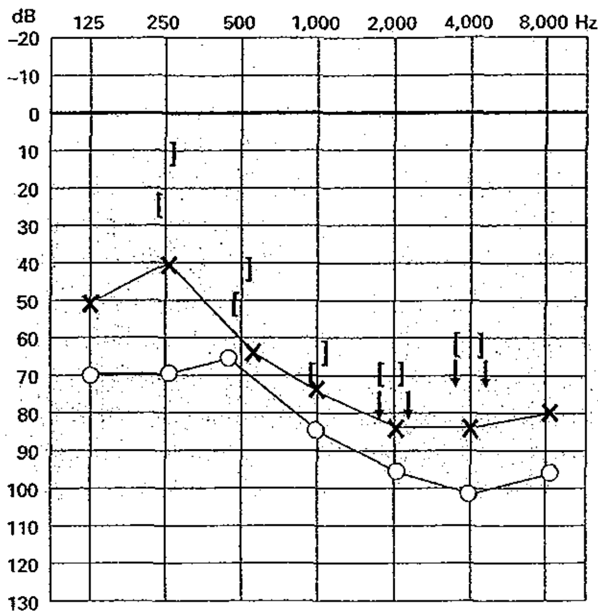

Fig. 3. Typical audiogram of a patient with EVA.

and nitrate ions [Scott et al., 1999; Scott and Karniski, 2000]. In the inner ear, *PDS* is expressed in the endolymphatic duct and sac, the nonsensory area of the utricle and saccule, and the external sulcus region [Everett and Green, 1999]. On the basis of these observations, pendrin is thought to participate in the regulation of endolymphatic fluid composition. *PDS*-knockout mice, with deafness and vestibular dysfunction, display severe endolymphatic dilatation after embryonic day 15 [Everett et al., 2001].

Our population screening revealed that there is a specific spectrum of *PDS* mutations including novel mutations in the Japanese population [Tsukamoto et al., in preparation]. We are currently evaluating whether the H723R mutation, the most common *PDS* mutation in the Japanese population, may have a founder effect. At present, among many screening methods, denaturing high performance liquid chromatography (DHPLC) is thought to fulfill the requirements for mass screening with high throughput, high sensitivity, accuracy, semiautomation, and low cost. A mutation detection system using DHPLC is now being developed for *PDS* screening [Koda, in preparation].

Unfortunately, there is no effective treatment for fluctuating and sometimes progressive sensorineural hearing loss associated with EVA. These patients should receive prescriptive, well-fitted hearing aids to utilize any residual hearing. Cochlear implantation was recently successfully done for a profound hearing loss patient with this anomaly [Bent et al., 1999].

Mitochondrial 1555A→G Mutation: Preventing Hearing Loss

Mitochondrial mutations have been demonstrated to be responsible for syndromic as well as nonsyndromic hearing impairment [Van Camp G, Smith RJH: Hereditary Hearing Loss Homepage: http://www.uia.ac.be/dnalab/hhh].

Studies of familial aggregation of aminoglycoside-induced hearing loss have indicated that some families may have a constitutional susceptibility for cochlear damage from aminoglycoside antibiotics. Aminoglycoside-induced hearing loss has also been found to be exclusively maternally transmitted, suggesting mitochondrial inheritance. Genetic analysis has revealed that this hereditary susceptibility is caused by the 1555A→G point mutation in the mitochondrial 12S ribosomal RNA gene [Prezant et al., 1993].

Concerning phenotype viability, although there is a wide range of hearing levels from normal to severe in affected subjects, they usually have in common bilateral, symmetric hearing loss that mainly affects the high frequencies [Usami et al., 1997; Tsuiki et al., 1997]. Figure 4 shows overlapping audiograms of the average hearing levels. Progressive hearing loss is sometimes detected [Usami et al., 1997; Tsuiki et al., 1997]. Within families, individual variations are seen and permanent tinnitus is usually associated with the hearing loss [Usami et al., 1997; Tsuiki et al., 1997]. A number of reports have indicated that the 1555A→G mutation can cause a usually milder hearing loss even without aminoglycoside injection [Usami et al., 1997; Estivill et al., 1998] (fig. 4). Individuals with the 1555A→G mutation may be genetically sensitive to various environmental factors and therefore at risk for hearing impairment, even without exposure to aminoglycosides. Detailed anamnestic evaluation has shown that the hearing loss of these patients is not congenital. Auditory testing has shown that the sensorineural hearing loss associated with the 1555A→G mutation may originate in the inner ear. Excellent auditory performance with a cochlear implant has been achieved in patients with the 1555A→G mutation [Tono et al., 1998, 2001], supporting the theory that hearing loss associated with this mutation is caused by injury to the inner ear rather than to the cochlear nerve and its central connections.

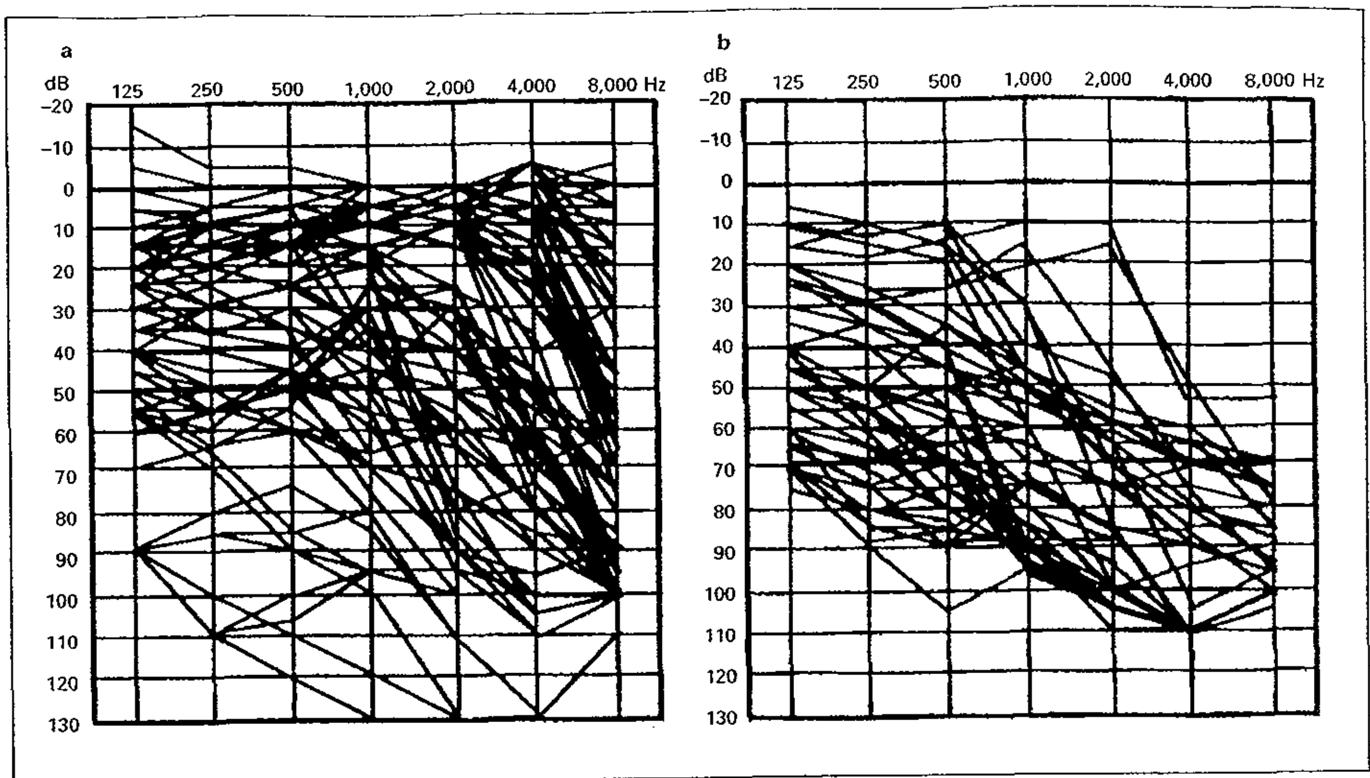

Fig. 4. a Overlapping audiograms from subjects bearing the 1555A→G mutation but without a history of aminoglycoside injection. Although there is a wide variety of hearing impairment it is milder than that in **b**. **b** Overlapping audiograms from subjects with the 1555A→G mutation and aminoglycoside injection. High-frequency hearing impairment is evident. From Usami et al. [2000b].

