

THESIS FOR PH.D. DEGREE

**NON-SYNDROMIC HEREDITARY HEARING
IMPAIRMENT**

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1. INTRODUCTION

The „Human Genome Project” was founded in 1988 as the first worldwide bioscientific project. The aims were to sequence and build a genetic map of the human genome, to identify new genes and to determine the sequences of model organisms. The main part of these aims has already been reached today.

In our society, the ability to hear is taken for granted as hearing is an essential part of oral communication. However, millions of people throughout the world are affected by the very common sensory disability that hearing impairment (HI) is. The consequences of this disorder are still highly underestimated. Indeed, difficulties in hearing are not life threatening, but they may drastically limit the quality of life of those affected, as they may impose severe social and professional burdens. During the past few decades, this last notion has become increasingly important, especially since profound changes in the structure of our society have resulted in a major shift from non-communicative labor to mainly communicative professions.

The cause of hearing impairment are mostly multifactorial, but with better control of environmental and infectious factors, the portion attributed to hereditary steadily increases. In regard to the different forms of hereditary hearing impairment this difficulty becomes particularly apparent. It is generally known that the prevalence of hearing impairment increases with age. About one in thousand newborns suffer from congenital hearing impairment. It is presumed that in approximately 60% of patients a genetic component is involved in the development of hearing impairment. The underlying genetic defect can be detected only in rare cases. Because of the extensive inner ear research 100 to 150 genes and gene products are estimated to play a role in the inner ear and in the procedure of hearing in general. The pattern of inheritance is autosomal recessive in about 75% of cases, autosomal dominant in about 20%, X-linked in approximately 5%, and mitochondrial in less than 1%. Non-syndromic forms outnumber syndromic forms of hearing impairment with regard to the frequency of occurrence, since the former account for approximately 70% of genetic hearing impairment. Late onset hearing impairment is more frequent, but also the least understood.

Up until now, hearing impairment genes have been identified by positional cloning or by the positional candidate gene approach. Both techniques are preceded by linkage analysis, so that an initial localization of the gene of interest is already known. The positional cloning strategy uses linkage analysis in large families to locate the mutation that compromises the normal function of the gene, and this mutation is thus responsible for the hearing

impairment. The larger the family and the more markers used, the more likely it will be that the mutated gene is located accurately. Once the disease gene is mapped, this gene needs to be cloned and finally the gene product can be identified. In most instances, after the identification of a deafness gene, there still remains a world of understanding and the overall process may require several years. The positional candidate gene approach, on the other hand, uses – after linkage – the phenotype to predict the genotype and is therefore dependent on previously acquired knowledge. Indeed, knowledge about one gene can trigger the identification of another.

It is to be expected that the more thorough phenotype studies are carried out, the more criteria will emerge. Since phenotypic expression of hearing impairment can be very similar for different genotypes. Phenocopies are subjects with a clinical presentation identical to carriers, but due to a cause other than the mutation present in these carriers. On the other hand, a person shown to carry the particular mutation may exhibit a different kind of hearing impairment. Indeed, a specific mutation in a given gene can lead to a wide variety of phenotypes. This phenotypic variability can be attributed to environmental factors, genetic background or the genotype at another locus.

Using families with inherited recessive hearing impairment, more than 70 loci and more than 30 genes responsible for non-syndromic hearing impairment have been mapped on the human chromosomes. The DFNB genes are found in autosomal recessive and hearing impairment is usually congenital and stationary. The most frequently involved DFNB gene, *GJB2*, segregates to the DFNB1 locus on 13q12 and it codes the gap junction protein connexin 26. *GJB2* expression was shown in several tissues and in the cochlea. Gap junctions may play an important role in the recirculation of K^+ ions from hair cells to the marginal cells of the stria vascularis. This gene is responsible for approximately 60% of prelingual, non-syndromic, recessive hearing loss in the Caucasian population with a carrier rate varying from 2 to nearly 5% depending on ethnic group. More than 70 mutations have been described for the *GJB2* gene. The most common mutation associated with DFNB1 hearing loss is a deletion of one out of six subsequent guanines in the coding region of the *GJB2* gene (35delG). In addition, there are many other *GJB2* defects, including nonsense and missense mutations, insertions and deletions.

