Investigation of the genetic background of sensorineural hearing loss

PhD thesis

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1. Introduction

Congenital sensorineural hearing loss affects 1-3 children per 1000 newborns, and among the premature infants this ratio could reach 4-5‰. About 70% of the congenital sensorineural hearing loss cases are attributed to genetic factors, while some well characterized infectious diseases and the aminoglycoside induced hearing loss have major shares as aetiological factors as well.

Furthermore, there are more than 100 genes already have been detected in the background of sensorineural hearing loss. More than two third of them show autosomal recessive inheritance, 25% are autosomal dominant and only 1-2% of them are X-linked or inherited by mitochondria. At least 40% of the non-syndromic sensorineural hearing loss is caused by the alterations of the gap junction ß-2 (*GJB2*) gene. In Europe the c.35delG mutation is the most common alteration of this gene which inhibits the expression of the connexin 26 protein. The c.35delG homozygous genotype results in prelingual, profound sensorineural hearing loss in the vast majority of cases while in case of other mutations that are combined in compound heterozygous form a wide spectrum of severity of hearing impairment has been observed.

The *GJB2* sequence analysis has become a routinely used tool nowadays in hearing loss diagnostics. However, the correlation between the *GJB2* alterations and the clinical manifestation has not yet been clearly defined. With the technological development and the appearance of next generation sequencing technique in the last decade multiple new genes have been described as potential causal factors of hearing loss. The functional studies of these genes constitute the scope of several research groups lately. We aimed to investigate the correlation between *GJB2* alterations and clinical manifestation in this study. Selected patients with familiar sensorineural hearing loss have been investigated using next generation sequencing to identify other pathogenic gene alterations that might play a causal role.

Mitochondrial mutations have been described in the pathogenesis of the aminoglycoside induced hearing loss that could increase the risk of related hearing impairment. Nevertheless, aminoglycoside antibiotics are routinely used in severe infection in intensive care and perinatal intensive care. The well-known side effects such as ototoxicity, vestibulotoxicity and nephrotoxicity evoked the need of genetical testing for the mitochondrial mutations before the application of these drugs. In this study we investigated the m.1555 A>G and m.1494 C>T mutations allele frequency in the Hungarian population. Furthermore, we attempted to formulate a clinical recommendation whether mitochondrial genetical testing would be required as part of routine diagnostics before applying these antibiotics or not.

2. Objectives

We investigated selected patients treated between 2012 and 2018 at the Department of Otorhinolaryngology, Semmelweis University and at the Institute of Genomic Medicine and Rare Disorders, Semmelweis University. Our objectives were:

1) to investigate the aetiological factors of profound sensorineural hearing loss in patients under 18 years of age who had undergone cochlear implantation,

2) to investigate the prevalence of mutations of the *GJB2 gene* in patients with non-syndromic sensorineural hearing loss and healthy controls,

3) to study the correlation between the found mutations of *GJB2* gene and the severity and onset of the hearing loss; and furthermore, to elucidate the role of the *GJB2* heterozygous genotype in the background of hearing loss,

4) to find further possible pathogen genes in patients with familiar hearing loss who had heterozygous *GJB2* or wild type *GJB2* genotype using next generation sequencing technique,

5) to investigate the prevalence of mitochondrial m.1555 A>G and m.1494 C>T mutations in patients with non-syndromic sensorineural hearing loss and in selected neurological patients with normal hearing,

6) to formulate a suggestion which patient groups were indicated to have genetical testing and to state whether mitochondrial genome tests were required routinely before applying aminoglycoside antibiotics.

3. Materials and Methods

Patient selection

We created three patient groups. Firstly, we investigated 89 patients retrospectively who underwent unilateral or bilateral cochlear implantation under the age of 18 to identify the prevalence of aetiological factors of profound sensorineural hearing loss. Secondly, for the investigation of the *GJB2* gene we enrolled 239 patients with sporadic or familiar, non-syndromic, unilateral or bilateral sensorineural hearing loss and 160 healthy controls. Five families with familiar hearing loss and heterozygous mutation or no mutation in the *GJB2* were examined with next generation sequencing. Finally, the mitochondrial m.1555 A>G and m.1494 C>T mutations were researched in 269 non-syndromic sensorineural hearing loss patients and in 128 control individuals from the Institute of Genomic Medicine who had other neurological conditions with normal hearing.

