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SPECTRUM AND FREQUENCY OF THE *GJB2* GENE MUTATIONS AMONG LATVIAN PATIENTS WITH PRELINGUAL NONSYNDROMIC HEARING LOSS

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Mutations in the GJB2 *gene (connexin 26) are the most common cause of congenital nonsyndromic severe-to-profound hearing loss. Sixty-five hearing impaired probands from Latvia were tested for mutations in the* GJB2 *gene to determine the percentage of hearing loss attributed to connexin 26 and the types of mutations in this population. A total of 62% of patients tested had* GJB2 *mutations. Four different mutations in the* GJB2 *gene were identified in Latvian patients with nonsyndromic sensorineural hearing loss: 35delG, 311-324del14, 235delC and M34T. The most prevalent mutation is 35delG (47% of all probands were homozygous and 8% compound heterozygous). Our findings support the conclusion that the 35delG mutation is the most prevalent* GJB2 *mutation and that it is the common cause of hereditary nonsyndromic hearing loss in populations of European descent.*

Key words: *hearing loss,* GJB2*, connexon, gap junction, DFNB1 – deafness autosomal recessive type 1.*

INTRODUCTION

Hearing loss is the most common birth defect and affects about 1 in 500 newborns (Morton and Nance, 2006). It is the most frequent sensory defect and has significant effect on life quality. Hearing is critical to the normal development and acquisition of language. Prelingual hearing loss is either present at birth or begins before the age of five years, when language has normally been acquired. Hearing loss is a highly heterogeneous disease with many diverse causes. In developed countries about 60% of the prelingual cases are of genetic origin and 40% are due to environmental causes (prenatal infections, prematurity, bacterial meningitis, ototoxic drugs are the most frequent ones of them) (Petit *et al*., 2001). Hearing impairment can be classified according to the degree of severity of the hearing loss for the better hearing ear: mild (hearing level is 26–40 dB), moderate (41–55 dB), moderately severe (56–70 dB), severe (71–90 dB) and profound (≥90 dB) (Smith and Van Camp, 2008). The various forms of deafness are divided in two categories, syndromic and nonsyndromic (isolated). Syndromic deafness contributes to about 30% of the cases of prelingual deafness. Syndromic disorders are those where hearing impairment is not the only clinical feature. More than 300 different syndromes (Alport, Pendred, Treacher Collins, Usher, Waardenburg, etc.), where hearing loss is one of the clinical manifestations, have been identified (Petit *et al*., 2001; Anonymous, 2009).

The majority of hereditary deafness cases (~70%) are nonsyndromic, where hearing deficit is the only clinical sign. Up to date, more than 140 nonsyndromic deafness loci have been reported (Van Camp and Smith, 2009). Nonsyndromic hearing loss is categorised by the mode of inheritance. Approximately, 75% of non-syndromic hearing loss cases have an autosomal recessive mode of inheritance (DFNB loci), ~20% autosomal dominant (DFNA loci), 2% X-linked (DFNX loci), and 1% to modifier (DFNM loci), Y-linked (DFNY loci) and mitochondrial inheritance each (Feldmann *et al*., 2004, Van Camp and Smith, 2009). The first locus for an autosomal recessive form of deafness DFNB1 (MIM 220290) was mapped to chromosomal region 13q12-13 (Guilford *et al*., 1994).

Presently, 26 genes responsible for autosomal recessive hearing loss have been identified, but most of them are causative in only a small percentage of patients. However, one particular gene *GJB2*, which corresponds to DFNB1 locus, was found to be responsible for up to 50% of the cases with autosomal recessive nonsyndromic hearing loss. Gene

GJB2 encodes connexion 26, a gap junction protein β-2. Connexin 26 is one member of a family of more than 20 related gap junction channel (connexons) forming proteins. All connexins have a common structure with four transmembrane domains, two extracellular loops, one intracellular loop and C- and N-terminal ends (Fig. 1) (Petit *et al*., 2001; Anonymous, 2009). Most cell types express more than one connexin species, which can form homomeric or heteromeric connexons. Intercellular channels in the auditory system are formed predominantly by connexion 26 but also by connexins 30 and 31. Connexin 26 has an important role in maintaining a high extracellular electrical potential in the cochlea by recycling of K^+ ions back to endolymph following depolarization hair cells (Falk and Gilula, 1998; Petit *et al*., 2001).

