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Original scientific article

PREVALENCE OF VARIANTS IN DFNB1 LOCUS IN SERBIAN PATIENTS WITH AUTOSOMAL RECESSIVE NON-SYNDROMIC HEARING LOSS

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Hearing impairment is the most common sensorineural disorder in humans and many genes have been identified as causable. Despite genetic heterogeneity, a single locus, DFNB1, that contains genes *GJB2* and *GJB6*, accounts for up to 50% of all cases. Aim of this study was to determine prevalence of identified variants in DFNB1 locus in patients from Serbia with autosomal recessive non-syndromic hearing loss (ARNSHL). In this study, PCR-ARMS and direct sequencing of the *GJB2* and *GJB6* genes was carried out in 54 probands and relatives from Serbia with nonsyndromic hearing loss (NSHL). In 31 patients a series of variants have been identified in the *GJB2* gene. Fully characterized genotype with bi-allelic mutations was observed in 40.74% of the probands (22/54). The remaining probands were either identified in the heterozygote form (9/54) or were identified with no (23/54) causing variants for the tested genes. A total of seven different mutations were found with following allele frequencies: c.35delG (31.48%), c.71G>A (6.48%), c.313_326del (5.56%), c.101T>C (1.85%), c.380G>A (1.85%), c.79G>A (0.92%) and c.269T>C (0.92%). The molecular basis of NSHL in patients from Serbia was analyzed for the first time in this study. The results have important implication to the development of the genetic diagnosis

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of deafness, genetic counseling, and early treatment in our country. Also, our findings contribute to the knowledge of geographic distribution of DFNB1 mutations.

Keywords: autosomal recessive non-syndromic hearing loss, *GJB2* gene, *GJB6* gene, variants

INTRODUCTION

Hearing impairment (HI) is the most common sensorineural disorder and affects more than ~ 70 million people worldwide with an incidence of 1/700-1000 newborns (ZHENG *et al*., 2015). Genetic causes account for at least 50% of all childhood hearing loss, while the remaining incidents are due to environmental and other factors. Such factors include rubella or CMV infection during pregnancy and premature or ototoxic medications (HOEFSLOOT *et al*., 2013). In 30% of genetically caused cases, HI can appear in syndomic form and relates to a variety of other malformations. Therefore, HI with the non-syndromic form, is being seen in 70% of all the rest of the cases (ZHENG *et al*., 2015; HOEFSLOOT *et al*., 2013).

Non-syndromic hearing loss (NSHL) is highly heterogeneous and more than 150 loci and 460 causative genes have been identified by now (The Hereditary Hearing loss Homepage. http://hereditaryhearingloss.org). Despite genetic heterogeneity, mutations in a single locus called DFNB1 (OMIM* 220290) accounts for up to 50% of these patients, all having mild to profound and generally unprogressive autosomal recessive form of the disease (HOEFSLOOT *et al*., 2013; SNOECKX *et al*., 2005). The DFNB1 locus contains the human gap junction β-2 gene (named *GJB2*-OMIM*121011) and the human gap junction β-6 gene (*GJB6*- OMIM*604418), which are related to hearing impairment. These genes encode the proteins being co-expressed in the inner ear, called connexin 26 (*GJB2* gene) and connexin 30 (*GJB6* gene).

To date, more than 150 different variants have been described in the *GJB2* gene (The Connexin- deafness Homepage. http://davinci.crg.es/deafness). In a significant number of patients only one pathogenic mutation in the *GJB2* gene coding region was detected (heterozygous form), therefore the possibility of digenic inheritance remains as a possibility for the development of the disorder. The most frequent mutations are located in the coding sequence of the *GJB2* gene and their frequencies vary among different populations. Such frequent mutations include the c.35delG, c.167delT, c.235delC and c.71G>A (UniGene Hs.524894) (HOEFSLOOT *et al*., 2013; SNOECKX *et al*., 2005; LUCOTTE and MERCIER, 2001; TEKIN *et al*., 2010; BATTELLINO *et al*., 2011; HALL *et al*., 2012).

According to population studies, a relatively frequent mutation is c.-23+1G>A (originally named IVS1+1G>A), that affects the splice site of the non-coding exon 1 of the *GJB2* gene. This variant has been reported as frequent in specific populations of central European origin (HOEFSLOOT *et al*., 2013; SNOECKX *et al*., 2005; TEKIN *et al*., 2010). In addition, two more large partial deletions in the *GJB6* gene were found in some populations in patients with autosomal recessive NHL: del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) (BATTELLINO *et al*., 2011; DEL CASTILLO *et al*., 2005; DEL CASTILLO *et al*., 2002). All three mutations mentioned above are detected in compound heterozygosity with *GJB2*-coding sequence mutations (DEL CASTILLO *et al*., 2005).