The patient selection was conducted in the Department of Otorhinolaryngology and the Institute of Genomic Medicine and Rare Disorders between 2012 and 2018. The genetical testing of the *GJB2 and* the mitochondrial DNA was conducted in The Institute of Genomic Medicine and Rare Disorders. The whole exome sequencing of the selected families was conducted at the Institute of Human Genetics, University of Würzburg as part of a collaboration project. Patients belonged to the Caucasian population. Written informed consent was applied before every genetical test. The research was approved by the Regional, Institutional Scientific and Ethical Committee of the Semmelweis University.

Audiological methods

A detailed history was taken from each patient with hearing loss focusing especially on the beginning of the hearing loss, the familiar origin and the co-morbidities (cardiovascular, ophthalmologic or internal medicine disorders) to exclude the syndromic and acquired aetiology. In case of early onset hearing loss we also paid attention to the perinatal period (gestational time, perinatal disorders, medication). Thorough ear-nose-throat examination was carried out in each case. Tympanometry was followed by audiological tests. In case of a normal tympanogram pure tone audiometry (PTA) and objective audiological tests e.g. brainstem evoked response audiometry (ABR), Auditory Steady State Response (ASSR) audiometry, otoacoustic emission (OAE) and stapedius reflex test were conducted to prove the degree of hearing loss. Depending on the cooperation of the patient, speech perception test was carried out as well. PTA constituted of clear sinus tone threshold measurement

from 250 Hz to 8000 Hz. The result was registered in decibels (dB) if three unequivocal and identical signalling was yielded from the patient. In case of lacking patient signalling on a particular frequency, the limit of the measuring range (120 dB) was registered.
The ABR and ASSR tests were conducted awake in adults under relaxed conditions in a perfectly silent audiometric room. On the other hand, children had it in spontaneous sleep or under total intravenous anaesthesia. Concerning ABR, the appearance of the 5^{th} wave, the latency time of the 5^{th} wave and the interpeak latency time was measured at different volumes (max. 105 dB). When retrocochlear lesion was suspected, a skull MRI was requested as well to rule out intracranial lesion. Results of the ABR were assessed together with the results of the ASSR. The *GJB2* hearing loss group was divided into 7 subgroups based on the average of the pure tone audiometry measurements on 500, 1000, 2000 and 4000 Hz ($PTA4^{0,5\text{-}4kHz}$) taking the better hearing ear's result as follows: mild (25-40 dB), moderate (41-55 dB), moderately severe (56-70 dB), severe (71-90 dB), profound (>90 dB), asymmetric and unilateral hearing loss subgroup. Asymmetric hearing loss was defined as greater than 10 dB difference between the two ears or as 15 dB deviation between ears at two distinct frequencies. In case only one ear was affected, we named it unilateral hearing loss.

Genetic methods

The DNA was isolated from blood, urine or muscle samples using the QIAamp DNA blood kit and QIAGEN DNA Tissue kit based on the instructions of the manufacturer (QIAgen, Hilden, Germany). The concentration of the DNA was measured at 260 nanometre (nm) absorbance with UV spectrophotometer. The degree of purity of the DNA was determined with the ratio of the measurements at 260 nm and 280 nm.

The whole coding region of the *GJB2* was analysed with Sanger sequencing method. The exon region was amplified employing polymerase chain reaction with special primers of the *GJB2* and was sequenced bidirectionally. The received sequences were compared with the sequence of the human reference genome (ENST00000382848.4; NM_004004) with the NCBI Blast Program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The variants were examined with ClinVar database, NCBI database, „The Connexin-deafness homepage" and Deafness Variation Database.

GJB2 control groups were created enrolling normal hearing patients of the Institute of Genomic Medicine and Rare Disorders who had previously underwent whole exome

sequencing because of other reasons. At the selection variations we focused on the *GJB2* alterations only.

Patients with familiar sensorineural hearing loss who did not have homozygous or compound heterozygous alteration in the *GJB2*, but whose genetic origin was obtained, had the opportunity for whole exome sequencing as part of a collaboration with the Institute of Human Genetics, University of Würzburg. In case any alterations of other gene were found, we validated it with Sanger sequencing and segregation tests using the DNA of first degree relatives.

Mitochondrial mutations were detected by restriction fragment length polymorphism (PCR-RFLP) technique. The size and intensity of the bands were determined using Quantity One (BIORAD) and ImageJ softwares.

In silico and statistical analysis

For the *in silico* analyses we used prediction softwares (PolyPhen2, MutationTaster, SIFT) to identify the conservation of the mutations and the possible protein changes. The found alterations were compared with international databases (HGMD, „The Connexin-deafness homepage", „Hereditary Hearing Loss Homepage", NHLBI Exome Sequencing Project (ESP), ClinVar, dbGAP, dbSNP, Iranome, Deafness Variation Database). The data of the Exome Aggregation Consortium (ExAC) database, the 1000Genom program and GenomID project were used for the analysis of the allele frequencies. At the categorisation of the variants the American College of Medical Genetics (ACMG) guideline was utilised.