Our series of screenings for the 1555A→G mitochondrial mutation suggested that this is the most prevalent mitochondrial mutation found in the hearing-impaired population: approximately 3% of outpatients and 33% of subjects with a history of aminoglycoside injection [Usami et al., 2000a]. It should therefore be noted that this mutation is the most likely one responsible for hearing impairment in patients with aminoglycoside exposure. Family history on the maternal side is a significant criterion for the selection of patients to undergo mtDNA analysis. The 1555A→G mutation has also been found at a high rate in profound hearing-impaired populations. The mutation was detected in 57% of patients who received cochlear implantation due to aminoglycoside-induced hearing loss [Usami et al., 2000a].

Aminoglycosides have serious side effects and, along with the development of viable alternatives, their usage has decreased in Japan and other developed countries. A new generation of aminoglycoside antibiotics with less severe side effects has recently been introduced in Japan and Europe. These, however, should also be used with caution as 2 cases of hearing loss after short-term exposure in patients with the 1555A→G mitochondrial mutation have been reported [Usami et al., 1998].

This mutation was initially reported within Asian populations, but subsequent reports demonstrated that it is also found in Europeans. Phylogenetic analysis of independent families with the 1555A→G mutation has suggested that it is sporadic and has multiplied through the evolution of the mtDNA [Hutchin and Cortopassi, 1997; Abe et al., 1998]. It is likely that there is a considerably large high-risk population worldwide and a rapid screening method as well as careful counseling should be established to prevent aminoglycoside-induced hearing loss in this group. We currently use the mutant allele specific amplification (MASA) method to detect this mutation [Usami et al., 1999a] and have also had success with mutational analysis using oligonucleotide microarrays [Asamura et al., in preparation]. We warn our patients with the 1555A→G mutation of the risks for them and their families of aminoglycoside antibiotic exposure.

Conclusion

Our knowledge of the hearing mechanism has recently been greatly improved through the identification of genes involved in hearing loss. The progress in molecular genetics has facilitated the development of a number of molecular diagnostic methods. These new tools enable ENT clinicians to more accurately diagnose hearing loss and to categorize patients into clinically distinct entities.

Acknowledgements

We would like to thank Dr. W.J. Kimberling for helpful comments and Ms. A.C. Apple-Mathews and Ms. C. Kawashima for help in preparing the manuscript. This work is supported by the Ministry of Health and Welfare, Japan (S.U.), and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (S.U.).

References

Abe S, Usami S, Hoover DM, Cohn E, Shinkawa H, Kimberling WJ: Fluctuating sensorineural hearing loss associated with enlarged vestibular aqueduct maps to 7q31, the region containing the Pendred gene. Am J Med Genet 1999;82:322–328.

Abe S, Usami S, Shinkawa H: Three familial cases of hearing loss associated with enlargement of the vestibular aqueduct. Ann Otol Rhinol Laryngol 1997;106:1063–1069.

Abe S, Usami S, Shinkawa H, Kelley PM, Kimberling WJ: Prevalent connexin 26 gene (*GJB2*) mutations in Japanese. J Med Genet 2000;37:41–43.

Abe S, Usami S, Shinkawa H, Weston MD, Overbeck LD, Hoover DM, Kenyon JB, Horai S, Kimberling WJ: Phylogenetic analysis of mitochondrial DNA in Japanese pedigrees of sensorineural hearing loss associated with the A1555G mutation. Eur J Hum Genet 1998;6:563–569.

Bent JP, Chute P, Parisier SC: Cochlear implantation in children with enlarged vestibular aqueducts. Laryngoscope 1999;109:1019–1022.

Estivill X, Govea N, Barceló A, Perelló E, Badenas C, Romero E, Moral L, Scozzari R, D'Urbano L, Zeviani M, Torroni A: Familial progressive sensorineural deafness is mainly due to the mtDNA A1555G mutation and is enhanced by treatment with aminoglycosides. Am J Hum Genet 1998;62:27–35.

Everett LA, Belyantseva IA, Noben-Trauth K, Cantos R, Chen A, Thakkar SI, Hoogstraten-Miller SL, Kachar B, Wu DK, Green ED: Targeted disruption of mouse Pds provides insight about the inner-ear defects encountered in Pendred syndrome. Hum Mol Genet 2001;10:153–161.

Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani E, Nassir E, Baxevanis AD, Sheffield VC, Green ED: Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). Nat Genet 1997;17:411–422.

Everett LA, Green ED: A family of mammalian anion transporters and their involvement in human genetic diseases. Hum Mol Genet 1999;8:1883–1891.

Griffith AJ, Arts A, Downs C, Innis JW, Shepard NT, Sheldon S, Gebarski SS: Familial large vestibular aqueduct syndrome. Laryngoscope 1996;106:960–965.

Hutchin TP, Cortopassi GA: Multiple origins of a mitochondrial mutation conferring deafness. Genetics 1997;145:771–776.

Kelsell DP, Di WL, Houseman MJ: Connexin mutations in skin disease and hearing loss. Am J Hum Genet 2001;68:559–568.

Kelsell DP, Dunlop J, Stevens HP, Lench NJ, Liang JN, Parry G, Mueller RF, Leigh IM: Connexin mutations in hereditary non-syndromic sensorineural deafness. Nature 1997;387:80–83.

Kimberling WJ: Genetic testing of hearing loss disorders; in Hashimoto I, et al (eds): Novel Findings of Gene Diagnosis, Regulation of Gene Expression, and Gene Therapy. Molecular Medicine. Amsterdam, Elsevier, 1999, pp 21–30.

Mafee MF, Charletta D, Kumar A, Belmont H: Large vestibular aqueduct and congenital sensorineural hearing loss. AJNR 1992;13:805–819.

Marazita ML, Ploughman LM, Rawlings B, Remington E, Arnos KS, Nance WE: Genetic epidemiological studies of early-onset deafness in the U.S. school-age population. Am J Med Genet 1993;46:486–491.

Morton N: Genetic epidemiology of hearing impairment. Ann NY Acad Sci 1991;630:16–30.

Prezant TR, Agapian JV, Bohlman MC, Bu X, Oztas S, Qiu WQ, Arnos KS, Cortopassi GA, Jaber L, Rotter JI, Shohat M, Fischel-Ghodsian N: Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. Nat Genet 1993;4:289–294.

Scott DA, Karniski LP: Human pendrin expressed in *Xenopus laevis* oocytes mediates chloride/formate exchange. Am J Physiol Cell Physiol 2000;278:C207–C211.