Considering the high genetic heterogeneity and ethnic diversity, for better understanding of the molecular basis of the disease it is essential for each population to determine its own frequency of pathogenic variants. The aim of this study was to identify and discuss the type and the frequency

of mutations in the DFNB1 locus, analyzed for the first time in children from Serbia with autosomal recessive bilateral sensorineural hearing impairment.

MATERIAL AND METHODS

Patients

All patients included in this study attended the Unit for Audiology and Neurootology, part of the Department of Otorhinolaryngology in the Mother and Child Health Care Institute of Serbia''Dr Vukan Cupic" (MCHCI). In each patient, history of hearing loss, family history and physical examination was obtained. Absence of developmental delay, growth failure and other characteristic features supported clinical diagnosis of non-syndromic form of hearing loss. Otolaryngological examination was done by pure tone audiometry and all probands were found to have mild to profound range of hearing loss.

From April 2016 to September 2017, 54 patients with non-syndromic bilateral sensorineural hearing impairment corresponding to autosomal recessive pattern of inheritance were analyzed (29 males and 25 females). The medium age of patients was 8.5 years, ranging from 3.5 months to 68 years. A total of 70 available family members of the probands were also examined, bringing the analyzed cases to 124. A genetic counseling session after the completion of the genetic testing was organized to all probands and family members.

The study was approved by Ethical Committee of the MCHCI (number 8/20, dated 29.06.2018.) and a signed informed consent was obtained from all participants/parents.

Mutation analysis

Molecular screening was initially done in the Laboratory of Medical Genetics of the MCHCI. Further screening of the *GJB2* and the *GJB6* genes was performed in the Department of Molecular Genetics, Function and Therapy at the Cyprus Institute of Neurology and Genetics.
Genomic DNA was extracted from peripheral blood samples, using GenJet™ Genomic DNA Purification Kit (ThermoFisher Scientific Inc, USA) as instructed by the manufacturer.

PCR Amplification-Refractory Mutation System (PCR-ARMS) for direct detection of c.35delG variant in the GJB2 gene

The patients were initially screened for the presence of c.35delG mutation, using a PCR-ARMS method (LUCOTTE *et al*., 2001; SCOTT *et al*., 1998). In two separate PCR reactions, three primers, one common reverse (R-Com), one normal forward (F-Nor) and one mutant forward (F-Mut) were used. The produced size of amplicon of the above reaction was 202bp. Two additional primers for amelogenin (size 360bp) were also used as an internal control (GRILLO *et al*., 2015).

Direct sequencing of exon 2 in the GJB2 gene

All patients found negative or identified in heterozygosity for the c.35delG were further screened by direct sequencing of exon 2 of the *GJB2* gene as previously described (NEOCLEOUS *et al*., 2006). Direct sequencing of the entire coding sequence of the *GJB2* gene was performed using BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, USA), according to manufacturer's procedure, on an ABI PRISM® 3130XL Genetic Analyzer (Applied Biosystems,

Foster City, USA). Internal primers were also used for the complete cover-up of the coding region of the *GJB2* gene, as described in NEOCLEOUS *et al*. (2006).

Direct detection of c.-23+1G>A variant in exon 1 of the GJB2 gene

All samples being either heterozygous or negative for *GJB2* mutations were further screened for the presence of the splice mutation c.-23+1G>A, located in exon 1 of the *GJB2* gene as previously described (NEOCLEOUS *et al*., 2014).

Multiplex PCR amplification for del(GJB6-D13S1830) and del(GJB6-D13S1854) mutations in the GJB6 gene

Finally, all patients without identified bi-allelic mutations of the *GJB2* gene were further screened for the presence of two deletions in the *GJB6* gene: 342kb deletion named del (GJB2-D13S1830) and 265kb deletion named del (GJB6-D13S1854). Analyses were performed using multiplex PCR assays, previously described by WU *et al*. (2003) and DEL CASTILLO *et al*. (2005).

