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| Citation       | Yan, D., G. Xiang, X. Chai, J. Qing, H. Shang, B. Zou, R. Mittal, et al. 2017. “Screening of deafness-causing DNA variants that are common in patients of European ancestry using a microarray-based approach.” PLoS ONE 12 (3): e0169219. doi:10.1371/journal.pone.0169219. http://dx.doi.org/10.1371/journal.pone.0169219. |
|----------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Published Version | doi:10.1371/journal.pone.0169219                                                                                                                                                                                                                             |
| Citable link    | http://nrs.harvard.edu/urn-3:HUL.InstRepos:32072006                                                                                                                                                        |
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Screening of deafness-causing DNA variants that are common in patients of European ancestry using a microarray-based approach

Denise Yan1‡, Guangxin Xiang2‡, Xingping Chai1, Jie Qing1, Haiqiong Shang1, Bing Zou1, Rahul Mittal1, Jun Shen4,5, Richard J. H. Smith6, Yao-Shan Fan7,8, Susan H. Blanton1,7, Mustafa Tekin1,7, Cynthia Morton4,9,10, Wanli Xing2,3, Jing Cheng2,3*, Xue Zhong Liu1,3,8*

1 Department of Otolaryngology, University of Miami Miller School of Medicine, Miami, Florida, United States of America, 2 National Engineering Research Center for Beijing Biochip Technology, Beijing, China, 3 Tsinghua University School of Medicine, Beijing, China, 4 Department of Pathology, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 5 Laboratory for Molecular Medicine, Partners Personalized Medicine, Cambridge, Massachusetts, United States of America, 6 Department of Otolaryngology - Head and Neck Surgery, Carver College of Medicine, University of Iowa, Iowa City, Iowa, United States of America, 7 Department of Pathology, University of Miami Miller School of Medicine, Miami, Florida, United States of America, 8 Dr. John T. Macdonald Department of Human Genetics and John P. Hussman Institute for Human Genetics, University of Miami Miller School of Medicine, Miami, Florida, United States of America, 9 Department of Obstetrics, Gynecology and Reproductive Biology, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 10 Division of Evolution and Genomic Science, School of Biological Sciences, Manchester Academic Health Science Center, University of Manchester, United Kingdom

‡ These authors are co-first authors on this work.
* xliu@med.miami.edu (XZL); jcheng@tsinghua.edu.cn (JC)

Abstract

The unparalleled heterogeneity in genetic causes of hearing loss along with remarkable differences in prevalence of causative variants among ethnic groups makes single gene tests technically inefficient. Although hundreds of genes have been reported to be associated with nonsyndromic hearing loss (NSHL), GJB2, GJB6, SLC26A4, and mitochondrial (mt) MT-RNR1 and MTTS are the major contributors. In order to provide a faster, more comprehensive and cost effective assay, we constructed a DNA fluidic array, CapitalBioMiamiOto Array, for the detection of sequence variants in five genes that are common in most populations of European descent. They consist of c.35delG, p.W44C, p.L90P, c.167delT (GJB2); 309kb deletion (GJB6); p.L236P, p.T416P (SLC26A4); and m.1555A>G, m.7444G>A (mtDNA). We have validated our hearing loss array by analyzing a total of 160 DNAs samples. Our results show 100% concordance between the fluidic array biochip-based approach and the established Sanger sequencing method, thus proving its robustness and reliability at a relatively low cost.