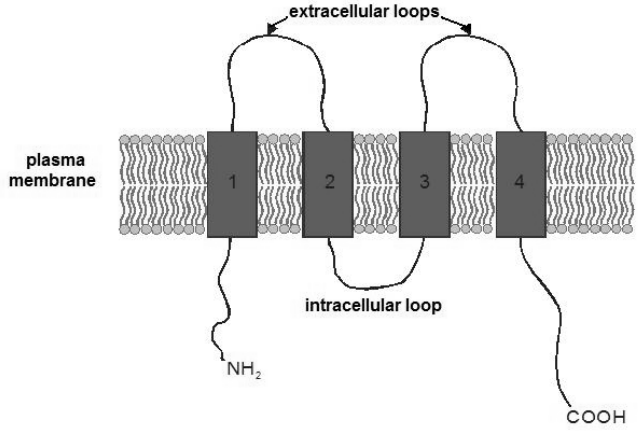

Fig. 1. Representation of the structure of connexins. Numbers 1 to 4 mark transmembrane domains.

Since the *GJB2* gene was discovered in 1997 (Kelsell *et al*., 1997), more than 110 mutations have been identified (mostly missense and frameshift in-dels). The majority (92) of all *GJB2* mutations are recessive, and the nature of ten mutations still remains unknown (Ballana *et al*., 2009). Although most mutations are not very frequent, one recessive mutation named 35delG has been found in more than 50% patients of Caucasian origin (Gasparini *et al*., 2000). This mutation deletes a guanine residue (G) in a sequence of six G extending from nucleotide position 30 to 35 which results in a premature stop codon at codon position 13 (Petit *et al*., 2001). The frequency of mutation 35delG among nonsyndromic hearing loss patients in Croatia, Italy, Spain, Greece, Turkey, France and Austria varies between 51.8% and 93% (Denoyelle *et al*., 1997; Estivill *et al*., 1998; Antoniadi *et al*., 2000; Baris *et al*., 2001; Frei *et al*., 2002; Pampanos *et al*., 2002; Roux *et al*., 2004; Sansovic *et al*., 2005). In Estonia, the mutation 35delG accounts up to 45% of prelingual nonsyndromic hearing loss cases (Teek *et al*., 2006). Mutation 35delG is responsible for 10% of all childhood hearing impairments and for 20% of hereditary hearing loss (if all environmental causes are excluded) (Kelley *et al*., 1998).

Carrier frequencies in Europe range from 0.5–4.5%, with the highest frequencies found in Estonia (Gasparini *et al*., 2000).

Two other mutations are particularly frequent in specific populations. The 167delT mutation is most frequent in Ashkenazi Jewish hearing impaired patients. This mutation deletes a thymine (T) residue in nucleotide position 167, which results in reading frame shifting and premature stop codon at codon position 56. The carrier frequency of this mutation among Ashkenazi Jews is reported to be 4% (Morell *et al*., 1998).

The mutation 235delC is common among hearing loss patients in Japan, Korea and China. This mutation deletes a cytosine (C) residue in a nucleotide position 235, which results in a reading frame shifting and premature stop codon at codon position 78. The carrier frequency of this mutation in China and Taiwan is estimated at l–2.8 % (Liu *et al*., 2002, Hwa *et al*., 2003). In European populations this mutation is rare.

The phenotype of the *GJB2* induced hearing impairment is variable even for the members of one family (Smith and Van Camp 2008). The degree of severity of deafness associated with *GJB2* defect varies from mild to profound (Petit *et al*., 2001).