In regard to daily medical practice the mitochondrial inherited hearing impairment appears to gain clinical relevance. It is estimated that mitochondrial defects cause non-syndromic hearing impairment. The A1555G mutation in the *12S rRNA* gene has been the

first mutation associated with aminoglycoside induced and non-syndromic sensorineural hearing impairment. The mitochondrial ribosome is the target of aminoglycoside ototoxicity since the natural target is the evolutionary related bacterial ribosome. Irreversible hearing impairment is the main complication of aminoglycoside antibiotics. The A1555G mutation occurs in a highly conserved region of the 12S rRNA molecule where aminoglycosid are known to bind and results in respiratory deficiency leading to reduced ATP production in the cochlea cells (hair cells and/or stria vascularis). These events have dramatic effects on the ion pumps and therefore the ion balance, which is essential for a normal hearing function.

Focus in this thesis is on the clinical presentation or phenotype, in relation to the genotype, of some forms of non-syndromic, autosomal recessiv and dominant, sensorineural hearing impairment. We report mutation analysis of the *GJB2* gene and *12S rRNA* gene in 500 healthy controls and 176 patients with moderate to profound hearing impairment from the Northeastern part of Hungary. The data are used to assess the impact of mutations of *GJB2* and A1555G mutation as the cause of congenital sensorineural hearing impairment in the Hungarian population.

We describe various phenotypic features in DFNA6/14 – *WFS1* and DFNA10 – *EYA4* families. These DFNA-families were genetically and clinically studied. In all families, phenotypic aspects were extensively investigated described with known mutations.

This study is a cooperation of the University of Debrecen Department of Otorhinolaryngology, University of Debrecen Department of Clinical Biochemistry and Molecular Pathology, University of Tübingen Department of Otorhinolaryngology, and University of Tübingen Department of Human Genetics.

2. PATIENTS AND METHODS

2.1. *GJB2*, *12S rRNA*

Subjects. The subjects were of Northeast Hungarian origin. The patients were recruited from DE OEC Department of Otolaryngology, University of Debrecen between Oktober, 1999 and September, 2002. Twenty families, in which two or more individuals were found to have a family history of congenital non-syndromic sensorineural hearing impairment greater than 40 dB, were used in this study. The 20 families comprised 84 people, 43 males and 41 females. With the exception of a single family (family 17), consanguinity could not be revealed in the patients' groups. The hearing disorder was sensorineural and bilateral in all cases. Ninety-two patients represented the single deaf child in their family and they were classified as non-familial (sporadic) cases. The non-familial cases included 41 males and 51 females. The age of the patients varied between 1 to 75 years (mean: 24.6 years). Information on the medical history and pedigree structure were obtained in personal interviews with the affected individuals or with their unaffected relatives. To determine the etiology of hearing defects, medical history and pedigree information were obtained through a questionnaire. Patients who had any of the following risk factors (syphilis, rubeola, toxoplasma, CMV, HIV, meningitis, ototoxic drugs, head and acoustic trauma, premature birth, fetal alcohol syndrome, thyroid disease, kidney disease, neurodegenerative disorders, other congenital and chromosomal abnormalities) were not included in this study. Written informed consent was obtained from all participants and from parents of patients younger than 18 years. The study was approved by the Ethics Committee of the University of Debrecen.

Audiology. Each patient underwent general and otoscopic examination and pure-tone threshold audiometry. Audiological assessment was performed by the same audiologist. Severity of hearing impairment was classified according to the better ear, as follows: normal hearing: 0-24 dB; mild hearing loss: 25-40 dB; moderate: 40-69 dB; severe: 70-94 dB; and profound hearing loss: greater than 95 dB (European Work Group on Genetics of Hearing Impairment). Tympanometry and otoacoustic emissions as well as caloric vestibular testing were done in a selected patient population.

Genetic analysis – Connexin 26. 4.5 ml anticoagulated venous blood was obtained from all affected patients and DNA was extracted according to standard protocols. DNA was tested for the common 35delG mutation by PCR based restriction endonuclease assay with use of primers (Primer1: GGTGAGGTTGTGTAACAGTTG, Primer 2:CTGGTGGAGTGTTCCTCCAC). The expected size of the PCR product was 207 bp. Mutation would appear due to a deletion of a guanine between nucleotides 30 and 35 (digestion site of BsiYI: CCN₅/N₂GG) reducing the band to 181 bp. The final products were visualised on a 6% polyacrylamide gel. Patients were divided into three subgroups, according to their allele status, wild type, heterozygous or homozygous for the mutation band. Individuals heterozygous for 35delG mutation and patients without 35delG mutation were characterised by direct sequencing of the encoding region of the *GJB2* gene.

The following primer pair was used for PCR of W24X mutation: GGTGAGGTTGTGTAACAGTTG (primer F), GAAAAATGAAGAGGACGGTGG (primer R). The primer set generates a PCR product of 178 bp. *AsuI* enzyme recognizes the sequence G/GNCC. The PCR product of individuals without mutation was cleaved by *AsuI* into products of 155 bp and 23 bp, while the mutant PCR product remained un-cleaved.