Introduction

In industrialized nations, one child in 1,000 is born deaf, and an additional one in 300 births will be diagnosed with a milder degree of hearing loss. A further 1/1,000 children become...
profoundly hearing disabled before adulthood [1, 2]. Overall, hearing loss affects up to 8% of the world’s population [3, 4]. Hearing loss can be due to environmental or genetic factors, or a combination thereof. At least 50% of prelingual hearing loss is due to genetic factors in industrialized nations [5]. Non-syndromic hearing loss (NSHL) is the most common form of neurosensory deafness, and account for 70% of inherited hearing impairment. To date, more than 80 genes, with approximately 1,000 pathogenic variants, and 150 loci have been identified for NSHL (http://hereditaryhearingloss.org/). The spectrum of deafness-associated genetic variants varies greatly among regions and ethnicities [6, 7]. Mutations in \textit{GJB2} are a major cause of autosomal recessive nonsyndromic hearing loss (ARNSHL) in many populations [8]. The most frequent pathogenic variants are c.35delG in European descent populations [9], c.167delT in Ashkenazi Jews [10], and c.235delC in East Asians (Japanese, Koreans, and Chinese) [11, 12, 13, 14]. In contrast, the \textit{GJB6} deletion mutation del(GJB6-D13S1830) is common in France, Spain, the United Kingdom and Israel, accounting for 5.9–9.7% of all DFNB1 alleles [15]. Pathogenic variants in pendrin encoded by \textit{SLC26A4} can cause both nonsyndromic (DFNB4) and syndromic deafness (Pendred syndrome; PDS). PDS is thought to be one of the most common forms of syndromic deafness and pathogenic variants of \textit{SLC26A4} were reported to be the second most frequent cause of ARNSHL worldwide [16]. However, there are considerable differences in reported percentages of individuals segregating \textit{SLC26A4} variants. The percentage of probands in whom biallelic variants are found varies accordingly, from a low of 13% [17] to a high of 62% [18]. This variation may be attributed to differences in selection criteria of each study and/or to the patient population being tested. Pathogenic variants in the mitochondrial (mt) 125 ribosomal RNA subunit gene (\textit{MT-RNR1}) have been associated with aminoglycoside ototoxicity in an estimated 2% of deaf individuals in the United States [19, 20]. One of the most common mitochondrial variants is the m.1555A>G substitution in \textit{MT-RNR1} which can be found in 0.6–2.5% of patients of European descent, 3–5% of Asians and as high as 17% of the Spanish population with NSHL [21]. The m.7444G>A mutations in the tRNA serine 1 gene (\textit{MTTS1}) has been found in patients with maternally inherited sensorineural hearing loss, but is less likely to cause hypersensitivity to aminoglycoside.

The extraordinary genetic heterogeneity of hearing loss has been a great challenge for molecular testing: analysis of multiple genes using conventional gel-based and traditional Sanger sequencing techniques is expensive, time-consuming and cumbersome. The DNA microarray, or biochip, is a hybridization-based genotyping method that allows simultaneous multi-gene variant analysis.

Multiplexed microarray platforms provide parallel detection capabilities that make it ideally suited to genotyping of genetically heterogeneous conditions such as deafness. To achieve an efficient method for a genetic diagnosis of hearing loss, we developed a genetic hearing loss DNA chip, CapitalBioMiamiOtoArray, that allows simultaneous analysis of the nine most common mutations in patients of European descent in the genes \textit{GJB2}, \textit{GJB6}, \textit{SLC26A4}, \textit{MT-RNR1} and \textit{MTTS1}. In this study we have validated our hearing loss biochip by analyzing a total of 160 DNA samples. Availability of an inexpensive and extensive screening test for common variants for hearing loss will result in affordable testing, improved diagnosis, more accurate genetic counseling, and eventually in improved management of hearing loss.

**Materials and methods**

**Subjects**

Genomic DNA was extracted from 136 whole blood samples and 24 dried blood spots. Of these, 101 were from deaf patients and 59 from normal hearing controls. For validation, 60 samples were pre-tested for the specific variants in \textit{GJB2}, \textit{GJB6} and \textit{SLC26A4} genes by Sanger
sequencing. Mitochondrial DNA (mtDNA) genes were subjected to PCR-RFLP (Restriction Fragment Length Polymorphism) analysis using specific primers [22]. The diagnosis of sensorineural hearing loss was established via standard audiometry in a sound-proofed room according to current clinical standards. HL was congenital or prelingual-onset with a severity ranging from moderate to profound. Clinical evaluation included a thorough physical examination and otoscopy in all cases. This study was approved by the University of Miami Institutional Review Board, USA (permit #20010415). The cohort of participants was recruited between 2003 and 2016. A signed informed-consent form was obtained from each subject or, in the case of a minor, from the parents.