Scott DA, Wang R, Kreman TM, Sheffield VC, Karnishki LP: The Pendred syndrome gene encodes a chloride-iodide transport protein. Nat Genet 1999;21:440–443.

Steel KP, Bussoli TJ: Deafness genes: Expressions of surprise. Trends Genet 1999;15:207–211.

Steel KP, Kros CJ: A genetic approach to understanding auditory function. Nat Genet 2001;27:143–149.

Tono T, Kiyomizu K, Matsuda K, Komune S, Usami S, Abe S, Shinkawa H: Different clinical characteristics of aminoglycoside-induced profound deafness with and without the 1555A→G mitochondrial mutation. ORL 2001;63:25–30.

Tono T, Ushisako Y, Kiyomizu K, Usami S, Abe S, Shinkawa H, Komune S: Cochlear implantation in a patient with profound hearing loss with the 1555A→G mitochondrial mutation. Am J Otol 1998;19:754–757.

Tsuiki T, Murai K, Murai S, Kitamura K, Tamagawa Y: Audiologic features of hearing loss due to the 1555 mutation of mitochondrial DNA. Ann Otol Rhinol Laryngol 1997;106:643–648.

Urman SM, Talbot JM: Otic capsule dysplasia: Clinical and CT findings. Radiographics 1990;10:823–838.

Usami S, Abe S, Kasai M, Shinkawa S, Moeller B, Kenyon JB, Kimberling WJ: Genetic and clinical features of sensorineural hearing loss associated with the 1555 mitochondrial mutation. Laryngoscope 1997;107:483–490.

Usami S, Abe S, Tono T, Komune S, Kimberling WJ, Shinkawa H: Isepamicin sulfate-induced sensorineural hearing loss in patients with the 1555A→G mitochondrial mutation. ORL 1998;60:164–169.

Usami S, Abe S, Shinkawa H, Inoue Y, Yamaguchi T: Rapid mass screening method and counseling for the 1555A→G mitochondrial mutation. J Hum Genet 1999a;44:304–307.

Usami S, Abe S, Weston MD, Shinkawa H, Van Camp G, Kimberling WJ: Non-syndromic hearing loss associated with enlarged vestibular aqueduct is caused by PDS mutations. Hum Genet 1999b;104:188–192.

Usami S, Abe S, Akita J, Namba A, Shinkawa H, Ishii M, Iwasaki S, Hoshino T, Ito J, Doi K, Kubo T, Nakagawa T, Komiyama S, Tono T, Kumune S: Prevalence of mitochondrial gene mutations among hearing impaired patients. J Med Genet 2000a;37:38–40.

Usami S, Abe S, Akita J, Shinkawa H, Kimberling WJ: Sensorineural hearing loss associated with the mitochondrial mutations; in Kitamura K, Steel KP (eds): Genetics in Otorhinolaryngology. Adv Otorhinolaryngol. Basel, Karger, 2000b, vol 56, pp 221–229.

Willems PJ: Genetic causes of hearing loss. N Engl J Med 2000;342:1101–1109.

Yoshinaga-Itano C, Sedey AL, Coulter DK, Mehl AL: Language of early- and later-identified children with hearing loss. Pediatrics 1998;102:1161–1171.

ELSEVIER

Auris, Nasus, Larynx 29 (2002) 231–239

AURIS NASUS LARYNX
INTERNATIONAL JOURNAL OF ORL & HNS

www.elsevier.com/locate/anl

High-throughput screening for *GJB2* mutations—its clinical application to genetic testing in prelingual deafness screening for *GJB2* mutations

Akemi Sugata [a], Kunihiro Fukushima [a,*], Ken-ichi Sugata [a], Syouichiro Fukuda [b], Nobuhiko Kimura [a], Mehmet Gunduz [a], Norio Kasai [a], Shinichi Usami [c], Richard J.H. Smith [d], Kazunori Nishizaki [a]

[a] *Okayama University Medical School, Department of Otolaryngology, Head and Neck Surgery, 2-5-1 Shikata Cho, 700-8558 Okayama, Japan*
[b] *Okayama Kanariya Gakuen, Okayama Hearing Training Center for Hearing Impaired Child, Okayama, Japan*
[c] *Department of Otolaryngology, Shinsyu University Medical School, Shinsyu, Japan*
[d] *Department of Otolaryngology, Head and Neck Surgery, The University of Iowa, Iowa, Japan*

Received 18 October 2001; received in revised form 30 January 2002; accepted 15 March 2002

Abstract

Objectives: Mutations in connexin26 (*GJB2*) are one of the most frequent causes of prelingual hearing impairment. Several different types of one-base deletions in exon2 were the most common type of *GJB2* mutation regardless of ethnicity, including 35delG in American–European populations, 235delC in Japanese population and 167delT in Ashkenazi Jewish population. Various types of one-base substitutions were also considered to be causative mutations of *GJB2* associated hearing impairment. This article describes a rapid and high-throughput screening procedure for the detection of one-base deletion/substitution in *GJB2* with less invasive sampling procedure in the implication for the clinical application. *Methods:* 53 hearing-impaired children and 50 healthy controls were admitted to take part in this study program. DNA samples obtained from buccal swab were used to amplify the exon2 of *GJB2*, and single run with an automated sequencer was used to identify the one-base deletion. Single-base substitutions were also screened by primer-extension procedure with dye terminators. The presence of both types of mutations was confirmed by direct sequence of the *GJB2* exon2. *Results:* Two of 50 controls (4%) included one-base deletion in *GJB2* as heterozygote. 14 of 53 hearing impaired cases (26.4%) contained deletion in *GJB2* either as homozygote (five cases) or heterozygote (nine cases) form. Sequencing analysis of whole exon2 of *GJB2* identified all these deletions as 235delC. Primer-extension analysis revealed additional mutations with single base substitutions in three cases with compound heterozygote with 235delC. *Conclusions:* Rapid screening procedure of *GJB2* can be potentially useful for the identification of prelingual deafness. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Connexin26; *GJB2*; Non-syndromic hearing loss; Screening method; Single nucleotide deletion; Single nucleotide substitution

1. Introduction

Approximately one newborn in 2,000 is affected with hereditary hearing impairment (HHI), most commonly presenting as autosomal recessive non-syndromic deafness (ARNSD) [1]. To date, 30 ARNSD loci have been identified and several of the relevant genes have been cloned [2]. The first locus to be mapped, DFNB1 (DFN, deafness; B, recessive; appended integer, locus in order of discovery) was localized to chromosome 13q11 in a Tunisian family [3]. Shortly thereafter, Maw et al. [4] reconstructed haplotypes in several families with ARNSD and found that in 19, segregation was consistent with linkage to DFNB1, raising the possibility that mutations in the DFNB1 gene were a significant cause of ARNSD.

This hypothesis was confirmed when *GJB2* was identified and found to be the cause of approximately half of severe-to-profound ARNSD in many countries around the world. To date, over 45 different mutations in *GJB2* have been reported in persons with ARNSD

* Corresponding author. Tel.: +81-86-235-7307; fax: +81-86-235-7308

E-mail address: kuni@cc.okayama-u.ac.jp (K. Fukushima).