DNA sequencing. DNA samples of all individuals heterozygous or wild type for 35delG mutation were analysed by direct sequencing. The whole encoding region was amplified by using primers 1F, 2F, 3R, 4F, 5R (Primer 1F: AGACTCAGAGAAGTCTCCCTG; Primer 2F: CCAGGCTGCAAGAACGTGTGC; Primer 3R: CTCATGTCTCCGGTAGGCCAC; Primer 4F: GCAGCATCTTCTTCCGGGT; Primer 5R: GGGCAATGCGTTAACTGGC). The PCR products were gel extracted (Gel Extraction Kit, Qiagen) and sequenced with five primers on an ABI 377 automated fluorescent sequencer. Detected mutations were confirmed at least two times and on both DNA strands. Sequences were compared with AF281280 using the DNAsis software (MWG).

Genetic analysis – 12S rRNA. DNA were tested for the A1555G mutation by a polymerase chain reaction (PCR) based restriction enzyme assay (*BsmAI*) with use of primers 1555Gf AGAAATGGGCTACATTTTCTACCC [position 1354-1377 mtDNA] and 1555Grev GTTCGTCCAAGTGCACCTTCCA [position 1580-1601 mtDNA]. The expected size of the PCR product was 197bp and 51 bp (restriction site *BsmAI*: GTCTCN/).

Mutation would appear due to a substitution of a adenin in position 1555 of the mtDNA resulting in a 248 bp band. The final products were visualised on a 6% polyacrylamide gel (PAGE).

2.2. LOCUS AND GENE IDENTIFICATION

Subjects. The families were ascertained at Department of Otolaryngology and Head & Neck Surgery, Medical and Health Science Center, Hungary. All the family members living in Hungary were invited to participate in a clinical and genetic linkage study. In “family 27.” thirty two family members of six generations were available for the analysis (17 males and 15 females) including 3 children under 10 years old age. The age ranged from 6 to 85 years, average 39.5 years. In “family 30.” comprised six generations and included 11 males and 11 females. In “family 27.” was found 11 affected and in “family 30.” 13 affected members with non-syndromic, postlingual, sensorineural hearing impairment. The diagnosis of hereditary hearing impairment was based on the standard protocol of clinical investigations: case history, family pedigree and general otorhinolaryngologic examinations (otoscopy, pure-tone audiometry, tympanometry, stapedial reflexe, electronystagmography, auditory brainstem responses, computerized tomography). This study is approved by the Ethics Committee of the University of Debrecen.

Medical history. Case histories were obtained using a questionnaire regarding the following aspects, with special attention to disease: age at onset of SNHL, hearing aids, symmetry of the hearing impairment, middle ear infections, medical treatment, noise damage, trauma, meningitis, ototoxic agents, tinnitus, vertigo and other clinical manifestation to exclude syndromic forms of hearing impairment and they were ruled out. The family members were generally in good health, with no features of other abnormalities.

Audiology. Threshold audiograms were obtained after otoscopic examination with pure-tone audiometry in a sound-treated room. The audiometric configuration, and other audiologic determinations were classified based on the definitions of the European Work Group on Genetics of Hearing Impairment. Acoustic impedance measurements were performed in a limited number of cases. Contralateral stapedial reflex thresholds were

elicited at 0.5 kHz, if abnormalities were observed at 1 kHz ipsilaterally. Auditory brainstem responses (ABR) was accomplished on only two affected members.

Vestibular examinations. In addition some affected family members were also referred for vestibular examination, included observation for spontaneous and positional nystagmus, gaze-evoked und caloric induced nystagmus as electronystagmography.

Other findings. In several affected people, high-resolution, axial-temporal bone computerized tomography (CT) was performed to evaluate inner ear anatomy. Magnetic resonance imaging (MRI) focused on the brainstem and cerebellopontine angle used to exclude CPA tumors.

Genetic analysis. A 15 ml venous blood sample was obtained from all family members participating in this study after informed consent. Genomic DNA was extracted from samples by using a DNA isolation kit. Individuals were genotyped in a genomewide linkage analysis using 400 microsatellite markers with an average spacing of 11cM. PCR-reactions were performed using manufacturers protocols. Semiautomated genotyping was performed by a MegaBACE-1000 analysis system. Data were analyzed by Genetic profiler Software 1.5. Two-point LOD score calculation was performed with the LINKAGE v5.2 program package. Most likely haplotypes were constructed with Simwalk2 v2.82. Mutation analysis was performed by genomic exon sequencing by using PCR primers. PCR products were purified with the Concert Rapid PCR Purification system and sequenced by using one of the amplification primers on an ABI3100 automatic sequencer. Detected mutations were compared with NCBI-Accession number 1364856 using DNAsis software (MWG).