The deafness fluidic microarray
The hearing loss fluidic array, CapitalBioMiamiOtoArray (CapitalBio Corporation, Beijing, China) was designed to detect the following nucleotide changes: GJB2 [c.35delG, p.W44C (c.132G>C), p.L90P (c.269T>C), c.167delT]; GJB6 c.309kb deletion; SLC26A4 [p.L236P (c.707T>C), p.T416P (c.1246 A>C)]; and mtDNA m.1555A>G and m.7444G>A mutations. The nine mutations were selected based on their high prevalence in patients of European descent. The 167delT is common in Ashkenazi Jews. The mtDNA changes 1555A>G and 7444G>A are frequently found in southern European populations [22, 23, 24, 25].

The Amplification refractory mutation system (ARMS) technique for detecting known point mutations described by Newton et al [26] was applied. The method requires that 3’-OH terminal mismatched primers are refractory to extension by the DNA polymerase and thus the terminal 3’-nucleotide only of a PCR primer be allele specific; the primer is synthesized in two forms. The wild type form is refractory to PCR on mutant template DNA and the ‘mutant’ form is refractory to PCR on normal DNA. We have previously described in details the design, optimization and validation of tagged primers and multiplex PCR conditions [27, 28, 29] using allele-specific amplification primers to the wild type (WT) and variant sequences (M) and common primers (S1 and S2 Tables).

The microarray is composed of a fluidic manifold made of polycarbonate glued on the glass slide to introduce a dynamic hybridization for signal enhancement and acceleration of reaction kinetics (Fig 1A). The tag probes contain a poly (dT) spacer and a 5’ amino group, are diluted in 50% dimethyl sulfoxide (DMSO) to a final concentration of 15 micromole and are spotted with a SmartArrayer 136 spotter (CapitalBio) on the aldehyde-functionalized glass slides. Each tag probe is spotted in triplicate. The diameter of each spot is approximately 150 micrometers. The pattern of the distribution of probes on the microarray is shown in the microarray layout (Fig 1B) (Fig 1A and 1B: Microarray design); the different numerals refer to different genetic loci, whereas upper case alphabetic letters W and M represent wild-type and mutant-type variant probes, respectively. A number of control probes are also represented: QC and NC are the positive and negative controls for the spotting of probes during array manufacture, PC and BC are the positive and negative controls for the hybridization reaction, IC is the internal control for the gene amplification, and MC is the positive control for magnetic beads. The QC, IC, BC and PC were spotted in 6 to 12 replicates.

Magnetic bead-based DNA microarray analysis. Mutations were detected using the nine common deafness mutations detection kit for populations of European descent (CapitalBio, Beijing). Each DNA sample was amplified in two 25-μl multiplex PCR reactions (A and B) as recommended by the manufacturer. Amplification primers were designed according to the principle shown in Fig 2 (Fig 2. The principle for design of the amplification primers). Each PCR reaction is prepared by combining 5 μl template DNA (15–150 ng), 10 μl mixture of amplification primer and 10 μl mixture of amplification reagent to the 25 μl reaction system.
For degradation of carryover contaminating molecules prior to amplification, we applied a procedure that makes use of dUTP incorporation instead of dTTP during amplification, which is preceded by treatment of PCR reaction mixtures with uracil-N-glycosylase (UNG) at 37˚C to degrade uridine-containing carryover products [30], and subsequent cleavage of apyrimidinic polynucleotides at elevated temperature (95˚C for 15 min) to remove contaminating U-DNA from the sample. The amplification was performed in a Master Cycler...
nexus X2e (Oppendorf) by setting the temperature rate using the RAMP function of the PCR instrument under the following conditions: 37˚C for 10min, 95˚C for 15 min; 96˚C for 1 min; then: 94˚C for 30 sec, ramp (0.4 C/s) to 55˚C, hold for 30 sec; ramp (0.2 C/s) to 70˚C, hold for 45 sec for 32 cycles; followed by 60˚C for 10 min, 4˚C soak. The total reaction time was about 3 hours and 20 minutes. Amplified products from the two tubes were mixed, and the amplicons were then captured by magnetic beads. Captured amplicons were denatured by NaOH, and only the single stranded DNA attached to the magnetic beads was collected and dissolved in hybridization buffer. Approximately 30 μL of the hybridization solution was then injected into the sample inlet to cover the central hexagonal array region. The sample inlet and the air outlet were then sealed and the chip was placed into the Easy Array 3A (CapitalBio Corporation) for a dynamic hybridization at 55˚C for 20 min followed by washing at 42˚C for 2 min. Due to the flowing reaction controlled by the instrument, the hybridization and washing process was about four times faster than the conventional static diffusion reaction. The total time required for PCR amplification, hybridization, washing and detection was about 4 hours.