0385-8146/02/$ - see front matter © 2002 Elsevier Science Ireland Ltd. All rights reserved.
PII: S0385-8146(02)00014-7

[5]. Of these mutations, 35delG has been reported most frequently [6–13]. Of newborns with ARNSD, two-thirds are homozygotes for this allele variant [14]. More detailed population-based studies, however, have shown that this figure is ethnically biased. 35delG is carried on a common genetic background, consistent with a founder effect about 10,000 years ago. In populations with little admixture with the northern European lineage, the 35delG mutation is rare. For example, among the Ashkenazim, 167delT is most common [9,15], and in the Japanese population, 235delC predominates, and the 35delG mutation has yet to be identified in spite of extensive screening [16,17].

Rapid screening procedures for *GJB2* deafness-causing mutations must be sensitive to these ethnic differences. In addition, because genetic testing may be offered to neonates and very young children, a minimally invasive procedure is needed. In this article, we describe a rapid high-throughput, minimally invasive procedure to identify *GJB2* deletions in very young children with ARNSD.

2. Material and methods

2.1. Subject identification and DNA extraction

The parents of 74 hearing-impaired infants followed at the Okayama University Medical School were asked to enroll in this study, and 53 of them were included with written informed consent. In participating families, cellular samples were obtained from hearing-impaired infants by brushing the oral mucosa with brushes used for cytological examination (Cytobrush™, Medscand, Hollywood, CA). All procedures were approved by the local Institutional Review Board.

Samples were collected from 20 cycles of brushing and washed from the brush by rinsing with 100 μl of double distilled water. Genomic DNA was obtained by phenol–chloroform extraction and ethanol precipitation. Previously identified samples were used as positive controls for the 35delG and 235delC allele variants. DNA samples from 50 healthy children were obtained by the same procedure.

2.2. Polymerase chain reaction

First round polymerase chain reaction (PCR) was completed with a primer pair covering exon2 in its entirety (primers A and B, Table 1), as previously described [18]. Briefly, each reaction contained 10 ng of genomic DNA, 2.0 pmol of each primer, 200 μM of each dNTP (Toyobo Inc., Osaka, Japan), 0.25 U of Taq DNA polymerase (Takara Shuzo Inc., Tokyo, Japan), and 1 μl of 10 × buffer (Takara Shuzo Inc.) in a total volume of 10 μl. After the initial denaturation step at 94 °C for 2 min, samples were amplified under the following thermal conditions: 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, for 25 cycles, with an additional extension time at 72 °C for 10 min. PCR products were purified using the QIAquick™ PCR purification kit (QIAGEN Inc., Valencia, CA) and stored for later use in the following procedures.

Table 1

Cox26 exon2	primerA	5'-gcattcgtcttttccagagc-3'
	primerB	5'-cctcatccctctcatgctgt-3'
235delC	primerC	5'-cacgctgcagacgatcct-3'
	primerD	5'-cctcttcttctcatgtctccggta-3'
Y136X	primerE	5'-ggctccctgtggtggaccta-3'
R143W	primerF	5'-acacaagcagcatcttcttc-3'
G45E	primerG	5'-caaagtcggcctgctcatct-3'

2.3. Screening for single nucleotide deletions

Primers for the screening PCR (second-round PCR) amplified a 241 bp region of exon2 that includes the 35delG, 165delT and 235delC mutations; the primers were labeled with blue fluorescent dye 5-carboxyfluorescein (5-FAM) (primers C and D, Table 1). Actual synthesis and primer labeling were done by Applied Biosystems (ABI, Foster City, CA). The above-described conditions for PCR conditions were used. PCR products were visualized using the ABI PRISM 310 Genetic Analyzer (ABI). Injection was performed at 15 kV for 12 s; electrophoresis was performed at 13.0 kV for 35 min at 60 °C.

2.4. Screening for single nucleotide substitution

PCR products obtained from the first PCR were also used as the template of the single nucleotide substitution screening. First PCR products were treated by 2 U of shrimp alkaline phosphatase (SAP) (New England BioLabs (NEB), Hertfordshire, UK) and 2 U of *ExoI* endonuclease (NEB) at 37 °C for 1 h. Second, 0.15 pmol of primers and 0.15 pmol of template DNA were added to reaction. Primers used for the detection of known single-base substitutions were synthesized as summarized in Table 1. Reaction mixture contained buffers, R6G-labeled ddATP, TAMRA-labeled ddCTP, R110-labeled dGTP and ROX-labeled ddTTP, supplied by the manufacture (SnaPshot™ kit, ABI). Thermal cycling was then conducted with following conditions: 96 °C for 10 s, 50 °C for 5 s and 60 °C for 30 s. The products were then treated by 0.5 U of SAP at 72 °C for

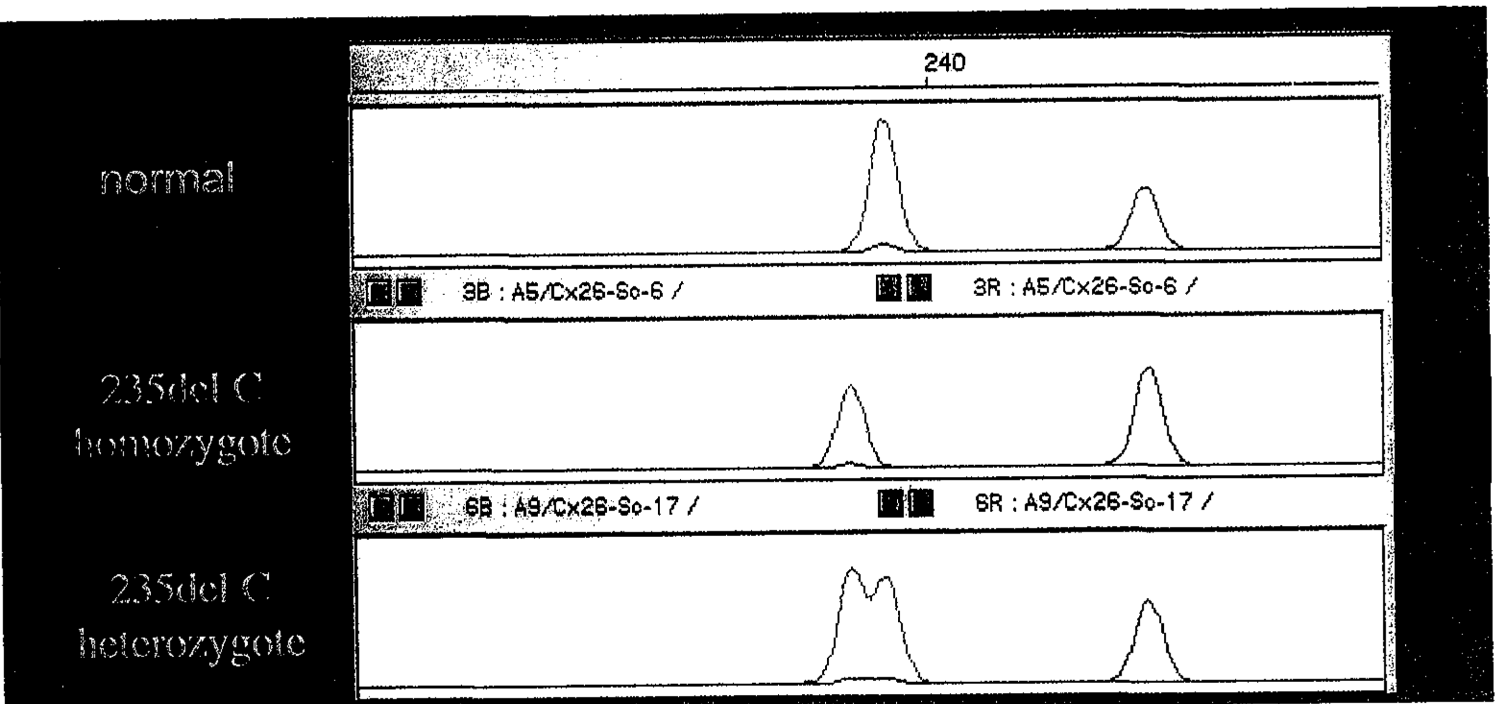

Fig. 1. Detection of 235delC in chromatogram. Red marker indicates the size marker. PCR product obtained from 235delC homozygotes formed a single different peak, which was shifted to the left side from that of normal controls. PCR product obtained from 235delC heterozygotes formed a two different peaks, each of which was as same as peak of normal control, or 235delC homozygotes.

