Hearing impairment in Estonia: An algorithm to investigate genetic causes in pediatric patients

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ABSTRACT

Purpose: The present study was initiated to establish the etiological causes of early onset hearing loss (HL) among Estonian children between 2000-2009.

Methods: The study group consisted of 233 probands who were first tested with an arrayed primer extension assay, which covers 199 mutations in 7 genes (GJB2, GJB6, GJB3, SLC26A4, SLC26A5 genes, and two mitochondrial genes – 12S rRNA, rRNA\textsubscript{Ser(UCN)}). From probands whose etiology of HL remained unknown, DNA analysis of congenital cytomegalovirus (CMV) infection and G-banded karyotype and/or chromosomal microarray analysis (CMA) were performed.

Results: In 110 (47%) cases, the etiology of HL was genetic and in 5 (2%) congenital CMV infection was diagnosed. We found mutations with clinical significance in GJB2 (100 children, 43%) and in 2 mitochondrial genes – 12S rRNA, rRNA\textsubscript{Ser(UCN)}). From probands whose etiology of HL remained unknown, DNA analysis of congenital cytomegalovirus (CMV) infection and G-banded karyotype and/or chromosomal microarray analysis (CMA) were performed.

Conclusion: This practical diagnostic algorithm confirmed the etiology of early onset HL for 115 Estonian patients (49%). This algorithm may be generalized to other populations for clinical application.

Key words: Sensorineural hearing loss; GJB2 gene; congenital cytomegalovirus; chromosomal microarray analysis

INTRODUCTION

Hearing loss (HL) is a sensory condition affecting millions of people worldwide, and although not life-threatening may impact social and professional life [1]. The global prevalence rate of children born with HL is approximately 1 to 2 per 1000 [2-5]. The etiology of HL is extremely heterogeneous. While environmental factors such as congenital cytomegalovirus (CMV) infection, prenatal rubella infection, prematurity and meningitis are thought to be the cause of 40-50% of HL cases, the remainder are genetic and result from mutations involving any one of numerous loci [2,6]. The incidence of genetic HL is increasing because acquired impaired hearing
from meningitis is decreasing as a consequence of improved prenatal and neonatal care, antibiotic therapy and vaccination programs [2,7-9]. Current research estimates that 1% of the 30,000—50,000 human genes are necessary for hearing, of which more than 95 independent genes and 170 loci have been identified as causes of HL [8-10].

The majority of deaf children are born to normal hearing parents (90-95%) and in most of these families there is no history of HL [7,8,11,12]. It is caused by the fact that the autosomal-recessive forms of HL are most common and usually more severe than the other forms of sensorineural hearing loss (SNHL) [13]. Mutations in the GJB2 gene, which have been mapped to 13q11-q12 and encode the gap junction protein connexin 26 (MIM 121011), represent a major cause of pre-lingual, non-syndromic, recessive deafness [13-15]. One specific mutation, c.35delG, accounts for the vast majority of the GJB2 mutations detected in Caucasian populations and represents one of the most frequent disease-associated mutations identified so far [13].

To identify the genetic basis of HL, mutation screening of certain genes is offered to patients with HL. In children with early onset HL, GJB2 and GJB6 gene are tested most often [13]. During the last fifteen years, major achievements have been made in detecting new deafness genes. Unfortunately, most diagnostic tests are still performed using the classical sequencing technology, which is expensive and time consuming. For this reason, only a very small set of genes is routinely screened for mutation with result that in a large percentage of individuals with HL, no genetic cause is identified [5]. An additional problem for extended diagnostic screening is that most genes for autosomal-recessive SNHL lead to congenital severe-to-profound hearing impairment (HI) that is indistinguishable between different genes [5].

The present study, was initiated to establish the genetic and congenital causes of early onset HL among Estonian children. Due to very heterogeneous genetic etiology of early onset HL, we decided to work-out diagnostic algorithm, which has been practically applied to care for children who are deaf or hard of hearing in Estonia.

**MATERIAL AND METHODS**

**Study group**
The overall study group consisted of 233 probands (children ranging in age from 0-18 years) who were referred to genetic evaluation between 2000-2009 from the whole of Estonia, with early or childhood onset HL as a main complaint. The diagnosis of HL was confirmed by audiologists in the hearing centers of Estonia. All the probands were selected from children who were referred to an otorhinolaryngologist due to a suspicion of HL or were selected from the newborn hearing screening (NBHS) program.