**Results**

DNA from 101 deaf patients was used to validate the deafness chip. Of these, 60 subjects were pre-screened and known to be positive for one or two specific variants (s) present on the chip; 18 had one \(\text{GJB2}\) variant, 21 had two \(\text{GJB2}\) variants (homozygous or compound heterozygous), four were digenic for \(\text{GJB2-}\text{c.35delG}\) and \(\text{GJB6-309kb}\) deletion. For \(\text{SLC26A4}\), 10 of the subjects were heterozygous and two were homozygous for variants on the chip. All the five individuals tested positive for the m.1555A>G variant in \(\text{MT-RNR1}\) are homoplasmic as well as two deaf subjects among the 41 cases of unknown molecular genetic etiology (Table 1).

The results showed 100% concordance between the fluidic array biochip-based approach and the established PCR protocol. It did not detected any unreported variant in the validation set as expected. Of 10 individuals found with one \(\text{SLC26A4}\) variants, two were biallelic based on Sanger Sequencing, because the c.1001+1 G>A and c.412G>T variants in these two individuals were not present on the chip (Table 1). There were no false-positive results in 59 negative controls.

**Table 1. Mutations detected in the deaf individuals.**

| Gene      | Genotype            | Number of Subjects |
|-----------|---------------------|--------------------|
| \(\text{GJB2}\) | 35delG/35delG       | 11                 |
| \(\text{GJB2}\) | 35delG/WT           | 10                 |
| \(\text{GJB2}\) | 167delT/167delT     | 2                  |
| \(\text{GJB2}\) | 167delT/WT          | 2                  |
| \(\text{GJB2}\) | L90P/L90P           | 5                  |
| \(\text{GJB2}\) | L90P/WT             | 6                  |
| \(\text{GJB2}\) | 35delG/L90P         | 3                  |
| \(\text{SLC26A4}\) | L236P/WT           | 5                  |
| \(\text{SLC26A4}\) | T416P/T416P        | 2                  |
| \(\text{SLC26A4}\) | T416P/WT          | 1                  |
| \(\text{SLC26A4}\) | L236P/c.1001+1 G>A | 2                  |
| \(\text{SLC26A4}\) | T416P/c.412G>T     | 2                  |
| \(\text{MT-RNR1}\) | 1555A>G/1555A>G   | 7                  |
| \(\text{GJB2}/\text{GJB6}\) | 35delG/309kb   | 4                  |
|           |                     | 62                 |

doi:10.1371/journal.pone.0169219.t001
Discussion

The profound genetic heterogeneity of deafness has proved a challenge for genetic testing using conventional approaches. It is neither financially or nor practically feasible to screen for causative variants in many cases, because they can be in one of several hundred genes, many of which have many exons. Demand for genetic testing for deafness has risen dramatically over the last decade, in part due to identification of many genes associated with hearing loss and to the introduction of Universal Newborn Hearing Screening Programs in many countries. There is therefore a need for simpler, cheaper, and more comprehensive methods for screening for variants associated with hearing loss.

Patterns of high frequency pathogenic variants in diverse populations and their population-wide distributions make it efficacious to target ethnically restricted disease variants in screening tests. In the present study, we have used an oligonucleotide-array based chip approach for simultaneous analysis of nine mutations in five genes. Using this chip, rapid screening for specific variants at different genetic loci can be performed. The technology can also easily be expanded or modified, according to further epidemiological surveys of carrier rate and the spectrum of genetic variants associated with hearing loss in certain ethnic backgrounds. We have validated the hearing loss chip on DNA obtained from 60 deaf individuals who were previously screened and known to carry one or two pathogenic variants included on the chip. Analysis detected all known variants in the correct zygotic and plasmic state in nuclear and mitochondrial genes. These results prove the accuracy and reliability of the custom capture experiment.