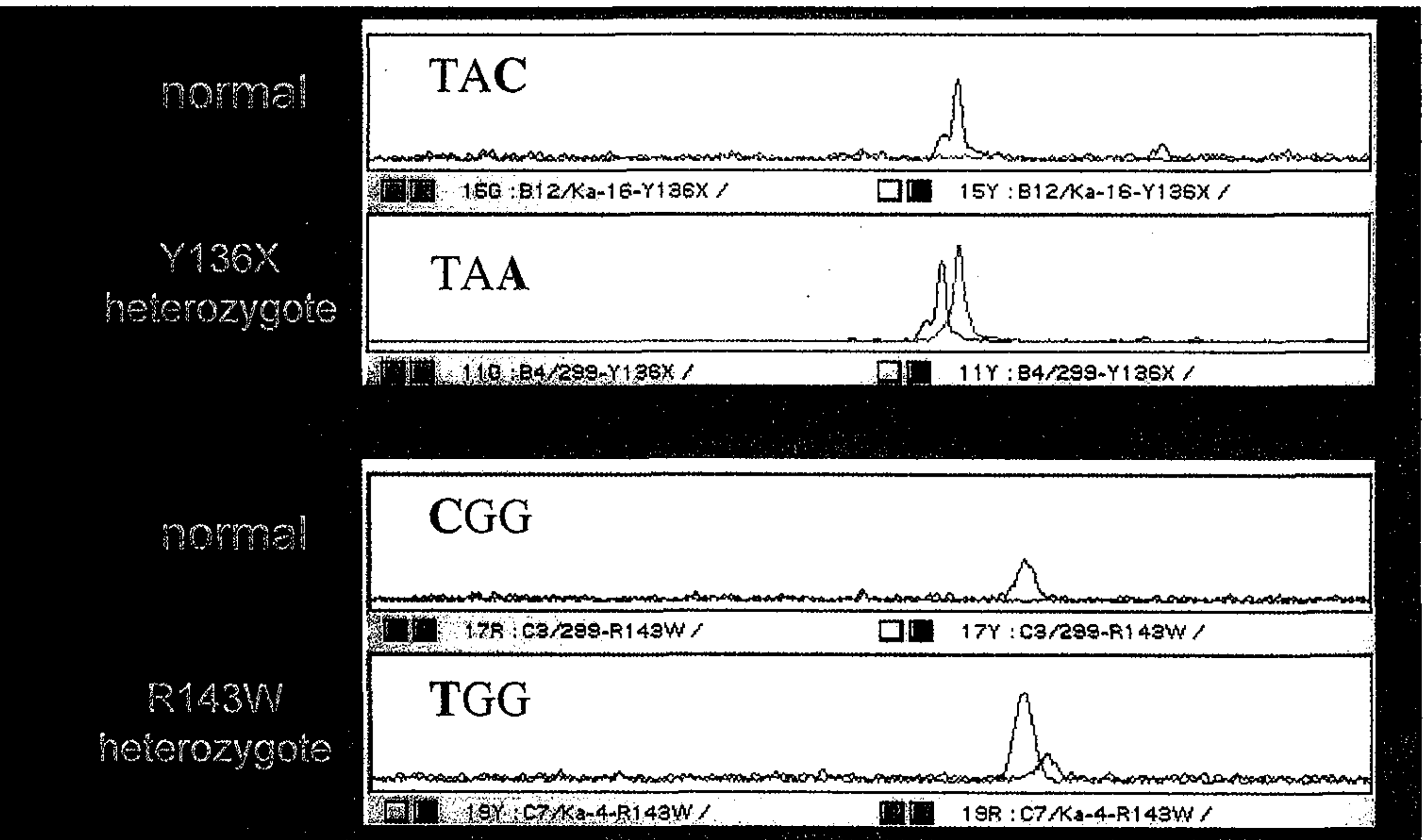

Fig. 2. Screening for single nucleotide substitution. The substitutions of single nucleotide was screened by adding labeled dideoxynucleotides (ddNTP) to the primer-extension step. Incorporation of ddNTP directly ceased the extension step, and the difference of incorporated ddNTP can be easily detected by automated sequencer (ABI 310). Upper columns indicate the result of the Y136X screening. The samples of normal control only formed one black peak (normal). Additional green peak, together with the black peak, was observed in the heterozygous Y136X case, showing C–A substitution at 408 position (Y136X heterozygote). Lower columns indicate the result of R143W screening. The result with normal control again demonstrated single black peak and additional red peak indicated the presence of C–A substitution at 427 position (R143W heterozygote).

15 min. The products were then visualized and evaluated by ABI 310 as described above.

2.5. Sequence analysis

An aliquot of the first-round PCR product from all study participants was directly sequenced to confirm the efficiency and accuracy of our screening method. A second aliquot was subcloned into pBluescript (Stratagene, La Jolla, CA) and sequenced to confirm allelic differences in compound heterozygotes. Sequencing was completed with BigDye™ terminator cycle sequencing ready kit with Amplitaq™ DNA polymerase FS (ABI). PCR products were loaded and run on the ABI PRISM 373S Genetic Analyzer (ABI). Injection was performed at 15 kV for 12 s. Electrophoresis was performed at 2.8 kV for 18 h.

3. Results

3.1. Screening for single nucleotide deletions

Samples with known single nucleotide deletions were examined first. Chromatograms of the PCR products from 35delG and 235delC homozygotes resulted in single peaks, distinct from controls, while in heterozygotes two discrete peaks were apparent (Fig. 1). These samples were later used as external positive controls to identify shifted peaks.

Two of 50 (4%) normal hearing controls proved to be 235delC heterozygotes. Of the hearing-impaired children, 14 of 53 (26.4%) carried at least one 235delC allele. Five (35.7%) children were 235delC homozygotes and the other nine (64.2%) were 235delC heterozygotes.

3.2. Screening for single nucleotide substitution

All first-round PCR products were screened for single nucleotide substitutions. No additional mutations were identified in samples from normal hearing controls (zero of 50, 0%). Among the 53 hearing-impaired children, three mutations were found (G45E, Y136X and R143W) always segregating opposite the 235delC allele variant (compound heterozygotes) (Fig. 2). These missense mutations have been reported previously (see the Connexin-deafness homepage, http://www.iro.es/cx26deaf.html).

3.3. Sequence analysis

To evaluate the accuracy of our mutation detection methodology, all samples were directly sequenced and results between methods were compared. Direct sequencing identified two 235delC heterozygotes in the control population with normal hearing. No other mutations were identified in this group. In the group of hearing-impaired children, 235delC was identified in the 14 samples. In three of the nine heterozygotes, additional deafness-causing allele variants of *GJB2* were also detected (G45E, Y136X and R143W).