RESULTS AND DISCUSSION

Results

Fifty-four patients of Serbian descent with non-syndromic bilateral sensorineural hearing impairment and 70 relatives were screened for the presence of mutations in the *GJB2* gene and the presence of large deletions in the *GJB6* gene. Twenty-two of 54 probands (40.74%) were fully informative as their genotype was determined (being homozygous or compound heterozygous for the disease causing *GJB2* variants) (Table 1). Five variants of various clinical significance were detected in the heterozygous state in 9 probands (16.67%) (Table 1). The remaining 23/54 patients (42.59%) were identified with no mutations in the *GJB2* gene.

Table 1. GJB2 mutation spectrum detected in 31 out of 54 Serbian patients with nonsyndromic hearing loss

GJB2 genotype	N^0 of homozygote or compound heterozygote carriers (n=22)	Percentage (%)
c.[35delG];[c.35delG]	14	45.16
c.[35delG];[c.313_326del]	3	9.68
c.[71G>A];[c.71G>A]	3	9.68
c.[71G>A];[c.313_326del]	1	3.22
c.[313_326del];[c.313_326del]	1	3.22
GJB2 genotype	N^0 of heterozygote carriers (n=9)	Percentage
c.[35delG];[=]*	3	9.68
c.[101T4C];[=]*	2	6.45
c.[380G>A];[=]*	2	6.45
c.[79G>A];[=]*	1	3.22
c.[269T>C];[=]*	1	3.22
Total	31	100

*[=] unidentified responsible mutation in gene for autosomal recessive hearing loss other than DFNB1 locus (*GJB2* and *GJB6* genes)

A total of 10 different genotypes were found in a group of 31 patients that carried either a single or two mutations. As expected c.35delG was the most frequent defect and was observed in homozygosity in 14/31 patients tested (45.16%) (Table 1.).

As depicted in Table 1, the most frequent mutation was found to be c.35delG (p.Gly12Valfs). The c.35delG was identified in 14/20 and in 3/20 tested patients in the homozygous and heterozygous state, respectively. Six other different alterations were also identified in tested subjects and consisted of: c.71G>A (p.Trp24Ter), c.313_326del (p.Lys105Glyfs), c.101T>C (p.Met34Thr), c.380G>A (p.Arg127His), c.79G>A (p.Val27Ile) and c.269T>C (p.Leu90Pro) and their frequencies are presented in Table 2.

None of the patients was identified with the severe splice mutation c.-23+1G>A of non-coding exon 1 in neither the *GJB2* gene, nor the deletions del (GJB2-D13S1830) and del (GJB6-D13S1854) in the *GJB6* gene.

Table 2. Allele frequencies of variants in the GJB2 gene detected in a group of 54 patients (108 alleles) from Serbia

Variant			Nº of mutant alleles	Percentage (%)
DNA level	Protein level	Effect		
c.35delG	p.Gly12Valfs	Pathogenic	34	31.48
c.71G>A	p.Trp24Ter	Pathogenic	7	6.48
c.313_326del	p.Lys105fs	Pathogenic	6	5.56
c.101T>C	p.Met34Thr	Controversial	2	1.85
c.380G>A	p.Arg127His	Controversial	2	1.85
c.79G>A	p.Val27Ile	Polymorphism	1	0.92
c.269T>C	p.Leu90Pro	Pathogenic	1	0.92
Unknown			55	50.92
Total			108	100

As expected, the most frequent variant was c.35delG (31.48%, 34/108 alleles), followed by c.71G>A (6.48%, 7/108 alleles) and c.313_326del (5.56%, 6/108 alleles). A series of four other variants were also identified (Table 1). In total, seven variants mentioned above accounted for nearly half of the mutant alleles (49.08%), while the rest remained uncharacterized.

Genetic testing was also done in 70 parents and close relatives in order to confirm homozygosity/compound heterozygosity. Genetic counseling was recommended to all families at risk, considering possibilities of carrier testing and prenatal diagnosis if wanted.

DISCUSSION

As previously described, mutations in the DFNB1 locus are the most common cause of non-syndromic, prelingual hearing impairment (HOEFSLOOT *et al*., 2013). According to this, genetic testing of the *GJB2* and *GJB6* genes in this locus is the first step of molecular analysis in patients with autosomal recessive NHL. Although a large number of alterations in the *GJB2* gene have been published (with different clinical significance), the distribution and frequency of mutations in this

gene vary among populations (HOEFSLOOT *et al*., 2013; SNOECKX *et al*., 2005; STENSON *et al*., 2003; VIENNAS *et al*., 2017).