Population-based genetic testing has been proposed as a major vehicle for translating genetic and genomic advances into human health and disease. Testing for pathogenic variants in common deafness causing genes has several potential advantages over conventional hearing loss evaluation without genetic testing. Anticipated benefits from genetic screening include: 1) A need for more expensive or invasive procedures may be precluded, such as tests to identify prenatal infections, electrocardiograms and other heart and thyroid function tests. Inner ear imaging techniques such as magnetic resonance imaging (MRI) or a computed tomography (CT) scan can be avoided; 2) Earlier detection of hearing loss for making informed choices of interventions such as hearing aids, cochlear implants, or sign language proven to be effective in significantly improving language ability and quality of life outcomes can be realized. Research evidence has demonstrated the practicability, cost-efficiency and benefits of universal newborn hearing screening (UNHS) [1, 9, 31, 32, 33]. However, UNHS programs may suffer from inherent limitations such as failure to detect children with slight or mild hearing loss, because the target condition for the majority of UNHS programs is permanent hearing loss >35dB [34]. Additionally, children with late-onset or progressive hearing loss may be missed by UNHS, because their hearing is normal or near-normal at birth. Lifetime costs of all care related to deafness and lost productivity have been estimated to be $1.1 million USD per birth cohort of 80,000 children [35]; 3) Predictive information about possible progression of a hearing disorder may be provided, which can facilitate parents and professional teams in implementation of an individualized health-maintenance strategy; 4) Monitoring of associated clinical manifestations and complications, particularly for certain syndromic forms of hearing loss for early management may be enabled; 5) Obtaining an etiologic diagnosis provides the basis for precise genetic counseling including information on the chance of recurrence in the family that can inform reproductive decisions. Lastly, 6) Information pertinent to aminoglycoside antibiotic ototoxicity risk factors can guide medical care (e.g., avoiding administration of aminoglycoside antibiotics among those with the MTRNR1 m.1555A>G variant to prevent hearing loss.
Continuous advances in DNA sequencing technologies have allowed interrogation of very large numbers of variants in a highly rapid and inexpensive manner. As knowledge of the variant spectrum in specific populations increases, selection of variants in a population specific manner can be made. Routine screening can thus be performed at a reduced cost for a larger number of samples.

There are many reliable, relatively simple and inexpensive variant detection techniques available for a faster, more comprehensive and cost-effective approach for NSNHL mutations. These include the HHL APEX (Hereditary Hearing Loss Arrayed Primer Extension) microarray [36], that is comprised of 198 variants in six nuclear genes (GJB2, GJB6, GJB3, GJA1, SLC26A4 and SLC26A5) and two mitochondrial genes (MTRNR1 and MTTS1). Considering that the APEX array did not detect a single GJB3 or GJA1 sequence variant in any of the study subjects, these gap junction proteins do not seem to be a major cause of NSHL in populations of European descent. Inclusion of GJB3 for pre-screening is thus not warranted. For SLC26A5, the IVS2-2A>G (c.-53-2A>G) variant was found only in a heterozygous state, however, its clinical significance is unknown and it has been found at the same frequency in deaf individuals and in a control group and pathogenicity seems therefore now questionable [37, 38]. Resequencing microarrays have also been developed by Affymetrix [39, 40], allowing for both detection of previously reported hearing loss variants and discovery of new variants. But the Affymetrix resequencing array for variants screening is costly in comparison with the other methods, limiting its implementation in the routine laboratory setting [40, 41]. In addition, a key drawback of this technology has been a high false positive rate (~70%) among identified variants, thus requiring time-consuming and expensive [39]. The TaqMan OpenArray has also been described [42]. All of these technologies have in common the use of oligonucleotides probes applied on a solid support and fluorescent compounds. The APEX microarray as well as our fluidic array allow some versatility, because it can be easily modified to alter mutation composition and/or to increase the number of mutations. In contrast, for OpenArray and resequencing arrays, modifications cannot be made after manufacturing of the plates/chips.