The NBHS program started in Estonia in 2004 and by 2009 88% of all newborns were included in the program. In Estonia, we have three-stage NBHS. All children whose HI with a pure tone average (PTA) <40 dB in the better ear should be identified by NBHS. The definition of the degree and type of HI was based on the most recent audiogram available. The severity of HI was graded by the degree of HL in the better ear as mild (21–40 dB), moderate (41–70 dB), severe (71–95 dB) and profound (greater than 95 dB).

All children were evaluated in one of two tertiary education hospitals, Tallinn Children’s Hospital for northern and western Estonia, and Tartu University Hospital for south eastern Estonia based on investigation program presented in the Fig. 1. In all cases of HL, family histories were collected, focusing particularly on the potential occurrence of HL in multiple generations. The clinical examination was performed with particular attention to dysmorphic features including growth parameters, facial phenotype, external ears, neck, skin, hair, eyes and digits, to exclude syndromic causes of HL.

**Cytogenetic and molecular investigations**
The detailed pathway of all investigations is presented in Fig 1.

All probands were tested between 2005 and 2009 with an arrayed primer extension (APEX) assay (Asper Biotech, Tartu, Estonia) [16] in the Department of Genetics of the United Laboratories of Tartu University Hospital. This microarray is capable of simultaneous evaluation of 199 mutations: several connexin genes (GJB2, GJB6, GJB3), mutations in 2 SLC26 anion transport genes (SLC26A4 and SLC26A5), and mutations in 2 mitochondrial genes (12S rRNA and tRNA<sup>Ser(UCA)</sup>). A complete description of this APEX assay including a list of the 199 mutations covered is published by Gardner et al. [16] and by Teek et al. [17]. Thirty-two patients were analyzed before 2005 for c.35delG mutation in GJB2 gene by PCR analysis; if the patients were homozgyous for mutation c.35deG, APEX array analysis was not performed.

In 15 probands, who were heterozygous for c.35delG or p.M34T mutation in GJB2 gene, the whole GJB2 gene was sequenced. Five probands, who had heterozygous mutation in SLC26A4 gene, the SLC26A4 gene was sequenced, and multiplex ligation-dependent probe amplification (MLPA) analysis was performed.

In 55 children, a geneticist decided that regular G-banded chromosomal analysis should be performed from peripheral blood lymphocytes.

From 126 probands whose etiology of HL remained unknown after DNA testing with the APEX method, 96 patients were chosen for the DNA analysis of CMV infection from neonatal screening cards—blood stored on Guthrie cards. Since the neonatal screening program for the whole of Estonia for phenylketonuria started in 1993, neonatal
screening cards were available for 85 (88.5%). DNA was extracted from a Guthrie test card specimen using QIAamp DNA Mini Kit (50) using the manufacturer’s instructions (Qiagen). The CMV copy number was established by Real-Time PCR method using Qiagen artus CMV Kit CE.

From 121 probands with HL of unknown etiology (normal results in APEX array, CMV testing and in some of them on regular karyotyping) we selected 24 children who, in addition to HL, had subtle facial dysmorphism, a failure to thrive and/or developmental or behavioral problems. They did not fit to any known dysmorphic syndrome. For exclusion of syndromic etiology we used the London Dysmorphology Database [18]. In order to detect DNA copy number changes in selected patients, chromosomal microarray analysis (CMA) was performed, using the Illumina HumanCytoSNP-12 version 1.0 BeadChip (for 4 samples Illumina Human CNV370-Duo BeadChip was used). HumanCytoSNP-12 BeadChip contains 220,000 markers targeting all regions of known cytogenetic importance, including subtelomeric regions, pericentromeric regions, sex chromosomes, and targeted coverage in ca. 400 additional disease-related genes. The signal intensity (log R ratio) and allelic composition (allelic frequency) of genotyped markers were analyzed using GenomeStudioV2010.1, KaryoStudioV1.0 (Illumina Inc.) and QuantiSNP v1.1 [19] software. The found regions were compared with the reference sample-set (1000 unrelated samples from Estonian Genome Center, University of Tartu) representing ca. 0.1% of Estonia’s total population [20] to exclude the population specific variations. Quantitative-PCR study was applied to confirm the detected aberrations and to verify if these are inherited or not.