The results of this study showed that in analyzed group of 54 patients, 22 were fully informative (40.74%), while 9 patients had mutations in the heterozygous form, which is similar to the worldwide data reported for inherited autosomal recessive NSHL. Unidentified mutations could be located in the regulatory region or in the intronic regions of the *GJB2* and *GJB6* genes, not covered by methods used. Or, in rare cases, causing variant can be located in genes other than the DFNB1 locus, recently reported in various studies (NEOCLEOUS *et al*., 2006).

A total of 7 variants with different clinical significance were identified and accounted for nearly 50% of mutated alleles in analyzed group of patients, with c.35delG being the most frequent (31.48%).

The c.35delG (p.Gly12Valfs) is known as a truncating mutation, resulting in a frameshift and premature protein termination (connexin 26), with the high prevalence in Caucasians, especially in Mediterranean and South Europe (DRAGOMIR *et al*., 2015). This could be explained by the founder effect phenomenon (VIENNAS *et al*., 2017). The overall frequency of 31.48% found in this study was similar with previously published frequencies in our neighboring populations: Croatia, Slovenia, Czech, Hungary, RN Macedonia, Greece, Italy (BATTELLINO *et al*., 2011; MEDICA *et al*., 2005; PAMPANOS *et al*., 2002; TÓTH *et al*., 2004; SEEMAN *et al*., 2005; SUKAROVA STEFANOVSKA *et al*., 2009). In addition, if the frequency of mutant alleles is calculated only in patients with detected bi-allelic mutations, allele frequency of c.35delG mutation was 70.45%, which is in an agreement with data available from studies done in Greece, Italy, Cyprus, Austria, France and Spain (55% up to 95.2%) (NEOCLEOUS *et al*., 2014; PAMPANOS *et al*., 2002; RABIONET *et al*., 2000; JANECKE *et al*., 2002; MARLIN *et al*., 2005).

The second most frequent variant found in this group of patients was c.71G>A (p.Trp24Ter), a common nonsense pathogenic mutation (DRAGOMIR *et al*., 2015). The prevalence of mutant allele was 6.48% (7/108 alleles), similar to data published for different populations in Pakistan, Iran, Bangladesh, India, Slovakia, Spain, RN Macedonia, Romania and Hungary (DRAGOMIR *et al*., 2015; TÓTH *et al*., 2004; SUKAROVA STEFANOVSKA *et al*., 2009). Since this mutation is the most often being identified in Roma families, it is considered that c.71G>A mutation have been introduced into Europe by migration Roma from South Asia (SUKAROVA STEFANOVSKA *et al*., 2009).

Third detected variant was c.313_326del (p.Lys105Glyfs), frameshift pathogenic mutation. It is discovered that this deletion eliminates the main functional domain of connexin 26 (two transmembrane domains-E2 and C-terminal) and are presumed to damage the topology of these polypeptides. In this study c.313_326del had total frequency of 5.56% (6/108 alleles), which is similar to higher frequencies found in some populations of Central and Eastern Europe (Austrian, Polish, Hungarian, German, French) (JANECKE *et al*., 2002; MARLIN *et al*., 2005).

One patient was heterozygote for the c.269T>C (p.Leu90Pro) variant, which affects transmembrane domain of protein and associate as causative mutation in recessive hearing loss (RABIONET *et al*., 2000). The c.269T>C was detected in population of France, while more common incidence was found in populations of East Europe (DRAGOMIR *et al*., 2015; JANECKE *et al*., 2002; MARLIN *et al*., 2005; WISZNIEWSKI *et al*., 2001).

In the analyzed group of patients three controversial variants difficult to interpret were found. The first controversial variant identified in the present study was c.101T>C (p.Met34Thr) and was found in two patients in the heterozygous state. The c.101T>C was first described as an autosomal dominant causing variant of the disease (HOEFSLOOT *et al*., 2013). Various other studies that followed have commented on the observed high carrier frequency of c.101T>C in Caucasians and stated that is probably a hypomorphic recessive allele (HOEFSLOOT *et al*., 2013; SNOECKX *et al*., 2005; SCOTT *et al*., 1998; DE CASTRO *et al*., 2013). Several recent studies reported homozygous/compound heterozygous state, and considered it as a cause of hearing loss; in patients with profound hearing impairment that share c.101T>C , other causes must be also considered (HOEFSLOOT *et al*., 2013; HALL *et al*., 2012; DE CASTRO *et al*., 2013).