3. RESULTS

3.1. *GJB2* – *CONNEXIN 26*

The most common *GJB2* mutation in the Hungarian population is the 35delG. It was detected in 58 out of 84 patients with familial hearing impairment. Thirty-seven patients (44%) were homozygous, 21 patients showed heterozygosity (25%) (allele frequency:

56.5%) and in 7 out of 21 heterozygous cases no second *GJB2* mutation was found. In 6 familial cases mutations other than 35delG were present on both alleles, in a single case a non-35delG mutation was present in heterozygous form, while in 20 patients (24%) with HI no mutation was found in coding exon of the *GJB2* gen.

In the group of non-familial cases with HI, 34 patients out of 92 (37%) were homozygous for the 35delG mutation. Seventeen patients (18 %) showed heterozygosity and 11 (11%) out of them revealed a second *GJB2* mutation. The allele frequency of 35delG mutation is 46.2% in this patient group. Two cases were homozygous and 6 cases heterozygous for non-35delG mutation. No mutation of this gene was found in 33 patients (36%) with non-familial HI.

In the control group composed of 500 individuals displaying normal hearing, the 35delG mutation was detected in 24 chromosomes (allele frequency: 2.4%). This value differs only insignificantly from that published in our earlier study on 200 patients.

A nonsense mutation, W24X, was revealed in 4 patients with familial HI in heterozygous form and in 6 patients, 2 homozygous, 4 heterozygous, among the non-familial cases. An allele frequency of 1.4% was demonstrated for this mutation in healthy controls.

Several non-35delG *GJB2* mutations were found in our patients (31del14, S19T, W24X, R32C, M34T, V37I, E47X, 167delT, 233-235delC, L90P, 313del14) that have already been published and unequivocally related to recessively inherited HI. The mutation R127H has also been described earlier and was considered non-pathogenic. In our study heterozygous carrier individuals of the mutation in the „family 17” showed normal hearing, whereas R127H homozygous children were affected by profound HI. The pathogenic importance of mutation A149T is questionable. In „family 6” four heterozygous carriers for the A149T mutation were detected. All three children displayed 35delG/A149T genotype but only two of them demonstrated profound hearing impairment. The mother was heterozygous for the pathogenic mutation M34T and for A149T. The former allele also contains mutation K223R, a non-pathogenic polymorphism.

Two new *GJB2* mutations resulting in amino acid replacements (E42D, G59V) a not yet described silent mutation (C/T at 108) were detected in the present study. One of them represents a G → T transversion in codon 59 resulting in an amino acid change from glycine to valine. This mutation was present in two members of „family 10”, both showing profound congenital hearing impairment. No second *GJB2* mutation was found in father I:1, while his daughter had 35delG/G59V genotype. In addition, the 233-235delC and K223R mutations

were detected in this family. The other two newly described mutations occurred in combination with 35delG mutation. A moderate hearing impairment was diagnosed in the patient with mutations E42D and 35delG. In this case family members were not available for investigation.

Phenotypic analysis. All our individuals diagnosed with hearing impairment and 35delG homozygous mutation have a significant prelingual hearing disorder. In most of the cases both ears present a similar degree of hearing impairment. The auditory deficit involves all frequencies and the morphology of the audiometric curves was flat in 47.9% of cases and slightly decreasing towards the high frequencies in 15 (52.1%) of cases. No other configurations were observed.

The patients' hearing deficit was mostly of severe to profound degree at the middle and higher frequencies, but 9 individuals (12.7%) with biallelic *GJB2* mutations showed moderate hearing loss. 28 of 71 homozygotes (39.4%) had a severe and 34 of 71 (47.9%) had a profound hearing loss. In addition, we noticed that the severity of hearing impairment was variable, even within the same family.

Asymmetric hearing impairment (more than 15 dB difference between ears in three frequencies) was found only in 13 cases (18.3%) in this study. Hearing impairment was generally not progressive, but 13 homozygous patients (23.6%) showed a progression of more than 15 dB during 10 years. A history of progression was not related to gender. Vestibular testing did not detect any anomalies.

3.2. *12S rRNA – A1555G*

In 72 patients showing sporadic non-syndromic severe to profound hearing impairment no A1555G mutation was found in the *12S rRNA gene* (frequency below 1.38%). While analyzing all control individuals showing normal hearing no mutation was found (frequency below 0.44%).