RESULTS

We screened 233 probands with early onset HL with APEX array analysis and found 100 patients (42.9%) with GJB2 bi-allelic mutations (Tab. 1). Our results include 73 (31%) who were homozygous for c.35delG mutation, 7 (3%) who were homozygous for the p.M34T mutation, and 5 (2%) who had c.35delG/p.M34T compound heterozygosity. The genotypes of those probands and the correlation with their phenotype were published previously [17]. There were ten c.35delG heterozygous patients and five p.M34T heterozygous patients, in whom the mutation in the other allele was not identified on GJB2 gene sequencing. Thus, heterozygosity of c.35delG and p.M34T may be coincidental finding as the heterozygote frequency of those mutations is high in Estonia – 1:22 and 1:17, respectively [17]. Several instances of heterozygosity of GJB2 mutations in HI patients have been reported, but the mechanism behind silencing the other GJB2 allele has not been established yet. In the Estonian population with a high carrier frequency it is impossible to discriminate between coincidental carrier (and an undetected genetic cause), or the above-mentioned mechanism.

We did not find any children with GJB3 and GJB6 mutations in our study group, including 2 large deletions in the GJB6 gene, del(GJB6-D13S1830) and del(GJB6-D13S1854). In 5 probands (2.2%) a heterozygous mutation in SLC26A4 (Pendred) gene was found; in 4 cases p.L597S and in 1 case p.F335L mutation (Tab. 1). The mutation in the other allele was not identified by sequencing or MLPA analysis of SLC26A4 gene sequencing in any of them. The identification of a single mutant allele in SLC26A4 gene was considered diagnostic [21,22].

We identified a heterozygous IVS2-2A>G (by HGVS c.-53-2A>G) change in the SLC26A5 gene in 4 patients (1.7%) with early onset HL. We did not find any instances of homozygosity for this splice variant in the probands or in their family members. We have concluded previously that the mutation does not seem to be a dominant one [23]. In other words, the SLC26A5 mutations are inherited in an autosomal recessive manner and that 2 mutations are necessary for a phenotype to occur.

In the study group we found 2 patients (0.9%) whose HL was caused by a mutation in the mitochondrial DNA; firstly the mutation m.1555A>G in the mitochondrial 12S r-RNA gene and secondly the mutation 7472insC (by HGVS m.7471dupC) in the mitochondrial serine tRNAUCN gene.
In probands whose etiology of HL remained unknown after DNA testing with the APEX method, we conducted DNA analysis of congenital CMV infection if the neonatal screening card was available. We found positive results in 5 patients (2%). All patients with CMV infection had bilateral or unilateral SNHL and in 2 typical white matter lesions were also found, which confirmed the CMV infection diagnosis on clinical grounds.

G-banded chromosomal analysis was conducted for 55 (23.6%) patients. The indication for chromosomal analysis was decided during clinical evaluation by a clinical geneticist. Four chromosomal abnormalities were diagnosed. One patient had Down syndrome [24]. In the 3 patients (Tab. 1) who had a balanced chromosomal aberration, we performed whole-genome genotyping using Human370CNV-Duo BeadChips to detect submicroscopic chromosomal rearrangements in the breakpoint region; we did not find any abnormalities. A search in the Hereditary Hearing Loss Homepage [10] showed some loci for autosomal dominant HL – DFNA (DFN stands for deafness and A designates the autosomal dominant locus), located to the broken chromosomal region in 2 patients. The first patient had karyotype 46,XY,t(2;7)(q21;q32); and DFNA50 was located to region 7q32.2 [25]. Autosomal-dominant HL is caused by point mutations in the seed region of MicroRNA-96 (MIR96) gene [26]. The third patient had karyotype 46,XY,t(6;7)(p21.1;q36); DFNA13 was located to 6p21 and the gene was COL11A2 [27,28], DFNA21 to 6p21 [29] and DFNA31 to 6p21.3 [30] – in last two loci, the genes were not known. However, the patients may have some rearrangements in the breakpoints, but any detailed molecular studies are not available for us at the present, therefore the possible changes are not detectable and not known to us. Further studies are needed for these three patients with chromosomal translocations.