In this present study we have used an allele-specific PCR-based universal array designed to simultaneously screen nine deafness associated variants in the GJB2, GJB6, SLC26A4, MTRNR1 and MTTS genes that are common in patients of European descent. The test results are determined based on the fluorescent hybridization signal and the distribution of microarray probe. The array results for the 60 pre-screened subjects were consistent with the previous molecular diagnostic testing data and there were no false-positive results in 59 negative controls, thus confirming the 100% sensitivity and reliability of the assay.

A direct comparison of cost-effectiveness of Miami-CapitalBio array mutation analysis with other array-based tests is not straightforward since the number of genes and mutations covered vary depending on platform used. Furthermore, laboratory practices and workflows, array designs, and analytical performance parameters, all of which impact test cost, can differ greatly. For genetically heterogeneous diseases such as hearing loss, testing laboratories must strive to increase clinical sensitivity by including more genes and minimizing test price and turn around time. An Affymetrix resequencing microarray capable of resequencing 13 deafness causative genes has been reported to allow an individual worker to produce a throughput of nearly 100 patients per month and has reduced the cost of testing of the 13 genes roughly in half compared to dideoxy capillary sequencing approaches [39, 43].

Use of the Miami-CapitalBio fluidic array is a good option as an initial screening tool for the most common variants within genes. The cost of routine research grade Sanger sequencing of each variant in GJB6, SLC26A4 and MTRNR1 is approximately US$5 per sample. The cost estimated for analysis of all the 9 variants per patient, including materials and reagents, is approximately US$ 30. Costs for technician labor were not calculated directly since salaries,
benefits, and overhead rates vary across laboratories, but we estimated that a single technician could process 50 samples per week including data analysis. Equipment and maintenance costs are expected to be minimal as the array protocol only requires equipment that is of current standard for molecular laboratories. Overall, we demonstrate that the use of CapitalBioMiamiOtoArray for analysis of common mutations in patients of European descent is cost effective and the genotypes produced are high quality and match those collected using the established Sanger sequencing at a rate of 100%.

Supporting information

S1 Table. Loci and allele-specific amplification primers to the wild type (WT) and variant (MT) sequences. Tag sequences are underlined and mismatched bases are highlighted. (XLSX)

S2 Table. Common primers used for amplification. F: forward; R: reverse; BT: bitotin. (XLSX)

Acknowledgments

XZL’s research is supported by R01 DC05575, R01 DC01246, and R01 DC012115 (National Institutes of Health/National Institute on Deafness and Other Communication Disorders). CCM and JS are supported by R01 DC015052 (National Institutes of Health/National Institute on Deafness and Other Communication Disorders) and acknowledge the support of David and Sharon Neskey. We thank Dr. Andrew Griffith and Parna Chattaraj (National Institute on Deafness and Other Communication Disorders) for providing the control DNA samples with the known GJB2 and SLC26A4 mutations.

Author Contributions

Conceptualization: DY GX MT YSF WX CM JC BZ RM XZL.
Data curation: DY GX JS SHB MT CM BZ RM XZL.
Formal analysis: DY GX JQ JS WX SHB MT CM XZL.
Investigation: DY GX JQ BZ RM JS.
Methodology: GX WX.
Resources: GX XC JS RJHS MT CM JC XZL.
Validation: DY GX JS CM YSF HS RJHS XZL.
Writing – original draft: DY GX JS SHB MT CM XZL.
Writing – review & editing: DY GX JS SHB MT CM BZ RM XZL.

References

1. Mason JA, Herrmann KR. Universal infant hearing screening by automated auditory brainstem response measurement. Pediatrics. 1998; 101: 221–228. PMID: 9445495
2. Parving A. The need for universal neonatal hearing screening—some aspects of epidemiology and identification. Acta Paediatr. 1999; 88: 69–72.
3. Petit C, Levilliers J, Hardelin JP. Molecular genetics of hearing loss. Annu Rev Genet. 2001; 35: 589–646. doi: 10.1146/annurev.genet.35.102401.091224 PMID: 11700295
4. Prevention of blindness and deafness. WHO. 2012. http://www.who.int/pbd/deafness/estimates/en/
5. Marres HA. Congenital abnormalities of the inner ear. In: Ludman H, Bath WT (eds) Diseases of the Ear. New York, NY: Arnold & Oxford University Press; 1998.