Many additional single base substitutions were identified. The most frequent were 455G > C and 456C > G, which were found in 10 of the 53 subjects. This mutation results in a conservative amino acid change (serine to a threonine) and does not affect the polarity of the amino acid residue. The frequency of this mutation suggests that it is a benign polymorphism. Sequencing analysis of exon2, including the entire coding lesion of connexin26, demonstrated no other mutation in exon2 in six deaf children heterozygous for the 235delC mutation.

3.4. Effectiveness of the screening procedure

The positive and negative predictive values of this screening method were 100%, although the number of examined samples was small. Each sample required 1 h for DNA extraction, 2 h for PCR-based amplification of the coding region of *GJB2* (exon2), and 3 h for mutation detection. Compared to direct sequencing, this method is faster and cheaper by a factor of three.

3.5. Clinical and epidemiologic evaluation

The clinical background of the hearing-impaired subjects is summarized in Table 2. In three children, increased serum anti-rubella IgG levels suggested the diagnosis of congenital rubella syndrome, and in three children, a conductive hearing loss was later identified. If the phenotype examined here was limited to non-syndromic sensorineural hearing impairment, eight cases (five, 235delC homozygotes and three, 235delC compound heterozygotes) of 47 were identified to be *GJB2*-associated deafness. As for six who are 235delC heterozygotes could have *GJB2* deafness caused by a mutation we have not detected. In these 14 cases, 11 of them were severe-to-profound deafness and three of them were moderate hearing loss. Five profoundly deafened cases have already received cochlear implant (CI) and the rest of them were the hearing aid users. Seven of them were identified to have a hearing loss in their siblings. All but one case typically showed non-syndromic hearing loss, and the deafness of one case combined with cleft palate and cleft lip. Karyotyping of this case revealed the balanced translocation of t(7:11) which is inherited from his/her mother.

4. Discussion

We found primer-extension analysis to be highly effective for the detection of single-base deletions and

Table 2
The clinical background of the total 53 hearing-impaired subjects

no.	AG	Inner ear anomaly	Others	*GJB2* mutation
1	S	None		ND
2	P	N/A		ND
3	P	None	Auricular anomaly	ND
4	S	None		Compound heterozygote
5	P	Mondini		ND
6	P	N/A	Ossicle anomaly Atresia auris	ND
7	M	None		ND
8	S	None	AD	ND
9	S	N/A		ND
10	S	Inner ear anomaly		ND
11	P	N/A	Heart anomaly, PNHR	ND
12	S	None	Rubella infection	ND
13	P	None	Rubella infection	ND
14	P	None		Compound heterozygote
15	P	None		Heterozygote
16	S	N/A		ND
17	M	N/A	Ring 13 anomaly	ND
18	S	None	Possible AR	ND
19	S	None	PossibleAR	ND
20	P	N/A	None	Heterozygote
21	P	None	AD cleft palate heart anomaly	ND
22	Mild	None	None	ND
23	S	None	None	ND
24	M	LSCC	None	ND
25	P	None	Accessory auricles	Homozygote

26	P	Mondini	None	ND
27	P	Ossified inner ear	PNHR	ND
28	S	N/A	AR	Homozygote
29	P	None	None	Heterozygote
30	P	None	Possible AR	Heterozygote
31	P	None	Balanced translocation t(7,11), cleft palate	Heterozygote
32	P	None	PNHR	ND
33	P	None	None	ND
34	S	Possible AR	None	Heterozygote
35	P	None	PNHR	ND
36	S	None	None	Compound heterozygote
37	P	N/A	Hearing impairment after meningitis	ND
38	P	None	None	ND
39	P	None	Atresia auris, branchial synd.	ND
40	m	None	None	ND
41	P	None	PossibleAR	Homozygote
42	P	Mondini	PossibleAR	Homozygote
43	P	None	PNHR, William synd.	ND
44	P	None	Abdominal rupture, jejunum atresia	ND
45	P	None	None	ND
46	P	N/A	Heart anomaly Rubella infection	ND
47	P	N/A	None	Homozygote
48	P	N/A	None	ND
49	P	N/A	None	ND
50	S	None	None	ND
51	S	None	None	ND
52	P	None	None	ND
53	P	None	PNHR	ND

AG, Audiogram pattern of hearing impairment; P, profound; S, severe; M, moderate; LSSC anomaly: lateral semicircular canal anomaly; N/A, not available; PNHR, perinatal high risk for hearing loss; AD, Autosomal Dominant.;

Possible AR (autosomal recessive), AR inheritance was assumed by the presence of hearing loss of siblings, but not of either of their parents. No consanguineous mating was identified among these parents.; ND, Not detected

single-base substitutions in *GJB2*. Although we screened for the presence of only five different *GJB2* mutations, the technique can be used to detect any *GJB2* mutations. Mutation selection should be based on the relative prevalence of a given mutation in the population being studied. For example, in the western Japanese population, one-step mutation detection using the five sets of primers we selected was highly effective.

The accuracy of primer-extension analysis is similar to that of direct sequencing (100%), reflecting the common background science of both procedures. However, primer-extension analysis is not a mutation screening methodology. It is a mutation detection technique that determines with high accuracy whether an individual carries a particular allele variant of *GJB2*. The presence or absence of other allele variants will remain unknown unless additional testing is done.

This limitation notwithstanding, primer-extension analysis is more rapid and cheaper than direct sequencing. It is also more robust than other mutation detection methods, including allele-specific PCR, single-stranded conformational polymorphism analysis (SSCP) and denaturant gradient gel electrophoresis (DGGE) analysis [5,19–23].

The establishment of a high-throughput, inexpensive screening procedure to identify *GJB2*-related deafness has important clinical applications. Primer-extension analysis meets these requirements and is also only minimally invasive. It is robust, requiring two PCR amplifications, possible with even relatively poor quality DNA template.

Prognostically, the diagnosis of *GJB2*-related deafness is a useful preoperative predictor of language outcome after CI. We have reported that *GJB2*-related deafness is a better prognostic indicator of language acquisition following implantation than other causes of hearing impairment—data that are helpful in counseling prospective CI recipients. Because implantation should be performed before the critical period of language acquisition, the ideal time of implantation may be as early as possible. If this is true, coupled with newborn hearing screening, genetic testing for *GJB2*-related deafness may be useful to identify babies with severe-to-profound deafness immediately after delivery.

According to Brunger et al. [24], the majority of normal hearing parents of deaf children believe that genetic testing for deafness is beneficial. Not only does this type of testing offer the possibility of identification of the cause of deafness, but it permits accurate recurrence chance calculation and prepares parents for having deaf children. More than 90% of these parents had a positive attitude toward pediatric genetic testing, regardless of their previous experience with genetic testing.

Acknowledgements

The authors give thanks to all the participants of this project. We also thank all the staffs of Kanariya-Gakuen (Auditory Training Center for Hearing Impaired Children, Okayama) for helping us to follow-up the affected children. This study is partly supported by a grant from the Ministry of Health and Welfare of Japan (KF) and by a grant for scientific research (B), 09470372 from Ministry of Education, Science, Sports and Culture (NK).