Among 24 investigated patients, who, in addition to HL, had subtle facial dysmorphism, a failure to thrive and/or developmental or behavioral problems, 4 potentially pathogenic regions in 4 separate patients with the loss of 1 allele were found (17%) in CMA analysis (Tab. 1).

**Case 1:** An 8.5-year-old girl with mild SNHL and a dysmorphic facial phenotype – upslanting palpebral fissures, hypertelorism, prominent glabella, broad nasal bridge, hypoplastic alae nasi, prominent cupid’s bow, high palate and dysmorphic ears (Fig. 2a and Fig. 2b). She had moderate intellectual disability, and mild supravalvular pulmonary stenosis diagnosed at the age of 2. Family history showed no occurrence of HL or dysmorphism. CMA identified ~2.94-Mb size deletion in chromosomal region 12q13.3-q14.1 (55,604,593-58,543,784 basepairs (bp), according to National...
Center for Biotechnology (NCBI) version 36/hg 18), which probably occurred de novo (this deletion was not found in her mother and her father was not available for investigation). Non-syndromic autosomal dominant HL locus, DFNA48 is located to chromosome 12q13-q14 [31]. The MYO1A gene, which is located within DFNA48 locus, is the first myosin I family member found to be involved in causing HL. Donauthy et al. [32] identified one nonsense and six missense mutations in MYO1A gene in unrelated patients who were affected by SNHL of variable degree, usually ranging from moderate to severe but never profound. In some families, the autosomal-dominant pattern of transmission with either or both variable penetrance and expression was documented. The MYO1A gene has a cochlear expression and is considered to play a major role in hair-cell function [32]. Therefore we can conclude that the haplo-insufficiency of the MYO1A gene was responsible for the development of SNHL in our Case 1.

Case 2: A 7-year-old boy with poor weight gain (-2.5 SD), severe microcephaly (-5 SD) and trigonocephaly. He had facial dysmorphism, symphalangism, contractures of large joints, hyperopia, strabismus, bilateral conductive HL, genital abnormality, psoriasis vulgaris and tracheoesophageal fistula. He had profound intellectual disability with stereotypic movements. Analysis with CMA detected a 5.9 Mb deletion in chromosome band 17q22–q23.2 with breakpoints between 48,200,000–48,300,000 bp and 54,200,000–54,300,000 bp (according to NCBI 36/hg 18). Haplo-insufficiency of the NOG gene has been implicated in the development of conductive HL, skeletal anomalies including symphalangism, contractures of joints, and hyperopia in this patient and may also contribute to the development of either or both tracheo-esophageal fistula and esophageal atresia. Detailed information (including Figures) was published previously [33].

Case 3: A 10-year-old girl with mild SNHL (Fig. 3a), failure to thrive (-2 SD) and a mildly dysmorphic face – small eyes, upslanted palpebral fissures, high palate, protruding ears (Fig. 2c and Fig. 2d). In addition, mild thorax deformation, long fingers and long toes were also noted. Patient’s mother also had HI, which developed in adult age. She had bilateral steeply sloping (mild to severe) SNHL (Fig. 3b). CMA was identified ~0.74-Mb size deletion in 3p26.2 region (2,861,527-423,392,008).

Figure 2. (a, b) a facial phenotype of case 1; (c, d) a facial phenotype of case 3; (e, f) a facial phenotype of case 4.
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3,602,205 bp, according to NCBI 36/hg 18), which she inherited from her father. The hearing of the patient's father was normal. To date, no genetic locus in the 3p25-pter region has been linked to dominant or recessive HL in humans [10] and as she inherited 3p26.2 microdeletion from her healthy father, we concluded that this microdeletion was not causing her phenotype.