3.3. *DFNA6/14 – WFS1*

The pedigree of “family 27” consists of 32 relatives with 3 branches spanning 6 generations. 15 affected individuals in four successive generations of the family have been documented. The sex ratio of the affected individuals were 5 men / 9 women (1:1.8). The age distribution varies between 6 and 85 years (mean age 39.5 years). All affected subjects had developed normal speech and language at an appropriate age.

The statistical studies presented below are based on 32 individuals. In 14 of these members strong evidence exists that they are suffering from a hereditary form of nonsyndromic hearing impairment. The bilateral hearing impairment in the affected member is inherited in an autosomal dominant mode.

According to the anamnestic data of the 14 genetically affected persons, the hearing impairment started before the age of 25 years. In 5 persons, the hearing loss began at 7-15 years of age. In 2 persons the hearing loss was discovered after the age of 40. Mean age of onset is in the second decade.

Audiology. All affected members showed a sensorineural, bilateral hearing impairment. Asymmetry of the hearing threshold (>10 dB hearing level difference between the ears in at least two frequencies) was observed in 2 of 14 affected family members. The severity of hearing impairment varies from mild to moderate with an ascending low-frequency sensorineural pattern less than < 70 dB. The affected family members younger than 20 years showed only mild hearing loss. The threshold levels at 0.125-1 kHz were worse than in higher frequencies, with a maximum elevation at 0.5 kHz. The mild and high frequencies threshold level decreased corresponded to age-related hearing impairment too.

Progression. There was a linear correlation between hearing levels and age for first estimate. The audiograms were a progressive loss mainly in the mid and high frequencies. As these people grow older, they show slight progression, especially in the low frequencies leading to moderate hearing impairment by the seventh decade.

Vestibular examinations. No equilibrium complaints occurred found in the case history. Oculomotor tests result were normal and spontaneous, positional, or gaze-induced nystagmus were absent in all tested persons. Bithermal caloric stimulation demonstrated normal responses in all cases measured by ENG.

Radiology. High resolution temporal bone CT scan in four affected family members showed normal external, middle and inner ear without any osseous malformation in the temporal bone. MR image of the temporal bone of brainstem could not reveal malformations either.

Genetic analysis. In „family 27.” we found that the T699M mutation in *WFS1* gene segregated completely with the affected haplotype. None of the unaffected family members showed the respective mutation and it was not found in 176 control chromosomes. Surprisingly, one affected patient harbored a second missense substitution (R818C). R818C changes an evolutionary conserved, positively charged arginine-residue into a polar cysteine and has been described as a Wolfram-syndrome mutation. We therefore analyzed the segregation of R818C in part of the family but found that it did not segregate with the disease. Because we found the R818C variant in two out of control persons, it is most likely represents a polymorphism.

3.4. *DFNA10 – EYA4*

The medical history of the “family 30.” A total of 22 family members were examined. Twelve affected individuals in four successive generations of the family have been documented, and the pattern of affected individuals in the pedigree is consistent with autosomal dominant inheritance. Members expect that of the proband did not provide any clue for a syndromic genetic hearing loss. In two of 12 there may have been another explanation for hearing impairment than a genetic defect. The sex ratio was 5 men / 6 women (1:1.2). In each of the 11 affected cases examined, the hearing impairment was noticed before the age of 20 years.

Audiology. All affected members showed a sensorineural, bilateral hearing impairment first noted at 8 and 12 years of age. The severity of hearing impairment varies from mild to severe, related to age. The threshold level at 1-2 kHz was about 35 dB and at the lower (0.125-0.5 Hz) and higher (4-8 kHz) frequencies the offset threshold was close to normal at a young age. The severity of hearing impairment varies from mild to profound mainly in the middle frequencies, but with increasing age all frequencies become affected.

Progression. The progressive nature of the hearing impairment was also evident by linear regression analysis. Affected persons have a rapid progressive sensorineural hearing impairment that begins in the first to third decades and leads to severe impairment (75-90 dB) by the sixth decade. Pure-tone averages calculated for each patient per ear, did not disclose any significant left-right differences.

Vestibular examinations. No other vestibular complaints could be found in the anamnestic data. There was absence of gaze and spontaneous or positional nystagmus on electronystagmographic test, and the bilateral caloric stimulation demonstrated normal responses in all cases.

Radiology. MR studies showed normal inner ear structures with normal signal intensities in the cochlea and labyrinth. No evidence of malformation or tumor was observed, and the eighth nerves appeared completely normal.