Lerer *et al.* (2001) reported a large deletion, named del(*GJB6*-D13S1830), in the DFNB1 locus that truncates gene *GJB6* encoding connexin30. It was suggested that this recessive mutation caused hearing loss in individuals that are double heterozygous for the deletion and mutation in the *GJB2* gene, either due to a digenic mode of inheritance or because the deletion abolishes control elements that are important in the expression of *GJB2* since both genes are close to each other on chromosome 13q12 (Castillo *et al*., 2002). In 2005, another *GJB6* truncating deletion del(*GJB6*-D13S1854) was reported (Fig. 2) (Castillo *et al*., 2005). The aim of our paper was to find spectrum and frequency of the *GJB2* gene mutations in patients with prelingual nonsyndromic hearing loss in Latvia.

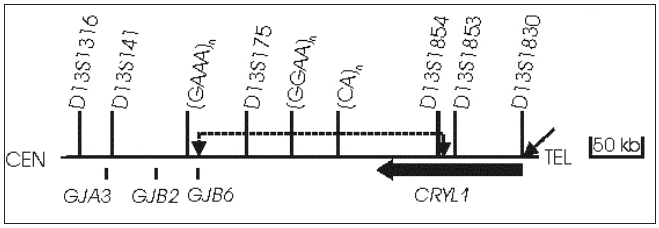

Fig. 2. Map of a 600 kb DNA segment on chromosome region 13q12 including the DFNB1 locus that contains genes *GJB2* and *GJB6*. Vertical bars above the map indicate the positions of polymorphic genetic markers. Genes in the region are depicted as vertical bars and arrow below the map. The two breakpoints of the del(*GJB6*-D13S1854) mutation are marked by vertical arrows, and the extent of the deletion is indicated by a dashed line. The single arrow indicates the distal breakpoint of the del(*GJB6*-D13S1830) mutation.

MATERIALS AND METHODS

We obtained DNA samples from Latvian patients with prelingual moderately severe to profound sensorineural hearing loss in whom syndromic forms and environmental

causes of deafness had been excluded; their relatives and individuals with hearing loss positive family history. Blood samples were obtained after obtaining written informed consent in accordance with regulations of the Central Medical Ethics Committee.

Genomic DNA was extracted by standard procedure from peripheral blood leucocytes.

Mutation 35delG was tested in all samples by a method previously described (Simsek *et al*., 2001). Genomic DNA was amplified by PCR, following enzymatic digestion with restriction endonuclease *Mva*I. The wild-type 89 bp fragment yielded two digested fragments of 80 and 29 bp. A mutation eliminates the *Mva*I site, thus the mutant 88 bp fragment remains uncut.

Samples with no or heterozygous mutation 35delG were screened for other mutations in the *GJB2* gene exon 2 by the automated sequencing method (Wu *et al*., 2002). The open reading frame of the *GJB2* gene was amplified from genomic DNA. Prior to cycle sequencing, PCR products were purified on Montage™ columns following the manufacturer's instructions ("Millipore", USA). The amplicons were sequenced using two partially overlapping sets of primers: first set from the initial amplification of whole open reading frame and second internal primers. Sequencing was performed using ABI PRISM BigDye Terminator Cycle Sequencing kit v.3.1 on an ABI310 Genetic Analyzer (Applied Biosystems, USA).

Samples that had only one detected mutation in *GJB2* had been tested for del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) using multiplex PCR assay (del Castillo *et al*., 2005). Genomic DNA was amplified using three sets of primers for the del(*GJB6*-D13S1830) breakpoint junction fragment, the del(*GJB6*-D13S1854) breakpoint junction fragment and for the fragment of *GJB6* exon 1 (to obtain a product in case of no deletion occurred). In case of wild type allele only 333 bp *GJB6* exon 1 fragment is amplified, del(*GJB6*-D13S1830) yielded 460 bp junction fragment and del(*GJB6*-D13S1854) yielded 564 bp junction fragment.

RESULTS

A total of 65 unrelated patients from Latvia who had bilateral moderately severe to profound prelingual nonsyndromic hearing loss, 79 of their relatives and seven individuals with a hearing loss positive family history were screened for the mutation 35delG in *GJB2*. For those probands who were found to be heterozygous or normal for mutation 35delG, the coding region of the gene was sequenced and examined.

Among the *GJB2* mutations in 65 probands (total of 130 chromosomes) we identified 77 mutated alleles.