References

[1] Morton NE. Genetic epidemiology of hearing impairment. Ann NY Acad Sci 1991;630:16–31.
[2] Chen AH, Stephan DA, Hasson T, Fukushima K, Nelissen CM, Chen AF, Jun AI, Ramesh A, Van Camp G, Smith RJ. MYO1F as a candidate gene for nonsyndromic deafness, DFNB15. Arch Otolaryngol Head Neck Surg 2001;127:921–5.
[3] Guilford P, Ben Arab S, Blanchard S, Levilliers J, Weissenbach J, Belkahia A, Petit C. A non-syndrome form of neurosensory, recessive deafness maps to the pericentromeric region of chromosome 13q. Nat Genet 1994;6:24–8.
[4] Maw MA, Allen-Powell DR, Goodey RJ, Stewart IA, Nancarrow DJ, Hayward NK, Gardner RJ. The contribution of the DFNB1 locus to neurosensory deafness in a Caucasian population. Am J Hum Genet 1995;57:629–35.
[5] Rabionet R, Zelante L, Lopez-Bigas N, D'Agruma L, Melchionda S, Restagno G, Arbones ML, Gasparini P, Estivill X. Molecular basis of childhood deafness resulting from mutations in the GJB2 (connexin 26) gene. Hum Genet 2000;106:40–4.
[6] Carrasquillo MM, Zlotogora J, Barges S, Chakravarti A. Two different connexin 26 mutations in an inbred kindred segregating non-syndromic recessive deafness: implications for genetic studies in isolated populations. Hum Mol Genet 1997;6:2163–72.
[7] Denoyelle F, Weil D, Maw MA, Wilcox SA, Lench NJ, Allen-Powell DR, Osborn AH, Dahl HH, Middleton A, Houseman MJ, Dode C, Marlin S, Boulila-ElGaied A, Grati M, Ayadi H, BenArab S, Bitoun P, Lina-Granade G, Godet J, Mustapha M, Loiselet J, El-Zir E, Aubois A, Joannard A, Petit C, et al. Prelingual deafness: high prevalence of a 30delG mutation in the connexin 26 gene. Hum Mol Genet 1997;6:2173–7.
[8] Kelley PM, Harris DJ, Comer BC, Askew JW, Fowler T, Smith SD, Kimberling WJ. Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss. Am J Hum Genet 1998;62:792–9.
[9] Lench N, Houseman M, Newton V, Van Camp G, Mueller R. Connexin-26 mutations in sporadic non-syndromal sensorineural deafness. Lancet 1998;351:415.
[10] Lench NJ, Markham AF, Mueller RF, Kelsell DP, Smith RJ, Willems PJ, Schatteman I, Capon H, Van De Heyning PJ, Van Camp G. A Moroccan family with autosomal recessive sensorineural hearing loss caused by a mutation in the gap junction protein gene connexin 26 (GJB2). J Med Genet 1998;5:151–2.
[11] Storm K, Willocx S, Flothmann K, Van Camp G. Determination of the carrier frequency of the common GJB2 (connexin-26) 35delG mutation in the Belgian population using an easy and reliable screening method. Hum Mutat 1999;14:263–6.
[12] Antoniadi T, Rabionet R, Kroupis C, Aperis GA, Economides J, Petmezakis J, Economou-Petersen E, Estivill X, Petersen MB. May high prevalence in the Greek population of the 35delG

mutation in the connexin 26 gene causing prelingual deafness. Clin Genet 1999;55:381–2.

[13] Morell RJ, Kim HJ, Hood LJ, Goforth L, Friderici K, Fisher R, Van Camp G, Berlin CI, Oddoux C, Ostrer H, Keats B, Friedman TB. Mutations in the connexin 26 gene (GJB2) among Ashkenazi Jews with nonsyndromic recessive deafness. N Engl J Med 1998;39:1500–5.

[14] Zelante L, Gasparini P, Estivill X, Melchionda S, D'Agruma L, Govea N, Mila M, Monica MD, Lutfi J, Shohat M, Mansfield E, Delgrosso K, Rappaport E, Surrey S, Fortina P. Connexin26 mutations associated with the most common form of non-syndromic neurosensory autosomal recessive deafness (DFNB1) in Mediterraneans. Hum Mol Genet 1997;6:1605–9.

[15] Sobe T, Erlich P, Berry A, Korostichevsky M, Vreugde S, Avraham KB, Bonne-Tamir B, Shohat M. High frequency of the deafness-associated 167delT mutation in the connexin 26 (GJB2) gene in Israeli Ashkenazim. Am J Med Genet 1999;86:499–500.

[16] Fuse Y, Doi K, Hasegawa T, Sugii A, Hibino H, Kubo T. Three novel connexin26 gene mutations in autosomal recessive non-syndromic deafness. Neuroreport 1999;10:1853–7.

[17] Abe S, Usami S, Shinkawa H, Kelley PM, Kimberling WJ. Prevalent connexin 26 gene (GJB2) mutations in Japanese. J Med Genet 2000;37:41–3.

[18] Fukushima K, Kasai N, Ueki Y, Nishizaki K, Sugata K, Hirakawa S, Masuda A, Gunduz M, Ninomiya Y, Masuda Y, Sato M, McGuirt WT, Coucke P, Van Camp G, Smith RJ. A gene for fluctuating, progressive autosomal dominant nonsyndromic hearing loss, DFNA16, maps to chromosome 2q23–24.3. Am J Hum Genet 1999;65:141–50.

[19] Kudo T, Ikeda K, Kure S, Matsubara Y, Oshima T, Watanabe Ki, Kawase T, Narisawa K, Takasaka T. Novel mutations in the connexin 26 gene (GJB2) responsible for childhood deafness in the Japanese population. Am J Med Genet 2000;90:141–5.

[20] Grifa A, Wagner CA, D'Ambrosio L, Melchionda S, Bernardi F, Lopez-Bigas N, Rabionet R, Arbones M, Monica MD, Estivill X, Zelante L, Lang F, Gasparini P. Mutations in GJB6 cause nonsyndromic autosomal dominant deafness at DFNA3 locus. Nat Genet 1999;23:16–8.

[21] Prasad S, Cucci RA, Green GE, Smith RJ. Genetic testing for hereditary hearing loss: connexin 26 (GJB2) allele variants and two novel deafness-causing mutations (R32C and 645–648del-TAGA). Hum Mutat 2000;16:502–8.

[22] Scott DA, Kraft ML, Carmi R, Ramesh A, Elbedour K, Yairi Y, Srisailapathy CR, Rosengren SS, Markham AF, Mueller RF, Lench NJ, Van Camp G, Smith RJ, Sheffield VC. Identification of mutations in the connexin 26 gene that cause autosomal recessive nonsyndromic hearing loss. Hum Mutat 1998;11:387–94.

[23] Estivill X, Fortina P, Surrey S, Rabionet R, Melchionda S, D'Agruma L, Mansfield E, Rappaport E, Govea N, Mila M, Zelante L, Gasparini P. Connexin-26 mutations in sporadic and inherited sensorineural deafness. Lancet 1998;351:394–8.

[24] Brunger JW, Murray GS, O'Riordan M, Matthews AL, Smith RJ, Robin NH. Parental attitudes toward genetic testing for pediatric deafness. Am J Hum Genet 2000;67:1621–5.

Association of Clinical Features With Mutation of *TECTA* in a Family With Autosomal Dominant Hearing Loss

Satoshi Iwasaki, MD; Daisuke Harada, MD; Shin-ichi Usami, MD; Mitsuyoshi Nagura, MD; Tamotsu Takeshita, MD; Tomoyuki Hoshino, MD

Background: The *TECTA* gene, which encodes α-tectorin, has recently been cloned. α-Tectorin is a major component of the noncollagenous matrix of the tectorial membrane. Nonsyndromic hearing impairment caused by *TECTA* mutations has been reported in Austrian, Belgian, Swedish, French, and Lebanese families. The phenotypes and genotypes were different among these families.