Genetic analysis. Microsatellite analysis revealed significant linkage of marker D6S1009 with the disease phenotype (max. two-point LOD score $Z_{\max} = 4.73$ at $\Theta_{\max} = 0.00$). By haplotype analysis a critical interval of 36.8 cM was determined between markers D6S262 and D6S305 which corresponds to the chromosomal region 6q23.2-q26 and contains the candidate gene *EYA4*. Sequencing of all 21 *EYA4* exons revealed an insertion of 4 bases (TTTG) in exon 13 (1558insTTTG). This insertion was detected in all affected family members. Mutation 1558insTTTG causes a frame shift beginning in codon 373, followed by amino acid substitutions and a premature termination codon (PTC) at position 379. This PTC is likely to cause degradation of the mutant transcript by nonsense-mediated decay, otherwise it would result in a nearly complete deletion of the *eya* homologous region of *EYA4*.

4. DISCUSSION

4.1. *GJB2* – Connexin 26

Hearing impairment is one of the most common sensorineural deficit in humans affecting more than 350 million people in industrialized countries (World Health Organization). Cx26 mutations may be responsible for up to 50% of cases of autosomal recessive non-syndromic hearing impairment. It is a gap junction protein, encoded by a gene localized on chromosome 13q11-12. Although we know that it is synthesized in the cochlea, its precise function is yet not clearly defined. Immunohistochemical analysis of Cx26 in rat cochlea showed that gap junctions in both epithelial and connective tissue cells regulate the fluid and ion balance. Defects in Cx26 lead to impaired hearing sensitivity. The prevalence of the 30delG-deletion in Cx26 varies in different populations and was reported as the most common form in Mediterranean families.

These thesis presented here confirm the high prevalence of *GJB2* mutations in Hungarian patients with hearing impairment. 35delG mutant allele in the *GJB2* gene accounts for 51.1% of all *GJB2* alleles in Hungarian patients with familial and non-familial non-syndromic HI. An allele frequency of 2.4% was observed in the general population, this number represents one of the highest frequency of *GJB2* mutations in Caucasians. For a second mutation, W24X, we found a mutation rate of 1.4% in the normal population and demonstrated 3.4% allele frequency in the combined patients group. Together, the 35delG and W24X mutations in the *GJB2* gene are responsible for approximately 58.8% of familial and 50.5% of sporadic cases of HI in the Hungarian population. In general, these patients showed a prelingual, sensorineural, bilateral, symmetric hearing impairment without progression. The audigrams demonstrated sloping as well as flat patterns. The phenotypic manifestation varied in 30% of all analyzed patients, making genetic counseling extremely difficult.

Sequence analysis revealed several further DNA changes (31del14, S19T, W24X, R32C, M34T, V37I, E47X, 167delT, L90P, 313del14) which had already been described as disease related. We also discovered the 233-235delC mutation in DNA samples derived from two Hungarian patients. This mutation is considered characteristic for the Asian population, however no Asian ancestry could be identified in our cases. A single patient possessed the S19T mutation and another single patient the M34T mutation and both mutations were considered to be the cause of HI of the respective individual. The mutation R127H was

detected in one consanguineous Hungarian family and in 5 sporadic cases. All affected persons belong to the Romany ethnic group. In our family both parents and two children heterozygous for this mutation were without disease while children displaying homozygosity were affected by profound HI. To our knowledge this is the first report on R127H homozygous patients and their hearing impairment suggests that the mutation somehow affects the function of gap junction channels. Two new mutations (E42D, G59V) with amino acid replacement were detected in Hungarian patients. The glutamic acid to aspartic acid substitution at codon 42 (E42D) maps to the first extracellular domain of *GJB2*. This mutation was detected in compound heterozygosity with 35delG in one patient associated with prelingual, non-syndromic moderate hearing impairment. Mutation G59V was detected in one Hungarian family. An autosomal dominant pattern of inheritance would explain the HI of father, who carries the G59V variant but no second *GJB2* mutation. His daughter with prelingual HI was also heterozygous for G59V but carried 35delG mutation, as well. Based on our results we suggest a clinical routine screening for *GJB2* mutations for patients with non-syndromic HI. As it has been shown in the study changes in this gene are the most common cause of HI in Hungary. Early molecular diagnosis of the genetic background of HI would result in more efficient genetic counselling and improved speech and communication development.