Statistical analysis was conducted using normality tests, one-way ANOVA test, Mann-Whitney-test and Pearson's chi-square test as well. The level of significance was always $p < 0.05$. Descriptive statistical methods were applied. The confidence intervals (CI 95%) were calculated employing standard formulas.

4. Results

The cochlear implantation group

Out of the 89 patients 71 was implanted before the age of 7 and 18 children were implanted after that age. The cause of hearing loss was detected in 56 cases (62.9%). The pathogenic alteration of the *GJB2* gene was found to be the most common of them, showing 9 homozygous [c.35delG/c.35delG] cases, in 1 homozygous [W24X/W24X] case, 3 compound heterozygous [c.35delG/W24X] and in 1 compound heterozygous [c.35delG/c.-23+1G>A] mutations. The c.35delG heterozygous genotype was detected in 3 patients. The c.35delG allele frequency was 38.8% in this patient group. Besides the genetic origin, 9 patients had infectious disease in the background of the hearing loss. In four patients congenital cytomegalovirus infection, in one case varicella-zoster virus infection, in three cases meningoencephalitis and in one child intrauterine herpes simplex infection was detected. Other origins such as ototoxic medication (4 cases), intrauterine complication (3 cases), epilepsy (1 case) and Down-syndrome (1 case) was also observed. In our cohort 13 patients were premature infant, and 4 of them presented with multifactorial aetiology.

The *GJB2* patient group

We examined 199 bilateral and 40 unilateral hearing loss patients. Based on the degree of hearing loss 46 mild, 27 moderate, 15 moderately severe, 5 severe, 63 profound, and 43 asymmetrical hearing loss patients were analysed. The diagnosis was made between newborn and 75 years of age (average age of onset was 19.28±18.63 years (CI95% 16.91-21.66 years)). Ninety-three patients (38.9%) had prelingual and 146 patients (61.1%) postlingual hearing impairment. Significantly more severe hearing impairment was detected in the prelingual cases (Kruskal-Wallis one-way ANOVA test, $p<0.001$) and a correlation of earlier onset of the disease in men was also found (Kruskal-Wallis one-way ANOVA test, $p=0.001$).

In the non-syndromic hearing loss group, we found 10 different alterations in the *GJB2* in 53 patients. The *GJB2* mutation allele frequency was 16.12% in the hearing loss group and 3.12% in the control group. The most common alteration was the c.35delG (p.12Vfs) mutation, in 20 cases homozygous (8.3%), 7 cases compound heterozygous (2 [c.35delG/W24X], 2 [c.35delG/c.313del14], 1 [c.35delG/L90P], 1 [c.35delG/Q80P], 1 [c.35delG/ c.-23+1 G>A]) and 13 cases heterozygous genotypes were detected. The

c.109G>A (p.V37I) and c.139G>T (p.E47X) mutations showed compound heterozygous form in 2 patients and in 11 cases the c.71G>A (p.W24X), c.269T>C (p.L90P), c.101T>C (p.M34T), c.439G>A (p.E147K), c.109G>A (p.V37I) mutations were presented in heterozygous form. The c.35delG allele frequency was 12.55%.
In the control group the c.35delG showed an 2.5% and the p.M34T showed an 0.62% allele frequency.

Analysing the geno-phenotype correlation, we found that the c.35delG homozygote genotype cause severe to profound sensorineural hearing loss in earlier age (Kruskal-Wallis-test, $p<0.010$). In three patients we observed a progression of the severity of hearing loss and in a few years it progrediated to profound hearing impairment. The clinical appearance of the compound heterozygous genotype depended on the effect of the mutation on the protein expression. Two inactivating mutations together, such as splice site mutation, nonsense mutation, insertion, duplication or deletion of less than 3 bases (c.35delG, W24X, c-23+1G>A, c.313del14) caused profound prelingual hearing loss, and a non-inactivating mutation, like a missense mutation or deletion of 3 bases (V37I, Q80P, L90P) combination with an inactivating mutation caused postlingual, moderate hearing loss. Every patient with compound heterozygous genotype had a disease onset before the 18th year of age.

