© J Hear Sci, 2017; 7(1): 33–40
DOI: 10.17430/903762

PREVALENCE OF DFNB1 HEARING LOSS AMONG COCHLEAR IMPLANT USERS ESTABLISHED WITH THE 3-STEP DFNB1 APPROACH

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Contributions:
A Study design/planning
B Data collection/entry
C Data analysis/statistics
D Data interpretation
E Preparation of manuscript
F Literature analysis/search
G Funds collection

Abstract

Background: Intensive studies have been conducted worldwide to elucidate the genetic basis of hearing impairment (HI). The aim of this study was to estimate the prevalence of DFNB1-related HI among patients with cochlear implants (CI).

Material and methods: We analyzed 1262 consecutive patients diagnosed with hearing loss who received a CI. At the time of writing this is the largest CI cohort tested for DFNB1 mutations. The search for mutations was done using our 3-step diagnostic approach to DFNB1 testing (3-step DFNB1 approach) comprising a range of molecular methods: multiplex PCR, PCR-RFLP, allele-specific PCR, Sanger sequencing, and real-time PCR with dedicated TaqMan probes.

Results: Our results show that DFNB1 deafness is present in 39.3% of Polish CI recipients. The most commonly detected causative variant in the study group was c.35delG within the *GJB2* gene. The majority of the revealed DFNB1 variants were truncating, and related to early HI onset as well as profound hearing loss.

Conclusions: The data conclusively show that mutations in the DFNB1 *locus* are the main cause of HI among CI patients, and that the proposed 3-step DFNB1 approach is a fast, effective, and economical method for DFNB1 screening.

Key words: *GJB2* • *GJB6* • DFNB1 • hearing loss • deafness • cochlear implants • mutation • gene

EVALUACIÓN DE LA PREVALENCIA DE LA HIPOACUSIA ASOCIADA A MUTACIONES EN EL *LOCUS* DFNB1 EN PACIENTES CON IMPLANTE COCLEAR, DETERMINADA POR EL TEST DIAGNÓSTICO DE TRES PASOS DFNB1

Resumen

Introducción: Actualmente en todo el mundo se están llevando a cabo estudios intensificados sobre la búsqueda de causas genéticas de la hipoacusia. El objetivo del presente trabajo fue evaluar la prevalencia de la hipoacusia condicionada por mutaciones en el *locus* DFNB1 entre pacientes con implantes cocleares (CI).

Material y métodos: El estudio fue realizado en un grupo de 1262 pacientes consecutivos con implante coclear. Cabe destacar que hasta la fecha es el grupo más grande de pacientes con CI caracterizado en cuanto a la aparición de mutaciones en el *locus* DFNB1. La búsqueda de mutaciones fue realizada utilizando el test diagnóstico de tres pasos DFNB1 (3-steps DFNB1 app.), basado en distintos métodos moleculares, tales como: multiplex PCR, PCR-RFLP, PCR alelo-específica, secuenciación de Sanger y PCR en tiempo real con sondas dedicadas de tipo TaqMan.

Resultados: La hipoacusia asociada a mutaciones en el *locus* DFNB1 se manifiesta en un 39,3% de pacientes polacos con implante coclear. La variante causal de hipoacusia en el grupo analizado detectado con más frecuencia fue la mutación c.35delG, localizada dentro del gen *GJB2*. La mayoría de las variantes de DFNB1 identificadas son mutaciones destructoras, asociadas a la hipoacusia con un inicio temprano e intensidad profunda.

Conclusiones: Los datos presentados indican claramente que las mutaciones en el *locus* DFNB1 son la principal causa genética de la hipoacusia en pacientes con implante coclear. El test diagnóstico de tres pasos constituye un método rápido, eficaz y económico para detectar la hipoacusia del tipo DFNB1.

