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ORIGINAL STUDY

Connexin 26 (GJB2) and connexin 30 del(GJB6-D13S1830) mutations in Slovenians with prelingual non-syndromic deafness

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Objective: To analyze the frequencies and clinical significance of connexin 26 (GJB2) mutations and connexin 30 (GJB6) del(GJB6-D13S1830) mutation in congenital deaf patients in Slovenia.

Materials and Methods: The frequency of the mutations in the connexin 26 gene and the frequency of del(GJB6-D13S1830) mutation in the connexin 30 gene were determined in a cohort of 218 deaf patients referred for evaluation in a tertiary referral university hospital.

Results: Among 218 congenital deaf patients 58 (26.6%) of them had mutations on both alleles of the GJB2 gene, with c.35delG being the most common. As in other neighboring countries we have not found the del(GJB6-D13S1830) mutation in our sample.

Conclusion: The c.35delG mutation in the GJB2 gene was the most common genetic cause of hearing loss in Slovenia. Homozygous c.35delG mutations (21.1%) and compound heterozygotes (4.55%) were established among Slovene patients with congenital hearing loss. As in other neighboring populations, none of the Slovenian patients carried the del(GJB6-D13S1830) mutation.

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Introduction

Hearing loss is the most common sensory deficit in humans, and more than 60% of the cases have genetic background[1-5]. About 70% of genetic deafness is nonsyndromic and in 80% of these cases the mode of transmission is autosomal recessive[1,6,7]. Genetically caused deafness, congenital and later developed, is highly heterogeneous[2,5]. One in 2000 newborn children has genetic hearing impairment[2], and later in life 0.32% of adults will develop genetically caused hearing loss[8]. The number of genes involved in hearing loss (DFNB genes) has been estimated between 36 and 102[6]. So far, 30 genes involved in recessive inherited deafness (DFNB 1-30) have been mapped on human chromosomes (Hereditary hearing loss homepage, http://www.dnalab-www.uia.ac.be/dnalah/hhh/). Mutations in the GJB2 gene encoding gap junction protein connexin 26 (Cx26) at locus DFNB1 on chromosome 13q are responsible for as much as 50% of pre-lingual recessive deafness. The most common reason for genetic cause for deafness in European and Mediterranean population is the deletion of a single guanine nucleotide in a series of six guanines (c.35delG) in the GJB2 gene[9-11]. This mutation may account for 70% of mutant GJB2 alleles[10]. There is also a high prevalence of the c.35delG mutation in healthy population[12]. There are some differences among various populations, notably a lower carrier frequency in northern Europeans compared to southern Europeans[13]. Connexin related hearing loss can range from medium to profound[14].

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Finding of a large number of affected subjects with only one GJB2 mutant allele complicates the molecular diagnostics of the DFNB1 deafness. It has been shown that a deletion del(GJB6-D13S1830) that truncates the neighboring GJB6 gene (encoding for connexin 30, Cx30), might accompany a mutation in a single GJB2 allele in some populations[15,16]. Both GJB2 and GJB6 are found in the same DFNB1 locus and both proteins are co-expressed in the inner ear. The second mutation truncating GJB6 gene, del(GJB6-D13S1854) was found in Spain, Italy, United Kingdom and Brazil[15,16].

There are no reports on frequencies of mutations in the GJB2 and GJB6 genes in the hearing impaired in Slovenia. The aim of the present study was to analyze the frequencies of the connexin 26 (GJB2) mutations and connexin 30 (GJB6) del(GJB6-D13S1830) mutation in a cohort of 218 patients with congenital deafness in Slovenia.

Materials and Methods

Patients

Patients were recruited from the Ljubljana Cochlear implant program and the National newborn hearing Screening Program at University Medical Centre Ljubljana, Department of Otorhinolaringology and cervicofacial surgery, and from Clinical Institute of Medical Genetics, Department of Gynecology and Obstetrics, from February 2003 to March 2010. The protocol of this prospective study was approved by the national Medical ethics committee, No. of approval: 34/04/07, and informed consent was obtained from all subjects and/or parents of minors.