6. Liu XZ, Xia XJ, Ke XM, Ouyang XM, Du LL, Liu YH, et al. The prevalence of connexin 26 (GJB2) mutations in the Chinese population. Hum Genet. 2002; 111:394–397. doi: 10.1007/s00439-002-0811-6 PMID: 12384781

7. Usami S, Wagatsuma M, Fukushima H, Suzuki H, Tsukada K, Nishio S, et al. The responsible genes in Japanese deafness patients and clinical application using Invader assay. Acta Otolaryngol. 2008; 128: 446–454. doi: 10.1080/00016480717850406 PMID: 18368581

8. Kennerson A, Van Naarden Braun K, Boyle C. GJB2 (connexin 26) variants and nonsyndromic sensorineural hearing loss: a HuGE review. Genet Med. 2002; 4: 258–274. doi: 10.1097/0125817-0020709000-00004 PMID: 12172392

9. Zelante L, Gasparini P, Estivill X, Melchionda S, D’Agruma L, Govea N, et al. Connexin26 mutations associated with the most common form of non-syndromic neurosensory autosomal recessive deafness (DFNB1) in Mediterraneans. Hum Mol Genet. 1997; 6: 1605–1609. PMID: 9285800

10. Morell RJ, Kim HJ, Hood LJ, Goforth L, Friderici K, Fisher R, et al. Mutations in the connexin 26 gene (GJB2) among Ashkenazi jews with nonsyndromic recessive deafness. N Engl J Med. 1998; 339: 1500–1505. doi: 10.1056/NEJM19981119392103 PMID: 9819448

11. Abe S, Usami SI, Shinkawa H, Kelley PM, Kimberling WJ. Prevalent connexin 26 gene (GJB2) mutations in Japanese. J Med Genet. 2000; 37: 41–43. doi: 10.1136/jmg.37.1.41 PMID: 10633133

12. Park HJ, Houn Hahn S, Chun YM, Park K, Kim HN. Connexin26 mutations associated with nonsyndromic hearing loss. Laryngoscope. 2000; 110: 1535–1538. doi: 10.1097/00005537-20000923-00023 PMID: 10983956

13. Yan D, Park HJ, Ouyang XM, Pandya A, Doi K, Erdenetungalag R, et al. Evidence of a founder effect for the 235delC mutation of GJB2 (connexin 26) in East Asians. Hum Mol Genet. 2000; 37: 41–43. doi: 10.1097/j.mgg.0000078026.01140.68 PMID: 10577941

14. Pandya A, Xia XJ, Erdenetungalag R, Amendola M, Landa B, Radnaabazar J, et al. Heterogenous point mutations in the mitochondrial IRNA Ser(UCN) precursor coexisting with the A1555G mutation in deaf students from Mongolia. Am J Hum Genet. 1999; 65: 1803–1806. doi: 10.1086/302658 PMID: 10577941

15. Van Hauwe P, Everett LA, Coucke P, Scott DA, Kraft ML, Ris-Stalpers C, et al. Two frequent missense mutations in Pendroye syndrome. Hum Molec Gene. 1998; 7: 1099–1104.

16. Morell RJ, Kim HJ, Hood LJ, Goforth L, Friderici K, Fisher R, et al. Mutations in the connexin 26 gene (GJB2) among Ashkenazi Jews with nonsyndromic recessive deafness. N Engl J Med. 1998; 339:1500–155525. doi: 10.1056/NEJM19981119392103 PMID: 9819448
26. Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res. 1989; 17:2503–2516. PMID: 2785681

27. Li CX, Pan Q, Guo YG, Li Y, Gao HF, Zhang D, et al. Construction of a multiplex allele-specific PCR-based universal array (ASPUA) and its application to hearing loss screening. Hum Mutat. 2008; 29:306–314. doi: 10.1002/humu.20622 PMID: 18161878

28. Zhang GB, Li CX, Lu Y, Hu H, Xiang GX, Liang QZ, et al. Validation of a mobile phone-assisted microarray decoding platform for signal-enhanced mutation detection. Biosens. Bioelectron. 2011; 15:4708–4714.