Palabras clave: *GJB2* • *GJB6* • DFNB1 • hipoacusia • implante coclear • mutación • gen

ОЦЕНКА ЧАСТОТЫ ПОЯВЛЕНИЯ ТУГОУХОСТИ, ОБУСЛОВЛЕННОЙ МУТАЦИЯМИ *LOCUS* DFNB1, СРЕДИ ПАЦИЕНТОВ С КОХЛЕАРНЫМ ИМПЛАНТАТОМ, ОПРЕДЕЛЁННАЯ С ИСПОЛЬЗОВАНИЕМ ТРЁХЭТАПНОГО ДИАГНОСТИЧЕСКОГО ТЕСТА DFNB1

Изложение

Введение: В данный момент во всём мире проводятся интенсивные исследования над поиском генетических причин тугоухости. Целью представляемой работы была оценка частоты появления тугоухости, обусловленной мутациями *locus* DFNB1, среди пациентов с кохлеарными имплантатами (CI).

Материал и методы: Исследования проводились в группе 1262 консекутивных пациентов с кохлеарным имплантатом. Следует подчеркнуть, что на настоящий момент это самая большая группа пациентов с CI, которая была охарактеризирована с точки зрения наличия мутации *locus* DFNB1. Поиски мутации были проведены с применением разработанного трёхэтапного диагностического теста DFNB1 (3-steps DFNB1 app.), опирающегося на разных молекулярных методах, таких как мультиплексная ПЦР, ПДРФ-анализ, аллель-специфическая ПЦР, метод Сэнгера и ПЦР в реальном времени с использованием зондов типа TaqMan.

Результаты: Тугоухость, связанная с мутациями *locus* DFNB1, имеет место у 39,3% польских пациентов с кохлеарным имплантатом. Движущим вариантом для тугоухости, который обнаруживался чаще всего в исследующейся группе, является мутация c.35delG, находящаяся в районе гена GJB2. Большинство идентифицированных вариантов DFNB1 - это разрушающие мутации, связанные с тугоухостью с ранним началом и глубокой степенью интенсивности.

Выводы: Представленные данные однозначно указывают, что мутации *locus* DFNB1 является главной генетической причиной тугоухости среди пациентов с кохлеарным имплантатом. Разработанный трёхэтапный диагностический тест - этот быстрый, эффективный и экономный метод обнаружения DFNB1-зависимой тугоухости.

Ключевые слова: *GJB2* • *GJB6* • DFNB1 • тугоухость • кохлеарный имплантат • мутация • ген

OCENA CZĘSTOŚCI WYSTĘPOWANIA NIEDOSŁUCHU UWARUNKOWANEGO MUTACJAMI *LOCUS* DFNB1 WŚRÓD PACJENTÓW Z IMPLANTEM ŚLIMAKOWYM, OKREŚLONA PRZY UŻYCIU TRZYETAPOWEGO TESTU DIAGNOSTYCZNEGO DFNB1

Streszczenie

Wprowadzenie: Obecnie na całym świecie prowadzone są intensywne badania nad poszukiwaniem genetycznych przyczyn niedosłuchu. Celem przedstawianej pracy była ocena częstości występowania niedosłuchu uwarunkowanego mutacjami *locus* DFNB1 wśród pacjentów z implantami ślimakowymi (CI).

Materiał i metody: Badania wykonano w grupie 1262 konsekutywnych pacjentów z implantem ślimakowym. Należy podkreślić, iż jest to największa do tej pory grupa pacjentów z CI scharakteryzowana pod kątem obecności mutacji *locus* DFNB1. Poszukiwanie mutacji zostało przeprowadzone z zastosowaniem opracowanego trzyetapowego testu diagnostycznego DFNB1 (3-steps DFNB1 app.) opartego na różnych metodach molekularnych, takich jak: multiplex PCR, PCR-RFLP, allelospecyficzny PCR, bezpośrednie sekwencjonowanie Sangera i PCR w czasie rzeczywistym z dedykowanymi sondami typu TaqMan.