Forty of the 65 probands (62%) carried mutations in *GJB2*, including 31 homozygotes for mutation 35delG (47%), four compound heterozygotes for mutations 35delG and 311-324del14 (6%), one compound heterozygote for mutations 35delG and 235delC (2%), one compound heterozygote for mutations 35delG and M34T (2%), two heterozygotes for mutation M34T (3%) and one heterozygote for mutation 35delG (2%).

Two causative *GJB2* mutations were found in 37 patients (56%), 25 patients had no *GJB2* mutations (38%), three patients were heterozygous for one *GJB2* mutation (6%) and the cause of impairment remained unclear.

Patients with only one *GJB2* mutation identified were tested for two deletions that truncate gene *GJB6*: del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854). No deletions were found.

Four of five affected relatives of the screened probands were found to be 35delG homozygotes, one case was detected prenatally. One affected relative is 35delG and 311-324del14 compound heterozygote. Forty-one from 74 unaffected relatives of the patients were determined to be 35delG carriers, two were 235delC carriers and one has a311-324del14 mutation. Thirty unaffected relatives had no mutations in the *GJB2* gene.

Seven unaffected individuals with a positive family history were tested for mutations in *GJB2*. Two of them were found to be *GJB2* mutation carriers: one for mutation 35delG and one for mutation 51del12insA.

Four different mutations in the *GJB2* gene were identified in Latvian patients with nonsyndromic sensorineural hearing loss: 35delG, 311-324del14, 235delC and M34T. One heterozygous 51del12insA mutation was identified in an unaffected individual with positive family history (Table 1).

All probands with biallelic truncating mutations in *GJB2* have severe or profound hearing loss, and the proband whose genotype was 35delG/M34T had moderate hearing loss. Probands with no or one *GJB2* mutation had moderate to profound hearing loss.

Table 1

FREQUENCIES AND CHARACTERISATION OF MUTATIONS FOUND IN GENE *GJB2*

Mutation name	Codon number	Nucleotide change	Mutation character	Frequency among patients
35delG	10	ATCCTGGGGGgTG	frameshift, nonsense	53%
311-324del14	103	AGAggaagttcatcaagGGGGA	frameshift, nonsense	3%
235delC	77	ATGGGCcCTGC	frameshift, nonsense	1%
M34T	34	ATG → ACG	missense	2%
51del12insA	17	TC(del)caccagcattgg(ins)aAA	frameshift, nonsense	–

DISCUSSION

The aim of this study was to determine the spectrum and frequency of the *GJB2* gene mutations among Latvian patients with prelingual nonsyndromic hearing loss. Our study included patients with moderately severe to profound hearing loss.

Studies in many countries have shown that mutation in the *GJB2* gene is the major cause of the nonsyndromic prelingual hearing loss. About 60% of deafness is due to genetic factors and ~ 20% of all hereditary cases are caused by mutations in the *GJB2* gene. By the end of 2008, there were 6,000 hearing loss patients registered at the Centre for Hearing Impaired Children of Latvia. *GJB2* mutations might be the cause of impairment for about 1,200 of them. Unfortunately, there is a high level of resistance from patients and their relatives to participate in genetic studies. The consanguineous marriage is frequent among the people with hearing loss; they become a part of the so-called "deaf culture and think that their culture will be threatened by genetic studies. That is why our study group was rather small, which hampered a statistical analysis of the acquired data.

All patients were clinically classified by a referring physician. The age of the hearing loss diagnosis established varied from four months to three years. Sixty-five prelingual nonsyndromic hearing loss patients referred by Centre for Hearing Impaired Children of Latvia were tested to determine whether the *GJB2* gene mutation was responsible for their disease.

Five different *GJB2* genotypes were identified:

- 31 patients (47%) had genotype 35delG/35delG

- 4 patients (6%) had genotype 35delG/311-324del14

- 1 patient (2%) had genotype 35delG/235delC

- 1 patient (2%) had genotype 35delG/M34T

- 2 patients (3%) havd genotype M34T/N or X

- 1 patient (2%) had genotype 35delG/N or X

- 25 patients (38%) had no mutations in the *GJB2* gene.