In each patient, a complete medical and family history was obtained to determine the age at onset of deafness and to exclude the possibility of environmental causes, such as intrauterine infection, perinatal complications[17], meningitis, mumps, prenatal or postnatal ototoxic drug exposure, other inflammations, metabolic, vascular disorders and cranial and acoustic trauma. The deaf subjects underwent an otolaryngological examination with a systematic search for signs of a syndromic form of deafness. Infants underwent ophthalmologic and neurologic examination. Babies underwent transitory otoacoustic emission tests (TOAE), behavioral pure tone audiometry, auditory brain stem responses (ABR), electrocochleorgaphy (EcochG) and electrically evoked auditory bran-stem responses (EABR). Children older than 3,5 years and adults underwent pure tone audiometry with a diagnostic audiometer in a sound proof room. Before pure tone audiometry tympanometry was performed. Air-conduction pure tone average thresholds in the conversational frequencies (0.25, - 8 kHz) were measured and bone conduction (0,25-4 KHz) were measured. EcochG was performed in majority of cases. The morphological causes of deafness were excluded with imaging of the temporal bone: high resolution computer tomography was done supplemented with magnetic resonance imaging in all patients.

Mutation analysis of the GJB2 and GJB6 genes

Genomic DNA was extracted from peripheral blood with the FlexiGene DNA kit (Qiagen, Hilden, Germany). The coding exon for connexin 26 was amplified with two pairs of primers that produced two overlapping PCR products. Four primers used were Cx26F1 (5'-tgtgtgcattcgtcttctcc-3'), Cx26R2 (5'-cctcatccctctcatgctgt-3') chosen with Primer3Input0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and Cx26F2 (5'-ccaggctgcaagaacgtgtg-3'), Cx26R1 (5'-gacacgaagatcagctgcag-3') previously described[18].

PCR products were purified with QIA quick PCR purification kit (Qiagen, Hilden, Germany) and directly sequenced with BDTv1.1 sequencing kit and ABI PRISM 310 automated sequencer (Applied Biosystems, Norwalk, USA). Nucleotide sequences were compared to the sequence of the normal gene (Gene Bank Access No AY280971).

Screening for del(GJB2-D13S1830) mutation was performed by previously described PCR assay amplifying break-point junction[15].

Results

Phenotypic analysis

Among 218 congenitally deaf patients included into the study 133 (61%) were females. The medium age of the patients was 22 years, ranging from 6 months to seventy-two years. Among them, 81 patients were younger than 3.5 years. The exclusion criteria were presence of intrauterine and perinatal infections, perinatal jaundice, use of ototoxic drugs, meningitis and trauma and congenital syndromic deafness. In infants the deafness was confirmed by TAOE,

behavioral audiometry, negative ABR at 105 dBnHL and negative EcochG at 105 dB. In patients older than 3.5 years tympanometry was normal, air-bone gap was less then 5dB and the air conductive values on pure tone audiograms measurements were lower than 90 dB in speech frequencies. EcochG was negative in all cases performed. Computer tomography imaging of the temporal bones were done in all patients and in twenty-six patients the magnetic resonance imaging was additionally performed. No morphological changes in the inner ear were detected in any of the patients included in this study.

Mutational analysis

Mutations in the GJB2 gene were established in 72 out of 218 congenital deaf patients (33,0%), in 58 (26,55%) on both alleles and in 14 on one allele (6,45%) (Table 1).

Table 1. GJB2 mutation spectrum detected in 72 out of 218 congenital deaf Slovenian patients (mutations are described as cDNA change of ORF of the GJB2 gene and as affecting the protein coding sequence

GJB2 genotype	Type of mutation	No. of patients	Percentage
p.Gly12fx c.35delG	frameshift	46	21,1
p. Trp24X c.71G>A	nonsense	2	0,9
p.[Gly12fx]+[Val37Ile] c.[35delG]+[109G>A]	frameshift /missense	5	2,3
p.[Gly12fx]+[Ile182Met] c.[35delG]+[246C>G]	frameshift/missense	1	0,45
p.[Gly12fx]+[Leu90Pro] c.[35delG]+[269T>C]	frameshift/missense	1	0,45
p.[Gly12fx]+[Gln124 X] c.[35delG]+[370C>T]	frameshift/nonsense	2	0,9
p.[Gly12fx]+[Glu47X] c.[35delG]+[139G>T]	frameshift/nonsense	1	0,45
p.[Arg184Pro]+N c.[551G>C]+N	missense/N	1	0,45
p.[Gly12fx]+N c.[35delG]+N	frameshift/N	13	6,0
		72	33,0

The most common mutation was c.35delG mutation present in 69 (31,6 %) subjects. Mutation c.35delG was present in 46 patients in homozygous state (21,1% of all tested patients) and in 23 subjects in heterozygous state (10,5 %). Known pathogenic mutation on the second allele of the GJB2 gene was detected in 10 subjects with heterozygous c.del35G, namely p.Val37Ile, p.Ile82Met, p.Leu90Pro, p.Gln24X and p.Glu47X. Mutation on the second allele was not detected in 13 patients. Additionally, two known pathogenic mutations in homozygous state were detected, namely p.Trp24X in two subjects and p.Arg184Pro in one subject.