Wyniki: Niedosłuch powiązany z mutacjami *locus* DFNB1 występuje u 39,3% polskich pacjentów z implantem ślimakowym. Najczęściej wykrywanym wariantem sprawczym dla niedosłuchu w badanej grupie jest mutacja c.35delG zlokalizowana w obrębie genu *GJB2*. Większość zidentyfikowanych wariantów DFNB1 to mutacje niszczące, powiązane z niedosłuchem o wczesnym początku i głębokim stopniu nasilenia.

Wnioski: Przedstawione dane wskazują jednoznacznie, iż mutacje *locus* DFNB1 są główną genetyczną przyczyną niedosłuchu wśród pacjentów z implantem ślimakowym. Opracowany trzyetapowy test diagnostyczny to szybka, skuteczna i ekonomiczna metoda wykrywania niedosłuchu DFNB1 zależnego.

Słowa kluczowe: *GJB2* • *GJB6* • DFNB1 • niedosłuch • implant ślimakowy • mutacja • gen

Introduction

Hearing impairment (HI) is the one of the major public health problems, and due to its high prevalence a lot of effort has been made in understanding its causes. HI can be initiated by environmental as well as genetic factors or can be multifactorial [1]. Determining the genetic cause of HI is an important and challenging task. The genetic background of HI is complex and the occurrence of this defect may correspond to malfunction of many different genes [2].

To date over 100 *loci* related to both recessive and dominant HI have been determined (http: //hereditaryhearingloss.org/), but mutations in the deafness autosomal recessive 1 (DFNB1) *locus* (MIM #220290), encompassing two genes [i.e. gap junction protein beta 2 (*GJB2*) and gap junction protein beta 6 (*GJB6*)], account for the majority

DOI: 10.17430/903762

of hereditary isolated, prelingual deafness [2–6]. In a recessive disorder, such as DFNB1, both copies of a gene are mutated, and this is a prerequisite for the development of the disease.

GJB2 encodes connexin 26, which participates in the cochlear ion homeostasis as a gap junction protein. Connexin 26 – mediated potassium recycling is essential for maintenance of the endocochlear potential which plays a critical role in hearing [7]. A broad spectrum of mutations in the *GJB2* gene cause dysfunction of this protein. The prevalence of particular *GJB2* mutations differs among populations [8,9], but c.35delG is responsible for most of the autosomal recessive HI in Caucasians [6,10–12]. Mutations in *GJB6*, the second DFNB1 gene, cause HI mostly due to two common large deletions (D13S1830, D13S1854) [13], which most likely deprive *GJB2* of its regulatory element [14,15].

Using electronic prostheses (cochlear implants, CIs) certain groups of auditory nerve fibers are stimulated by miniature electrodes implanted in the cochlea. In this way, the activity of non-functional or missing hair cells of the organ of Corti is restored by electric pulses to the electrodes. The entire implant system consists of two modules: an external module positioned behind the ear composed of a microphone, speech processor, and transmitter; and an internal module permanently positioned in the cranial bone consisting of a receiver with stimulating electrodes inserted into the cochlea [16].

The world history of this first restoration of a human sense by use of a multichannel device began in the mid-1980s [17]. In Poland, pioneering cochlear implantation surgery was performed by Skarżyński in 1992, and since then the technique has been constantly improved [18], leading to a significant broadening of selection criteria for surgery. Among patients with residual hearing, electro-natural (ENS) or electro-acoustic (EAS) stimulation of the inner ear is used. This approach (partial deafness treatment, PDT) involves the use of a CI complemented by natural hearing (for ENS) or a hearing aid (for EAS) [19–22].

Currently, the genetic background of HI is an area of intensive research. The aim of our study was to estimate the prevalence of DFNB1-related HI among Polish CI patients, as well as to assess the usefulness and efficiency our 3-step DFNB1 testing.

