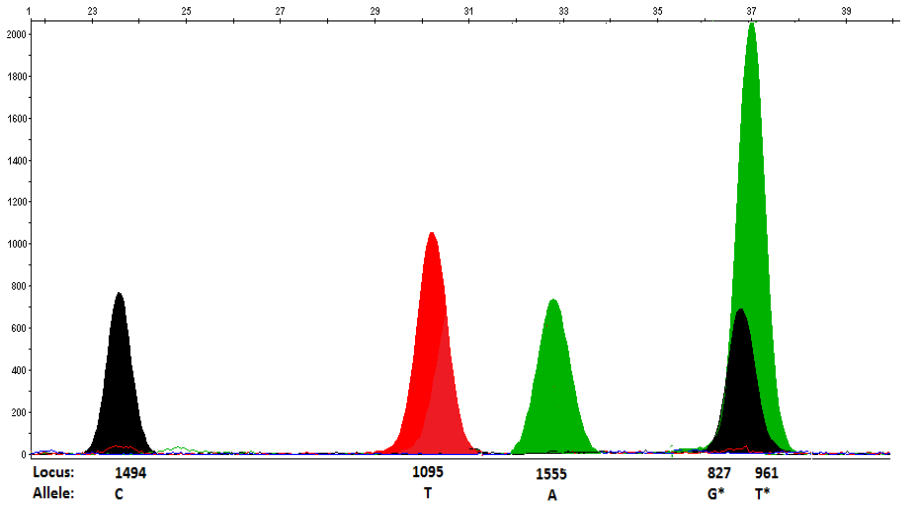

Fig. 1b. Patient with homoplasmic mutation 827A>G

The putative pathogenic mutation at position of 961 (m. 961 T>C) was detected in two cases with profound deafness, both carrying only one *GJB2* variant. One patient had the c.79G>A p.V27I pathogenic variant, while in the other a p.P175T pathogenic variant was determined. In the first case a history of aminoglycoside antibiotics usage during infancy was recorded. The mutation was confirmed by direct sequencing. This mutation was first described by [24] in five patients with distinct sets of mtDNA polymorphisms. Insertion or deletion at this position has been found to be associated with aminoglycoside-induced deafness in several genetically unrealted families as well indicating the pathological effect of nucleotide change at this position.

In conclusion, the high prevalence of c.35delG and p.W24* mutations among our patients (Caucasians and Gypsies, respectively) warrants screening for these two mutations among the deaf population in our country. The introduction of a newborn screening programme should also be considered. Also, our results suggest that mitochondrial DNA mutations do not represent a substantial risk factor for sensorineural deafness in Macedonian population, but mtDNA variants could influence the severity of deafness in cases with only one *GJB2* variant.

Conflict of interest statement. None declared.

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