In this study, c.380G>A (p.Arg127His) variant, missense mutation with controversial phenotypic effect, was present in two patients in the heterozygous state. Earlier published studies indicate the non-pathogenicity of c.380G>A (RABIONET *et al*., 2000). According to literature data, some of the authors consider this variant as a polymorphism, because functional analyses showed no affection of the function of connexin 26, or because in some populations was detected a high frequency of carriers (DRAGOMIR *et al*., 2015; SUKAROVA STEFANOVSKA *et al*., 2009). Other studies showed that c.380G>A variant affects the function of connexin 26 at different levels of expression (TÓTH *et al*., 2004).

In heterozygosity c.79G>A (p.Val27Ile) variant and was also reported as a common polymorphism in Asian and Brazilian populations (DE CASTRO *et al*., 2013). Various studies reported c.79G>A to be coinherited on the same allele with c.341A>G (p.Glu114Gly) polymorphism (TEKIN *et al*., 2010). A patient of the present study identified with c.79G>A in heterozygosity was not found to coinherit c.341A>G on the same allele.

In this study, we did not detect the splice mutation c-23+1G>A, commonly found in different populations (TEKIN *et al*., 2010; DEL CASTILLO *et al*., 2005; DEL CASTILLO *et al*., 2002; DRAGOMIR *et al*., 2015; SEEMAN and SAKMARYOVA, 2006). Also, none of the two common deletions del (*GJB6*-D13S1830) and del (*GJB6*-D13S1854) in the *GJB6* gene, were found in Serbian patients. The absence of these two most common deletions from the genetic pool of Serbian population is similar to data published from other neighboring populations of countries such as e.g. Slovenia, Austria, Croatia, Cyprus, Poland, Turkey and Italy (BATTELLINO *et al*., 2011; DEL CASTILLO *et al*., 2005; DEL CASTILLO *et al*., 2002; NEOCLEOUS *et al*., 2014; MEDICA *et al*., 2005; SEEMAN *et al*., 2005).

The 50.92% of alleles in analyzed group of Serbian patients remained uncharacterized. Therefore, further analyses using high through-put Next Generation Sequencing will most likely bring into the picture other genes that are involved in NSHL.

For Serbia this is the first study on patients with NSHL and will certainly have an important implication in the understanding of molecular basis of deafness in our country. Additionally, the findings of the present study will allow us to develop a strategy for a more appropriate use of genetic testing, counseling and possible treatment to the current and future patients.

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DETEKCIJA I UČESTALOST VARIJNTI DFNB1 LOKUSA KOD PACIJENATA SA AUTOZOMNO-RECESIVNIM NESINDROMSKIM OŠTEĆENJEM SLUHA IZ SRBIJE

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Izvod

Oštećenje sluha predstavlja najčešće senzorineuralno oboljenje sa mnogo identifikovanih gena kao uzročnika ovog oboljenja. Uprkos genetičkoj varijabilnosti, 50% svih slučajeva ima mutacije identifikovane u DFNB1 lokusu (u kome se nalaze geni GJB2 i GJB6). Cilj ovog rada je bio utvrđivanje učestalosti identifikovanih varijanti DFNB1 lokusa kod pacijenata iz Srbije sa autozomno-recesivnim nesindromskim oštećenjem sluha. Genetičke analize su sprovedene kod 54 pacijenta i njihovih članova porodice pomoću PCR-ARMS metode i sekvenciranja kodirajućeg regiona gena GJB2. Varijante gena GJB2 su identifikovane kod 31 pacijenta. Genotipovi sa bialelskim mutacijama su detektovani kod 40.74% pacijenata (22/54), dok su kod 9 ispitivanih pacijenata (9/54) detektovane uzročne varijante u heterozigotnoj formi. Kod 23 pacijenta (23/54) nisu detektovane varijante ispitivanih gena. U ovoj studiji identifikovano je 7 različitih varijanti sa sledećim učestalostima: c.35delG (31.48%), c.71G>A (6.48%), c.313_326del (5.56%), c.101T>C (1.85%), c.380G>A (1.85%), c.79G>A (0.92%) and c.269T>C (0.92%). U ovom radu je prvi put analizirana molekularna osnova nesindromskog oštećenja sluha kod pacijenata iz Srbije. Rezultati ukazuju na razvoj genetičkog testiranja, genetičkog savetovanja i što ranijeg lečenja ovog oboljenja u našoj zemlji. Takođe, rezultati ove studije doprinose geografskoj distribuciji mutacija DFNB1 lokusa.

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