Material and methods

Audiological assessment

Hearing levels were determined by pure tone audiometry (PTA) at frequencies of 0.5, 1, 2, 4, and 8 kHz. HI was defined as either mild (21–40 dB), moderate (41–70 dB), severe (71–90 dB), or profound (more than 90 dB).

Patients

From more than 10,000 patients consulted at the Genetic Outpatient Clinic of the Institute of Physiology and Pathology of Hearing (IFPS), a group of consecutive 1262 unrelated patients (614 males and 648 females) with a CI was selected for this study. All patients were diagnosed with non-syndromic sensorineural hearing loss (NSSNHL), except for one who had keratitis–ichthyosis–deafness syndrome (KID). No exclusions were made based upon age, age of HI onset (AO), phenotype, or the presence of environmental risk factors for HI. In the whole group the AO varied from 0 to 66 years (mean 3.9±8.3; median 1.0). For females the AO ranged from 0 to 60 y.o. (mean 4.7±9.2; median 0.7), whereas in males the AO varied from 0 to 66 y.o. (mean 3.1±7,2; median 1.5). Familial HI was identified in 29% of patients, whereas the remaining 71% of subjects did not have relatives with HI. The mean degree of HI for the whole studied group was 97±9.5 dB and classified as profound.

The study was approved by the local Ethics Committee (resolution no. IFPS: /KB/03/2012). Informed, written consent was obtained from the patients or their guardians prior to participation.

DNA isolation

DNA was isolated from peripheral blood by a standard salting-out method [23].

Molecular methods

The Genetic Laboratory of IFPS has worked out its own 3-step diagnostic approach for DFNB1 testing (3st-DFNB1app) to detect *GJB2* and *GJB6* mutations. In the first stage (DFNB1-base) the five most common *GJB2* gene (NM_004004.5) mutations in the Polish population (c.35delG, c.167delT, c.313_326del, c.334_335delAA, and c.358_360delGAG) are searched for. Briefly, the first step consists of multiplex PCR followed by PCR-RFLP [24]. The step also allows detection of other deletions or insertions in the studied regions (c.12-72, c.68-198, c.306-464) of the *GJB2* gene. For patients with two mutations detected in this step, the genetic cause of HI is determined and there is no need for additional tests.

Patients without an established genetic cause of HI after the first stage are further investigated for the *GJB6*-D13S1830 deletion (DFNB1-delGJB6) and c.-23+1G>A *GJB2* mutation (DFNB1-RT-IVS) in the second step. The aim of the DFNB1-delGJB6 is to detect or exclude the most common deletion of the *GJB6* gene and the test is carried out using an allele-specific PCR technique according to a modified method [14]. DFNB1-RT-IVS is aimed at detecting mutation in the noncoding part of the *GJB2* gene using a real time PCR technique with fluorescently-labelled TaqMan probes.

The third step is a direct Sanger sequencing of the whole coding region of the *GJB2* gene (DFNB1-seq). Its aim is to detect or exclude the presence of all pathogenic mutations located within the coding region of the *GJB2* gene [25].

Statistical analysis

AO for HI was compared between the groups using a Mann-Whitney U-test (Statistica StatSoft, Tulsa, USA) with $p<0.05$ considered as statistically significant.