Case 4: A 10-year-old boy with bilateral profound SNHL (Fig. 3c), delayed psychomotor development, muscular hypotonia, paresis of n. facialis on the right side, and mildly dysmorphic face – downsloanted palpebral fissures, widely spaced teeth, small mouth and thin upper lip (Fig. 2e and Fig. 2f). There were no other family members with hearing problems. CMA identified 0.54-Mb size deletion in 1p33 region (48,649,845-49,208,694 bp, according to NCBI 36). Analysis of DNA samples from his father and his mother’s sister revealed that the deletion in the patient appeared de novo. The SLC5A9 gene (solute carrier family 5, sodium/glucose co-transporter, member 9) was located in 1p33 region. The SLC5A9 gene possibly acts as a Mannose/1,5-anhydro-D-glycitol/fructose transporter in the intestine and kidney and is required mainly for protein glycosylation [34]. It is known that some other anion exchangers (for example SLC26 gene family) are multifunctional and play intriguing roles in normal physiology and human pathophysiology including in pathophysiology of HL [35]. Therefore, we might assume that the haplo-insufficiency of SLC5A9 gene may play a role in the development of profound SNHL in our case 3, but we cannot confirm it.

DISCUSSION

The etiology of early onset HL was confirmed in 115 cases (49%) and in 110 (47%) a genetic basis was identified. The most common were GJB2 mutations, which were identified on 2 alleles in 100 (42.9%) children. This is in line with the literature data; mutations in the GJB2 gene are responsible for as much as 50% of early onset HL in some European populations [13-15]. In our neighboring countries, in Russia molecular genetic analysis has detected mutations in GJB2 gene in 48.6% of children, which is similar to our results [36]. At the same time in northern Finland, GJB2 mutations were found in 21.1% children with HL [37]. However, genetic testing in North-America identified bi-allelic GJB2/GJB6 hearing loss-associated variants in 24.7% of infants with a significantly lower prevalence in Hispanic infants (9.1%) [38,39]. Controversially, mutations of GJB2 genes accounted for as much as 64.7% of non-syndromic HL in China [40]. Therefore, there is large variability of the prevalence of GJB2 mutations among different populations with inherited HL.

Two large deletions in the GJB6 gene, del(GJB6-D13S1830) and del(GJB6-D13S1854), are usually found in compound heterozygosity with a GJB2 coding mutation and cause HL that is significantly worse than most other GJB2 mutations, possibly because expression of both copies of GJB2 and one copy of GJB6 is abolished [5]. The del(GJB6-D13S1830) deletion is the second most frequent genetic cause of non-syndromic prelingual HL, after the c.35delG mutation in GJB2 in other populations [41]. We did not identify any children with GJB6 deletions in our study group. However, the del(GJB6-D13S1830) mutation is the most frequent in Spain, France, the United Kingdom, Israel, and Brazil (5.0–9.7% of all DFNB1 alleles; B1 designates the first autosomal recessive locus); occurs less frequently in the USA, Belgium, and Australia (1.3–4.5% of all DFNB1 alleles), and has not yet been found in Austria, Turkey, or China [41].

Mutations in the GJB3 gene have been pathologically linked to non-syndromic autosomal dominant (DFNA2) or recessive HL and erythrokeratoderma variabilis without HL [8,42]. We did not identify any occurrences of GJB3 mutations in our study among the patients with HL – confirming a low prevalence of GJB3 mutations in Estonia. Mutations in GJB3 have originally been reported as the cause of DFNA2 in Chinese patients [43]. Variations in the GJB3 gene have
also been linked to non-syndromic HL in Brazilian patients [42]. A previous study shows that while mutations in the GJB3 gene cause HL in Chinese and Brazilian populations, their prevalence is non-existent or extremely low in European populations and Caucasians in general [44].

Mutations in SLC26A4 gene are the second most frequent cause of autosomal recessive HL after GJB2 gene, which were found in 5-10% of childhood SNHL patients [45]. The associated phenotypic spectrum ranges from Pendred syndrome at one extreme to isolated non-syndromic HL with enlarged vestibular aqueduct at the other [5,21]. To date, more than 160 SLC26A4 mutations have been identified [21,46]. In northern Europe, 4 mutations (p.L236P, p.T416P, p.E384G and IVS8+1G>A) are found quite frequently [5]. In patients with 1 mutant allele of SLC26A4, HL and enlargement of the vestibular aqueduct may be associated with a second, undetected SLC26A4 mutation or epigenetic modifications of SLC26A4 [21,22]. Therefore, identification of a single mutant allele of this known recessive condition in our 5 patients (2%) confirmed hereditary HL.