29. Zhu J, Song XM, Xiang GX, Guo YJ, Feng ZD, Mei DY, et al. A rapid automatic processing platform for label-assisted microarray analysis: application for genetic hearing-loss mutation detection. J Lab Autom. 2014; 19:144–152. doi: 10.1177/2211068213491096 PMID: 23975388

30. Longo MC Berninger MS, Hartley JL. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene. 1990; 93:125–128. PMID: 22274241

31. Kennedy CR, McCann DC, Campbell MJ, Law CM, Mullee M, Petrou S, et al. Language ability after early detection of permanent childhood hearing impairment. N Engl J Med. 2006; 354:2131–2141. doi: 10.1056/NEJMoa054915 PMID: 16707750

32. Barsky-Firks L, Sun S. Universal newborn hearing screenings: a three-year experience. Pediatrics. 1997; 99: E4.

33. Vohr BR, Carty LM, Moore PE, Letourneau K. The Rhode Island Hearing Assessment Program: experience with statewide hearing screening (1993–1996). J Pediatr. 1998; 133:353–357. PMID: 9738715

34. Johnson JL, White KR, Widen JE, Gravel JS, James M, Kennalley T, et al. A multicenter evaluation of how many infants with permanent hearing loss pass a two-stage otoacoustic emissions/automated auditory brainstem response newborn hearing screening protocol. Pediatrics. 2005; 116:663–672. doi: 10.1542/peds.2004-1688 PMID: 16140706

35. Keren R, Helfand M, Homer C, McPhillips H, Lieu TA. Projected cost-effectiveness of statewide universal newborn hearing screening. Pediatrics. 2002; 110: 855–864. PMID: 12415021

36. Rodriguez-Paris J, Pique L, Colen T, Roberson J, Gardner P, Schrijver I. Genotyping with a 198 mutation arrayed primer extension array for hereditary hearing loss: assessment of its diagnostic value for medical practice. PLoS One. 2010; 5: e11804. doi: 10.1371/journal.pone.0011804 PMID: 20668867

37. Tang HY, Xia A, Oghalai JS, Pereira FA, Alford RL. High frequency of the IVS2-2A.G DNA sequence variation in SLC26A5, encoding the cochlear motor protein prestin, precludes its involvement in hereditary hearing loss. BMC Med Genet. 2005; 6:30. doi: 10.1186/1471-2350-6-30 PMID: 16086836

38. Teek R, Otmaa E, Krustuk K, Zordania R, Joost K, Raukas E, et al. Splice variant IVS2-2A.G in the SLC26A5 (Prestin) gene in five Estonian families with hearing loss. Int J Pediatr Otorhinolaryngol. 2009; 73:103–107. doi: 10.1016/j.ijpedit.2008.10.003 PMID: 19027966

39. Kothiyal P, Cox S, Ebert J, Husami A, Kenna MA, Greinwald JH, et al. High-throughput detection of mutations responsible for childhood hearing loss using resequencing microarrays. BMC Biotechnol. 2010; 10:10. doi: 10.1186/1472-6750-10-10 PMID: 20146813

40. Cremonesi L, Ferrari M, Giordano PC, Harteveld CL, Kleanthous M, Papasavva T, et al. An overview of current microarray-based human globin gene mutation detection methods. Hemoglobin. 2007; 31:289–311. doi: 10.1080/03630260701459366 PMID: 17654067

41. Cremers FP, Kimberling WJ, Kilm M, de Brouwer AP, van Wijk E, te Brinke H, et al. Development of a genotyping microarray for Usher syndrome. J Med Genet. 2007; 44:153–160. doi: 10.1136/jmg.2006.044784 PMID: 16963483

42. Martins FT, Ramos PZ, Svidnicki MC, Castilho AM, Sartorato EL. Optimization of simultaneous screening of the main mutations involved in non-syndromic deafness using the TaqMan OpenArray Genotyping platform. BMC Med Genet. 2013; 14:11238.

43. Waldmuller S, Muller M, Rackebrandt K, Binner P, Poths S, Bonin M, Scheffold T. Array-based resequencing assay for mutations causing hypertrophic cardiomyopathy. Clin Chem. 2008; 54:682–687. doi: 10.1373/clinchem.2007.099119 PMID: 18258667