DOI: 10.17430/903762

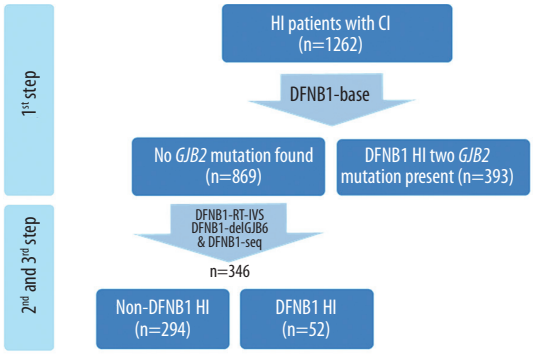

Figure 1. Study workflow

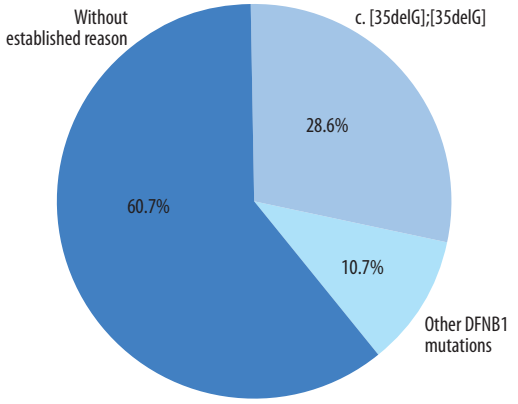

Figure 2. Percentage of CI patients with *GJB2* homozygous c.35delG mutations and other DFNB1 mutations detected in the 3-step DFNB1 approach

Table 1. Distribution of genotypes identified in the DFNB1 *locus* among CI patients

Genotype	Frequency [%]	Mutation status**
c.[35delG];[35delG]	28.6	T/T
c.[35delG(;)313_326del]	3.9	T/T
c.[35delG(;)167delT]	1.6	T/T
[GJB2: c.35delG];[GJB6: del(GJB6-D13S1830)]	1.2	T/T
c.[35delG(;)-23+1G>A]	1.0	T/T
c.[35delG(;)334_335delAA]	0.6	T/T
c.[35delG(;)235delC]	0.4	T/T
c.[35delG(;)269T>C]	0.2	T/N
c.[35delG(;)358_360delGAG]	0.2	T/N
c.[35delG(;)551G>C]	0.2	T/N
c.[167delT(;)313_326del]	0.2	T/T
c.[313_326del(;)-23+1G>A]	0.2	T/T
c.[35delG(;)101T>C]	0.2	T/N
c.[35delG(;)44A>C]	0.2	T/N
c.[35delG(;)139G>T]	0.2	T/N
c.[148G>A];[=]*	0.2	N/–
Total %	**39.3**	

* c.148G>A – dominant mutation responsible for keratitis-ichthyosis-deafness syndrome (KID); ** T – truncating; N – non-truncating.

Results

A total of 1262 CI patients were screened for the major *GJB2* mutations using the DFNB1-base method. After the first step, causative *GJB2* mutations were found in 35.2% (393/1262) of patients (Figure 1). Almost 91.8% of these patients were homozygous for the c.35delG mutation. The second most frequent mutation in this group was c.313_326del (5%), followed by c.167delT (2.3%), c.334_335delAA (0.7%), and c.358_360delGAG (0.2%). Apart from c.35delG homozygous mutations, the presence of other mutations detected by DFNB1-base was confirmed by DFNB1-seq.

Both the second (DFNB1-delGJB6, DFNB1-RT-IVS) and the third (DFNB1-seq) step were done in 346 patients. In this part of the study, causative mutations were identified in 15% (52/346) of the investigated patients. This group included mutations of the DFNB1 *locus* that were not covered by DFNB1-base, as well as mutations previously identified in the DFNB1-base step (except for homozygous c.35delG mutations). The most frequent in the latter group were GJB6: del(GJB6-D13S1830), GJB2: c.-23+1G>A, and GJB2: c.235delC. For calculation purposes, the genotype and allele frequencies of mutations detected in the second and third steps were extrapolated for the whole group of

DOI: 10.17430/903762

Table 2. Allele frequency of DFNB1 mutations detected among CI patients

Allele	Effect	Allele frequency [%]
c.35delG	p.Gly12Valfs*2	83
c.313_326del	p.Lys105Glyfs*5	2.2
c.167delT	p.Leu56Argfs*26	1
GJB6: del(GJB6-D13S1830)	no protein	0.6
c.-23+1G>A	impaired splicing	0.3
c.334_335delAA	p.Lys112Glufs*2	0.3
c.235delC	p.Leu79Cysfs*3	0.2
c.269T>C	p.Leu90Pro	0.1
c.358_360delGAG	p.Glu120del	0.1
c.551G>C	p.Arg184Pro	0.1
c.101T>C	p.Met34Thr	0.1
c.44A>C	p.Lys15Thr	0.1
c.139G>T	p.Glu47*	0.1
c.148G>A	p.Asp50Asn	0.1

HI patients with a CI. A schematic of the study workflow is shown in Figure 1.