The mutation m.1555A>G is the most common mitochondrial mutation associated with HL, found in many families worldwide and it can be found in 0.6-2.5% of the Caucasian clinical population with non-syndromic SNHL [47,48]. SNHL for this mutation may be triggered by the use of aminoglycosides, and may also occur without exposure to these drugs. The penetrance of mutation m.1555A>G differs between families; in the absence of aminoglycosides, the clinical phenotype may be variable even among family members [47,48]. We found only 1 patient with the mutation m.1555A>G. Nevertheless, there may be carriers of the m.1555A>G mutation among adults with late onset HL, but because genetic HL is thought to be early onset, and severe or profound, they are rarely referred to genetic evaluation for their HL.

The mutation 7472insC (by HGVS m.7471dupC) in the tRNA\textsubscript{Ser(UCN)} gene occurs more frequently in European populations than others [49]. Most individuals carrying mutation 7472insC have progressive SNHL, accompanied occasionally by one of a variety of widespread neurological diseases including ataxia, dysarthria, and myoclonic seizures [47]. The 7472insC mutation alone is usually sufficient to cause HL, and when present in very high levels can also lead to neurological dysfunction [47,49]. We found 1 child with 7472insC mutation in tRNA\textsubscript{Ser(UCN)} gene. Neurological symptoms in a patient and their mother with profound HL and carrying the same mutation were not known to us.

We performed CMA in 24 children with still unknown etiology of HL, and mild dysmorphism and/or other developmental complaints selected. Four chromosomal regions in 4 separate patients with the loss of one allele were found (17%). In cases 1 and 2 – 2.94-Mb size microdeletion in region 12q13.3-q14.1 and 5.9-Mb size deletion in 17q22-q23.2 region – we found clear connection between genotype and hearing phenotype (MYO1A gene and NOG gene, respectively). However, we did not find clear connection between 0.74-Mb size microdeletion in region 3p26.2 and 0.54-Mb size deletion in 1p33 region in cases 3 and 4. We had similar diagnostic yield in our small study group as it is shown in patients with intellectual disability, autism spectrum disorder, and multiple congenital anomalies of unknown causes – 15-20% [50-52]. Therefore, for patients who show developmental delay or congenital anomalies in addition to their main health problem i.e. HL, CMA is very much indicated.

The most common environmental (non-genetic) cause of HL is estimated to be congenital CMV infection [5] with overall birth prevalence at 0.64%. In our study group, in 5 (2.2%) probands, HL was caused by congenital CMV infection. Diagnosis is difficult, as only 11% of infected infants have non-specific symptoms at birth, and the definition of symptomatic varies between studies [53]. HL is a common sequelae of congenital CMV infection. Grosse et al. [54] found that HL occurs in 30-40% of children symptomatic with congenital CMV at birth and in 5-10% of children with asymptomatic infections. More than two thirds of children congenitally infected with CMV develop HL only months or years after birth, therefore HL may be missed by a hearing screening at birth [55,56].

In 24 cases (10%) abnormal findings were found, but there were co-incidental findings or there was no proven influence to the phenotype. Altogether, in 51% of cases the etiology of HL remained unknown. Next generation sequencing may lead to a genetic diagnosis in roughly 50% of unknown autosomal recessive deafness cases in the nearest future [57].

CONCLUSION

For specifying the etiology of early onset HL, we used different cytogenetic and molecular tests including APEX microarray and CMA. We were able to confirm the etiology of early onset HL in 49% of patients in our cohort. The APEX microarray is capable of simultaneously evaluate: 199 mutations in 7 genes (GJB2, GJB6, GJB3, SLC26A4, SLC26A5, 12S rRNA and tRNA\textsubscript{Ser(UCN)}). We found mutation(s) with clinical significance in GJB2 (100 patients), in SLC26A4 (5 patients) and in 2 mitochondrial genes (2 patients). APEX test was created in Estonia and was reachable for us with low cost and short diagnostic time. In the future it would be more practical to perform GJB2 gene sequencing first in the children with early onset HL. CMA improves significantly the diagnostic yield in patients with HL, dysmorphism and developmental delay. However, in at least half of the cases it was not easy to give a clear statement if the found submicroscopic chromosomal abnormalities are pathogenic or not.
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