None of the patients with none or single mutant allele was found to carry the del(GJB6-13S1830) mutation in either the homozygous or heterozygous state.

Discussion

Up to date, more than 100 GJB2 mutations are reported in HGMD database and are responsible for

mild to profound hearing loss with cases of incomplete penetrance and delayed onset of the disease. The commonest of them is the c.35delG mutation resulting in premature protein truncation at codon 13. It is responsible for up to 70% of the mutant alleles in Caucasians and Mediterraneans[10-12] and is the most important genetic cause of hearing impairment. It was reported that the frequency of the c.35delG mutation in different populations shows a south-to-north European gradient (Table 2), where the high prevalence of the mutation in Southern European populations is due to founder effect. Up today, there are no reports on the frequencies of the mutations in the GJB2 gene in the hearing impaired in Slovenia. In the cohort of 218 congenitally deaf patients in our study the mutation on both alleles of the GJB2 gene was established in 26.55%. Mutation c.35delG was the most common mutation, detected in 31.6% of subjects, either in homozygous or in compound heterozygous state. Here reported data on the c.35delG mutational frequency in Slovenian congenital deaf patients is similar as reported frequencies in neighboring Croatian and Italian populations (Table 2) and collaborates the reported geographic distribution of the mutation in Europe, confirming the south-to-north gradient.

Table 2. Frequencies of the homozygous and compound heterozygous c.35delG mutation.

Population	No. of patients	Pathological GJB2 genotype (% in total cohort)	Homozygous c.35delG (% in total cohort)	Heterozygous c.35delG (% in total cohort)
NE Hungary [31]	194	52,5	35,6	12,6
Czech [28]	156	48,1	28,8	5,1
Maroco [32]	81	43	35,8	3,7
Greece [33]	210	36,2	30	6,2
Austria[34]	69	34,7	11,5	11,5
Croatia[26]	63	33,3	25,4	7,9
Slovenia (this study)	218	33	21,1	10,5
Italy [35]	376	27,6	17,8	8,5
Germany [36]	228	22,8	8,1	8,7
Algeria [37]	116	20,7	13,8	6,9
France [38]	184	16,3	6,4	10,8

6.45% of the patients had established only one mutant GJB2 allele, which is slightly less comparing to reported 10 to 42%[14,19]. In those patients, additional mutation could be located in genetic regions not analyzed in this study.

The del(GJB6-13S1830) mutation was not found in any of investigated subjects.

It was shown that del(GJB6-13S1830) deletion truncating the GJB6 gene but not affecting the GJB2 gene frequently accompanies a mutation in a single GJB2 allele in some populations. The del(GJB6-13S1830) is reported to be the second most frequent genetic cause of nonsyndromic prelingual hearing impairment in Spain, with the first being c.35delG[16]. The del(GJB6-13S1830) deletion is also present with high frequencies in France, the United Kingdom, Israel, Brazil and Argentina[20-22]. In the United States two studies report different results, a moderate frequency or a low frequency of del(GJB6-13S1830)[21]. Its frequency is lower in Belgium, Denmark and Australia[21,23]. The deletion was not detected in Austrians, Croatians, in the northwest of Iran, Greek Cypriots, Chinese and Polish, and is very rare in Czechs and Italians[15,21,24-30] (Table 3).

Table 3. The del(GJB6-D13S1830) in European countries.

Country	del(GJB6-D13S1830) (%)
France [21]	8,2
Spain [21]	7.6-9.7
Great Britain [21]	5,9
Hungary [39]	4,3
Belgium [21]	1,4
Italy [21]	0
Cyper [27]	0
Croatia [26]	0
Austria [25]	0
Slovenia (this study)	0

Collaborating the data in patients from neighboring areas we have not detected the deletion del(GJB6-13S1830) in 218 Slovenian congenitally deaf patients in either homozygous or heterozygous state. This is confirming the del(GJB6-13S1830) deletion to be restrained to certain populations.

In conclusion, eight different mutations in GJB2 gene were identified in 33,0% of congenitally deaf patients, with c.35delG detected in 31,6%. Among those, 26.55% had homozygous or compound heterozygous mutation, confirming approximately half of the expected genetically caused nonsyndromic hearing loss. The del(GJB6-13S1830) mutation in connexin 30 gene was not identified in any of the investigated patients. Further studies of non-coding regions of the GJB2 gene or in other regions involved in nonsyndromic hearing loss are necessary to fully elucidate the genetic background of congenital nonsyndromic deafness.

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