4.2. *12S rRNA – A1555G*

In regard to daily medical practice one mitochondrially encoded gene appears to gain clinical relevance. In 1993 it was shown that mutations in the *12SrRNA* gene can lead to hearing impairment. In combination with aminoglycoside treatment one specific mutation, the A1555G mutation, predisposes for the development of HI and accounts for a large portion of hearing impaired patients worldwide. Therefore we screened for this mutation control individuals and corresponding Hungarian patients with non-syndromic mild to profound hearing impairment of unknown origin. In our patientsgroup the frequency of the A1555G mutation was below 1.4 %, whereas no mutation was detected in control individuals with normal hearing (frequency below 0.44%).

Already in 1992 was suggested a „two-locus model“ consisting of a mitochondrial and a nuclear gene in combination leading to the disease phenotype in a large family with maternal pattern of inheritance. Although the nuclear gene could not be identified up to

know, it was demonstrated that the region around marker D8S277 might contain a candidate modifier gene for the mitochondrial A1555G mutation. Nevertheless, the relationship between mitochondrial mutations and nuclear genes as well as the importance of environmental factors have not been clarified yet. Therefore it is important to screen patients with non-syndromic sporadic and aminoglycoside induced HI for the A1555G mtDNA mutation because it represents the most common mitochondrial mutation with frequencies around 0.4 % in Hungary.

4.3. *DFNA6/14 – WFS1*

With the development of genetic linkage techniques, it became possible to differentiate between several forms of hereditary hearing defect. Recently, heterozygous missense mutations in *WFS1* have been associated with low-frequency sensorineural HI. Recessive mutations, on the other hand, are responsible for Wolfram syndrome. Biochemical studies indicate that the protein may be an integral endoglycosidase-H-sensitive membrane glycoprotein that is predominantly localized in the endoplasmic reticulum. In this study, we have identified two mutations locating in the fifth intracellular domain in *WFS1* gene in one family with low-frequency HI. The T699M mutation was found in all affected members of family. In 3 cases we have found a second missense substitution (R818C) that has been reported as a Wolfram-syndrome mutation. However, it does not segregate with the disease in the family and is found in controls. One patients, who inherited two missense substitution, does not show signs of this syndrome. For these reason, we believe that the R818C change represents a benign polymorphism.

Affected members of this five-generational „family 27.” had been correctly identified on the basis of their audiograms. In most affected family members, despite the fact that the age at onset is variable, hearing impairment started before the third decade of life, but patients did not seek any contact with an audiologic clinic until midden age. Most of the family members do not require hearing aids. The severity of hearing impairment varies from mild to moderate, related in subjects aged < 80 years. The audiometric configuration is ascending audiogram, affects the lower frequencies (0.25, 0.5, 1 kHz). The low frequencies showed slowly increased threshold in the early stages, followed by the high frequencies in the fifth decade of life.

This finding suggests that mutations in this fifth domain affect only a limited number of functions and that this domain plays an important role in the function of the ear. However, functional studies are necessary to determine the physiological role of the WFS1 protein and to determine the pathogenic effect of mutations in this protein.

4.4. *DFNA10 – EYA4*

Autosomal dominant inherited hearing impairment is a genetically heterogeneous disorder. So far, 41 chromosomal loci have been linked to this disease and 17 genes have been reported. *EYA4* is orthologous to the *Drosophila* gene *eya* („eyes absent“), and is localized on 6q23. The encoded protein contains a highly conserved 271 amino acid C-terminus called the *eya*-homologous region (*eyaHR*, *eya* domain) and a more divergent proline-serine-threonine (PST)-rich transactivation domain at the N-terminus. We report a *DFNA10* family displaying non-syndromic, dominant, postlingual, initially progressive HI, that affected all frequencies with increasing age linkage to 6q23.2-q26. In 11 affected family members, despite the fact that the age at onset varied considerably, HI became apparent before the second decade of life. HI showed significant progression by an average about 10 dB / 10 years at all frequencies. All the affected family members use hearing aids. The optokinetic and caloric tests did not show any consistent deficit. None had any malformations indicative of a syndrome.

To our knowledge, this is the third *DFNA10* family revealing a mutation in the *EYA4* gene. The detected insertion of 4 bp (1558insTTTG) creates a frame shift and results in a PTC at position 379. The effect is either a complete degradation of the mutant messenger or a nearly complete deletion of the *eyaHR* in the *EYA4* protein. Based on the observations mentioned above, this region is of exceptional importance for the function of the protein. Therefore, it would be not surprising that, even if the mutant proteins were present in the cells, interrupting mutations within the *eyaHR* would lead to haploinsufficiency. The *eyaHR* contains several functional subregions with different tissue-specific importance. This would also serve as an explanation for the limited phenotype of *DFNA10*, showing no congenital abnormalities, despite the wide range of expression in early embryogenesis.