Considering the whole group of CI recipients analyzed in this study, HI was clearly related to DFNB1 in 39.3% of cases. Among the cohort we identified 28.6% of patients homozygous for the c.35delG mutation, while the remaining 10.7% of patients harbored other causative biallelic DFNB1 mutations (Figure 2 and Table 1). The first step of the 3st-DFNB1app allowed the genetic cause of HI (biallelic *GJB2* mutations) to be detected in 35.2% of CI patients. The remaining two steps increased the percentage of fully diagnosed patients by an additional 4.1%.

Allele frequency of the identified mutations is shown in Table 2. Additionally, in the whole CI cohort we identified 9.8% of heterozygous carriers of a DFNB1 mutation (data not shown).

Implementation of the 3st-DFNB1app method generated three clusters of patients: (i) a group homozygous for c.35delG mutation; (ii) a group with other DFNB1 mutations causative of HI; and (iii) a class of patients without an established cause of HI. The combined group (i) and (ii) was defined as DFNB1 HI, whereas the last group (iii) was classified as non-DFNB1 HI.

In the three groups, mean AO was 0.8, 1.3, and 4.2 y.o., respectively. There was no statistically significant difference in AO between group (i) and (ii). Further analysis revealed statistically significant differences in AO between DFNB1 HI and non-DFNB1 HI groups (i and ii vs. iii) (p<0.0001). Furthermore, groups (i) and (ii) tested individually vs. non-DFNB1 HI also showed statistically significant differences in AO (p<0.0001) and (p=0.01), respectively (Figure 3).

Mutations in the DFNB1 *locus* were classified as either protein-truncating (nonsense, frame shift, or splice site-disrupting mutations) or non-truncating (missense

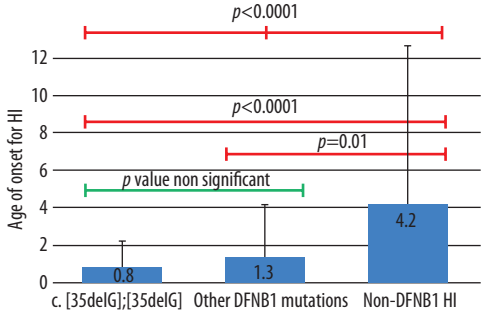

Figure 3. Comparison between mean age of onset for HI among three clusters of patients: homozygous for c.35delG mutation; with other DFNB1 mutations causative of HI; and without DFNB1 mutations

mutations). Based on this classification we identified two groups: one with bi-allelic truncating, and the second one with truncating/non-truncating mutations (Table 1). The mean AO for the group with biallelic truncating mutations was lower (0.8 y.o.) than in the group with truncating/non-truncating mutations (5 y.o.) but the difference was not statistically significant (p=0.08).

Discussion and Conclusions

Hearing loss is a genetically heterogeneous trait, although mutations in the DFNB1 *locus* represent the most common cause of HI, particularly in Caucasian patients with prelingual onset [6,10,26]. The DFNB1 *locus* comprises two genes (*GJB2* and *GJB6*), with respectively 385 and 28 different pathogenic mutations reported to date (The Human Gene Mutation Database 2016.4). As there are differences in the prevalence of DFNB1 mutations among ethnic groups, studies aimed at identifying the genetic background of HI should be performed based on data from a preliminary genetic analysis of the population of interest [27–30].