Our data shows that the *GJB2* mutations are a significant cause of nonsyndromic hearing loss in Latvia (62% of patients have *GJB2* mutations). The most frequent *GJB2* mutation is 35delG, of which 47% represented homozygous and 10% compound heterozygous cases. This is consistent with previous studies about the incidence and frequencies of the 35delG mutation (53.8–93 %) in nonsyndromic hearing loss patients in European and American populations (Denoyelle *et al*., 1997; Estivill *et al*., 1998; Cohn *et al*., 1999; Green *et al*., 1999; Antoniadi *et al*., 2000; Baris *et al*., 2001; Frei *et al*., 2002; Orzan *et al*., 2002; Roux *et al*., 2004; Sansovic *et al*., 2005).

All patients with biallelic deletions that truncate the synthesis of polypeptide and inactivate connexin 26 (genotypes 35delG/35delG, 35delG/235delC, 35delG/311-324del14) have bilateral prelingual stable nonsyndromic hearing loss; the degree of severity varies from severe to profound. Our results are consistent with previously reported data (Snoeckx *et al*., 2005). Five patients had delayed speech development that maybe due to late diagnosis. One patient with genotype 35delG/35delG had a vestibular disorder. Previous clinical study about phenotype correlation with mutations in *GJB2* identified that there is no associations between these mutations and vestibular defects (Cohn and Kelley 2000), which indicated another cause for this impairment.

One proband with a genotype of 35delG/M34T had moderate hearing loss. Mutation M34T is a missense mutation, it does not inactivate connexin 26, but reduces its activity. Current studies show that M34T causes a milder form of nonsyndromic recessive hearing loss and that it is not a common cause of severe-to-profound hearing loss (Snoeckx *et al*., 2005).

Patients with no or one *GJB2* mutation identified have moderate to profound hearing loss.

This study has direct clinical implication for the genetic counselling of hearing impaired patients. The challenge that remains is to identify the cause of the disease for 28 individuals screened who had one or no *GJB2* mutations. In our study we followed the testing strategy available at OMIM, where the first step is to detect mutations in the exon 2 of *GJB2*, and in case of only one *GJB2* mutation found, the second step is to detect *GJB6* deletions, del(*GJB6-D13S1830*) and del(*GJB6*-D13S1854).

Testing for DFNB1 mutations is the first step in identifying the cause of prelingual nonsyndromic hearing loss. A positive result for biallelic DFNB1 mutations in the DFNB1 should eliminate the need for expensive tests such as electroretinography and others, therefore, significantly reducing medical costs. Genotype data allow geneticists, physicians and audiologists to counsel affected individuals and their relatives more appropriately and to predict the risk to the future generations. A molecular diagnostic facility for DFNB1 mutation screening made prenatal testing available.

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GĒNA *GJB2* MUTĀCIJU SPEKTRS UN BIEŽUMS LATVIJAS PACIENTIEM AR PRELINGVĀLO NESINDROMĀLO VĀJDZIRDĪBU

Gēna *GJB2* mutācijas ir visbiežākais iemesls nesindromāliem sensoneirāliem dzirdes traucējumiem dažādās populācijās. Pētījumā tika veiktas analīzes 65 Latvijas Dzirdes centra pacientiem ar nesindromālo vājdzirdību, ar mērķi noteikt mutācijas gēnā *GJB2* un noskaidrot šo mutāciju spektru un sastopamības biežumu. 62% izmeklēto pacientu tika atrastas mutācijas gēnā *GJB2*. Latvijas pacientiem ar nesindromālo vājdzirdību ir atrastas četras mutācijas: 35delG, 311-324del14, 235delC un M34T. Biežākā gēna *GJB2* mutācija Latvijas pacientiem ar nesindromālo vājdzirdību ir 35delG (47% ir homozigoti un 8% ir kompaunda heterozigoti). Mūsu dati sakrīt ar citu pētījumu rezultātiem, ka mutācija 35delG ir biežākā mutācija *GJB2* gēnā un ir visizplatītākais vājdzirdības iemesls Eiropas populācijās.