Research into hereditary hearing impairment has become a hot topic in present day science. To date the number of „deafness genes” is still increasing and many more „new genes” are expected to follow. The increased knowledge on mutation frequencies of genes involved in the development of hereditary HI will lead to a more precise prediction concerning risk of affection, mode of inheritance and phenotypic specificity. Moreover, most of these genes are expressed in several tissues and organs. Indeed, mutations within these genes lead to exclusive impairment of the hearing organ in approximately 70% of all cases. These facts indicate that we deal with a complex, nested system containing highly sensitive components. Therefore molecular genetic hearing research does not only serve to detect new genes but also contributes to our improved understanding of the process of hearing and participating mechanisms.

PUBLICATION LIST

- 1./ **Tímea Tóth**, Susan Kupka, Hakan Esmer, Ulrike Zeissler, István Sziklai, Hans-Peter Zenner, Nikolaus Blin, Markus Pfister (2001) Frequency of the recessive 30 delG mutation in the GJB2 gene in Northeast-Hungarian individuals and patients with hearing impairment. *International Journal of Molecular Medicine* Aug; 8: 189-192. **[IF: 1,689]**

- 2./ **Tóth Tímea**, Pfister Markus, Sziklai István (2001) [Non-syndromic deafness causing by mutation in the Connexin 26 gene] *Fül-orr-gégegyógyászat* 47: 18-24. (Hungarian)

- 3./ Susan Kupka*, **Tímea Tóth***, Maciej Wrobel*, Jerzy Bal, István Sziklai, Nikolaus Blin and Markus Pfister (2002) Mutation A1555G in the 12S rRNA gene and its epidemiological importance in German, Hungarian and Polish patients. *Human Mutation* Mar; 19(3): 308-9. **[IF: 6,134]**

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- 9./ **Tímea Tóth**, Susan Kupka, Peter Nürnberg, Holger Thiele, Hans-Peter Zenner, István Sziklai und Markus Pfister (2002) Phenotypic characterization of a DFNA6

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family showing low-frequency sensorineural hearing impairment *HNO* (in press) (German) [IF: 0,62]

- 10./ **Tóth T**, Pfister M, Fazakas F, Blin N, Zenner HP, Muszbek L, Kupka S, Sziklai I: High frequency of GJB2 mutations in the Hungarian population. *European Journal of Human Genetics* (submitted)

PRESENTATIONS, POSTERS

- 1) *S Kupka, T Tóth, N Blin, I Sziklai, M Pfister*: Mutation analysis of the Cx26 gene in patients with moderate to profound hearing impairment. 10th International Congress of Human Genetics, Vienna, Austria, 2001. May 13-16. (poster)
- 2) *S Kupka, T Tóth, N Blin, I Sziklai, M Pfister*: Mutation analysis of the Cx26 gene in patients with moderate to profound hearing impairment. 51st Annual meeting, American Society of Human Genetics, San Diego, USA, 2001, Oktober. (poster)
- 3) *Tímea Tóth, István Sziklai*: The phenotypic manifestation and frequency of the recessive 30delG mutation in the *GJB2* gene in Northeast-Hungarian individuals and patients with hearing impairment. Medical Aspects of Mental Handicap, 3RD International Conference, Debrecen, Hungary, June 6-8, 2002. (presentation)
- 4) *Kupka S, Tóth T, Wróbel M, Szyfter W, Zenner HP, Sziklai I, Blin N and Pfister M*: Mutation A1555G in the *12S rRNA* gene and its epidemiological importance in German, Hungarian and Polish patients. European Human Genetics Conference, Strasbourg (France), 25-28 May, 2002. (poster)
- 5) *Haack B, Tóth T, Wróbel M, Sziklai I, Szyfter W, Zenner HP, Blin N, Pfister M, Kupka S*: Mutations in the *connexin 26* gene in German, Hungarian and Polish patients with hearing impairment. European Human Genetics Conference, Strasbourg (France), 25-28 May, 2002. (poster)
- 6) *T Tóth, S Kupka, N Blin, M Pfister and I Sziklai*: Hearing impairment related *GJB2* mutations in the Hungarian population. 39th meeting of the Inner Ear Biology Society, Liege, Belgium, September 8-10, 2002. (poster)
- 7) *Tóth T, Kupka S, Cryns K, Van Camp G, Zenner HP, Blin N, Pfister M and Sziklai I*: Phenotypic characterization of a DFNA6 Hungarian family showing low-frequency sensorineural hearing impairment ARO MidWinter Meeting, Daytona Beach, Florida, (USA) 23-27. February, 2003. (poster)