DOI: 10.17430/903762

According to the previously published data on the DFNB1 mutations frequency [24,31,32] in the Polish population, we have introduced the 3st-DFNB1app for the iterative diagnostic process in 1262 consecutive CI recipients. It should be emphasized that we did not exclude any CI patient from the study due to the fact that in individuals with potentially significant HI risk factors a genetic background of HI may be observed [33]. As far as we know, this is the largest CI cohort tested for the presence of DFNB1 mutations.

Considering the fact that, for 35% of CI patients, a full exhaustive molecular diagnosis was given after the first step of the proposed approach (without the need for more costly and time-consuming direct sequencing analysis), the 3st-DFNB1app is a fast, accurate, and inexpensive method for investigating the causes of HI in a dedicated group of patients (such as CI recipients). A full genetic diagnosis was established for almost 40% of patients with a CI, clearly emphasizing the significant role of the DFNB1 *locus* in the pathogenesis of HI in the Polish population. The percentage of fully (biallelic mutations) diagnosed CI patients in this study is substantially higher than in German, Chinese, or Iranian cohorts (40% *vs.* 13.4%, 7.2%, and 20%, respectively) [34–36], but it is similar to CI users from Slovakia, Romania, and Portugal. However, the spectrum of detected DFNB1 mutations, apart from c.35delG, in Slovakian, Romanian, and German patients [37–39] differs from ours, which strongly underlines the DFNB1 ethnic diversity. Although there is a high rate of DFNB1 mutations in the CI cohort, other causative genes as well as environmental risk factors should be taken into consideration in the remaining, genetically undiagnosed group.

A majority of DFNB1 mutations result in a congenital or very early AO of HI [40]; thus, a higher AO in the non-DFNB1 HI as compared to the DFNB1 HI group might be a prerequisite to consider participation of other genes as a cause of HI. Searching for other HI causative genes will be the subject of further study, and we predict that its results will substantially increase the ratio of CI recipients with an established genetic background of HI. The precise determination of HI background is important not only for proper genetic counseling and prediction of post-CI outcome, but in some cases also for adequate preparation and the way a surgical procedure is conducted [41].

Due to the fact that the ratio of DFNB1 monoallelic mutations among the tested cohort (9.8%) differs from that of the general Polish population (carrier frequency 3%) [31,32], there is a strong possibility that some of the CI patients may harbor a second pathogenic mutation within the *GJB6* gene or the non-coding regions within or encompassing the DFNB1 *locus*. Rather than the large deletions harboring the *GJB6* gene (because we excluded their presence in a large group of HI patients [32]), instead it may be that patients with only one pathogenic *GJB2* mutation may harbor other damaging variants across HI genes, which is in line with the theory of possible multifactorial or polygenic forms of inheritance in undiagnosed HI patients [42]. The vast majority of the detected DFNB1 causative variants were classified as truncating; moreover, we did not identify any individual with biallelic non-truncating mutations, which was to be expected due to the presence of profound HI and early HI onset in the whole CI cohort [40].

It is established that genetic backgroung is an important determinate of CI outcome. In particular, patients with causative mutations in genes expressed within the cochlea usually have a better post-CI score than those with mutations in genes expressed in the spiral ganglion [43,44]. Furthermore, patients with DFNB1-related HI reach significantly better CI outcomes than the group with acquired HI triggered by environmental factors [45]. It should also be emphasized that CI outcome also depends on other factors, such as the age at implantation, level of residual hearing, and period of device usage [46,47]. Thus, a thorough analysis of the correlation between detected genotype and CI outcome will be the subject of further studies.

Today, despite the wide availability of next generation sequencing (NGS), which allows for massive multigenic analysis, there is still a need for inexpensive, fast, screening methodologies. The 3st-DFNB1app should be the first preliminary test among CI patients prior to conducting a costly NGS analysis. The 3st-DFNB1app makes it possible to detect the genetic background of HI in as many as 40% of CI cases, thereby significantly reducing the number of patients undergoing an expensive NGS test.

Acknowledgements

This study was supported by NCN grant NCN 2011/03/D/NZ5/05592.

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