We did not find any correlation between the *GJB2* heterozygous genotype and the onset of the disease or the severity of hearing loss (Mann-Whitney test, $p=0.724$, $p=0.559$). The pathogenic *GJB2* alterations showed low incidence among the mild and unilateral hearing loss subgroups (chi-square test, $p<0.05$).

Results of whole exome sequencing

Five families were recruited with familiar hearing loss to the whole exome sequencing tests, and in 3 families (60%) we found other gene alterations which could lead to the hearing loss.

In the first family the autosomal dominant *DIAPH3* gene c.3125 G>A (p.R1042H) was detected. This rare alteration occured as a variant of unknown significance in the NCBI ClinVar Database. The *in silico* examination showed the possibly pathogenic function of the variant. The alterations of the *DIAPH3* gene have been described in auditory neuropathy.

In the second family the autosomal dominant *WFS1* gene c.2390A>T (p.D797V) heterozygous mutation was observed. This alteration has not yet been registered in public

databases (HGMD, ClinVar, NCBI, dbSNP, ExAc, 1000Genom, GnomAD), the *in silico* prediction analyses assumed the pathogenicity of this alteration. The *WFS1* gene probably caused autosomal dominant Wolfram-like syndrome in this family, which was implied by the severe sensorineural hearing loss and an optic neuropathy in the father. However, the index patients have not yet had a profound sensorineural hearing loss, something that a close follow up of these patients could identify in time as well as the role of this mutation.

In the third family the autosomal recessive *TRIOBP* gene's c.2581C>T (p.R861X) and c.5014G>T (p.G1672X) mutations were found in compound heterozygous form. The c.2581C>T mutation was a variant with unknown significance, whereas the c.5014G>T has not been described yet. Based on the recessive inheritance of the *TRIOBP* gene, these two alterations in compound heterozygous form could explain the hearing loss in this family.

The incidence of the m.1555 A>G and m.1494 C>T mutations of the mitochondrial DNA

Through the mitochondrial DNA analyses the m.1555 A>G mutation was found in one case in homoplasmic form. The m.1494 C>T mutation was not observed in our cohort. The mutant patient had progressive, bilateral asymmetric sensorineural hearing loss. This patient did not receive aminoglycoside antibiotics. The allele frequency of the m.1555 A>G mutation was 0.37% in the non-syndromic hearing loss group and did not appear in the control group.

5. Conclusion

Ethiological factors of the profound sensorineural hearing loss in cochlear implant patients:

1. Aetiology was identified in 62.9% of the hearing loss cases. The most common causes were the pathogenic alterations of the *GJB2* gene, the infectious origin and the aminoglycoside antibiotic exposure.

Conclusions of the genetical tests

2. The most common alteration of the *GJB2* gene was the c.35delG mutation, which showed the following allele frequencies: 12.55% in the non-syndromic hearing loss group, 38.8% in the prelingual profound hearing loss group. We found 9 other mutations in the *GJB2* gene.

3. The c.35delG homozygous genotype manifested in prelingual, profound sensorineural hearing loss, the compound heterozygous genotype caused moderate to profound sensorineural hearing loss depending on the associated mutation's effect on the protein expression.

4. The compound heterozygous genotype resulted in hearing loss before the 18^{th} year of age.

5. We suggest the *GJB2* gene analysis in bilateral, at least moderate hearing loss cases in adults. In case of unilateral or mild hearing loss in adults - except some special cases – we do not recommend the *GJB2* analysis. The genetical examination of the *GJB2* gene is highly recommended in childhood regardless of the severity of hearing loss.

6. In selected families with familiar hearing loss by whom the *GJB2* wild type or *GJB2* heterozygous genotype were found, the next generation sequencing identified pathogenic or likely pathogenic alterations in three genes (*DIAPH3, WFS1, TRIOBP)*. Our results suggest that the extended genetical tests with whole exome sequencing would be highly recommended in familiar hearing loss cases if the results of the *GJB2* analyses would not explain the ethiology of the hearing loss.

7. The mitochondrial mutations showed low incidence in the hearing loss group and the control group as well. The m.1555 A>G mutation's allele frequency was 0.37%, whereas the m.1494 C>T mutation was not found in our cohort. According to these results the

mitochondrial mutation analyses is not recommended before the usage of aminoglycoside antibiotics.

8. Finally, we propose a recommendation for the diagnostics and treatment of sensorineural hearing loss based on our results. This could help clinicians to find the correct indication and assessment of genetical testing as well as to utilise the the optimal therapy. Together with the results of the genetical tests the clinical progression can be predictable which is of upmost importance to achieve successful hearing rehabilitation.

6. Bibliography of the candidate's publications

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