Materials and Methods: Our study family displayed autosomal dominant hearing impairment through 3 generations. We sequenced the coding exons of the *TECTA* gene in 4 affected individuals, and we report the clinical features in a Japanese family with nonsyndromic hearing impairment and a mutation in the *TECTA* gene.

Results: The 5-frequency average of 250, 500, 1000, 2000, and 4000 Hz in 4 affected individuals was 42.2±3.7 (mean±SD) dB in the right ear and 42.3±4.5 dB in the left ear. The mean age at onset of hearing impairment was 5 years. The progression of hearing impairment was not confirmed for a 15-year period, from the age of 6 to 21 years, in 1 affected member. The 4 patients had a G→A missense mutation at nucleotide 6063 in exon 20. This mutation replaces arginine at residue 2021 with histidine (R2021H).

Conclusions: All 4 affected members showed symmetrical and stable bilateral mild to moderate hearing impairment in the midfrequencies. The mean threshold level of 2000 Hz was the worst among the 5 frequencies. All the affected members had normal vestibular function. The mutation in the *TECTA* gene, localized in the zona pellucida domain, was detected in all 4 affected individuals. The localization of the mutation in the different modules of the protein may have caused the different clinical features.

Arch Otolaryngol Head Neck Surg. 2002;128:913-917

THERE HAS been tremendous progress in the research of the genetic basis of deafness. It had always been assumed that single-gene defects were responsible for hearing impairment, but many different genes causing deafness, which probably account for more than 50% of the cases of childhood deafness, have recently been reported.[1] So far, 70 loci involved in nonsyndromic deafness have also been reported.[2]

The tectorial membrane is an extracellular gellike matrix leaf that attaches to the tallest row of the stereociliary bundles of the outer hair cells. The displacement of the tectorial membrane stimulates the outer hair cells, which open the transduction channels and lead to hair cell depolarization. The ultrastructural defects of the tectorial membrane are caused by mutations in 3 different points of genes, namely encoding α-tectorin (*TECTA*),[3] collagen 11-α_2 (*COL11A2*),[4] and otogelin (*Otog*),[5] which lead to human hearing impairment.[6] The tectorial membrane contains collagenase-sensitive and -insensitive proteins. The major components of the noncollagenous matrix are α-tectorin and β-tectorin, which interact with each other.[2]

The *TECTA* gene has recently been cloned and shown to be associated with nonsyndromic hearing impairment.[3] It encodes a protein of 2155 amino acids, 95% of which are identical to mouse α-tectorin, which has an aminoterminal hydrophobic signal sequence for translocation across the membrane and a carboxyterminal hydrophobic region characteristic of precursors for glycosylphosphatidylinositol-linked membrane-bound proteins.[7] An alteration in α-tectorin is likely to disrupt the structure of this matrix. DFNA8,[8] DFNA12,[9] and DFNB21[10] loci are mapped on chromosome 11q[11] and are all segregating alleles of *TECTA*. Nonsyndromic hearing impairment caused by *TECTA* mutations has been reported in Austrian,[3] Belgian,[3] Swedish,[12] French,[13]

From the Department of Otolaryngology, Hamamatsu University School of Medicine, Hamamatsu City (Drs Iwasaki, Nagura, Takeshita, and Hoshino) and Shinshu University School of Medicine, Matsumoto City (Drs Harada and Usami), Japan.

©2002 American Medical Association. All rights reserved.

and Lebanese[10] families. The phenotypes and genotypes were different among these families. In this study, we describe the clinical features in a Japanese family with autosomal dominant nonsyndromic hearing impairment and present a mutation analysis of the *TECTA* gene.

SUBJECTS AND METHODS

CLINICAL DIAGNOSIS

A family pedigree was constructed at Hamamatsu University Hospital, Hamamatsu City, Japan (**Figure 1**). Otoscopic and audiometric examinations were performed on all cooperative family members. Blood samples from the family members were obtained after informed consent was granted. Pure-tone audiometry was performed with air conduction at 125, 250, 500, 1000, 2000, 4000, and 8000 Hz and with bone conduction at 250, 500, 1000, 2000, and 4000 Hz. Audiograms were available for 4 members (III:2, III:3, II:2, and I:7) of the pedigree. A speech discrimination test, otoacoustic emissions screening (Capella; GN Otometrics, Tokyo, Japan), a caloric test, and computed tomography were also performed. For 1 member (II:2), we can chart the hearing impairment via audiometric examinations over a long term. His hearing impairment was detected during an examination in primary school when he was 6 years old, and he experienced head trauma while playing a sport at the age of 10 years. Congenital aural fistula of the right ear was confirmed in patient III:3, and her hearing impairment was noticed by her parents when she was 4 years old. Patient II:2 was diagnosed as having hypothyroidism (Hashimoto disease). Patient I:5 had a noise-induced hearing impairment. A hearing aid was used by patients I:4 and I:6. None of the 4 affected family members, all of whom underwent audiometric testing, had complained of tinnitus or vertigo.

MUTATION ANALYSIS

Intronic polymerase chain reaction (PCR) amplification primers flanking each exon[3] were used to detect mutations. Exons 1-20 of *TECTA* were amplified from genomic DNA samples by PCR. A 5-minute denaturation at 95° was followed by 35 three-step cycles (95° for 30 seconds, 55° for 1 minute, and 72° for 1 minute), followed by 72° for 10 minutes, and ending with a holding period at 4° in a thermal cycler (Perkin-Elmer Corp, Norwalk, Conn). The PCR products were directly sequenced after removal of unincorporated dinucleotide triphosphates and primers by incubation at 37° for 30 minutes with 50-to 100-ng PCR product with 0.1 µL of exonuclease I (Amersham Life Science, Cleveland, Ohio) and 1 µL of shrimp alkaline phosphatase (Amersham Life Science). The enzymes were heat inactivated at 80° for 15 minutes. An aliquot of 6 pmol of either the forward or the reverse primer was used in standard cycle sequencing reactions and run on a sequencer. DNA samples from 96 unrelated Japanese, who had normal hearing, were used as controls.

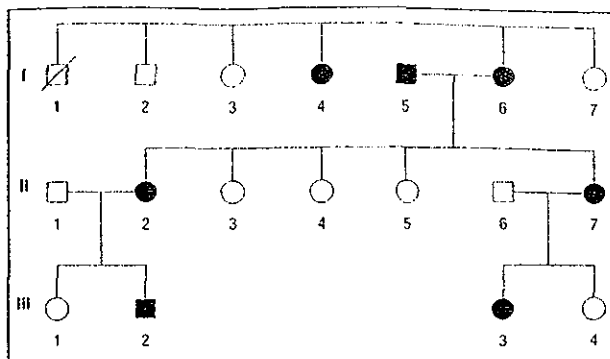

Figure 1. A Japanese pedigree with autosomal dominant hearing loss. Four affected family members (II:2, II:7, III:2, and III:3) were identified with *TECTA* mutation. Member III:3 had a congenital aural fistula in the right ear. Hypothyroidism (Hashimoto disease) was diagnosed in member II:2. Member I:5 had worked in a noisy environment.

RESULTS

Audiograms of the 4 affected members are shown in **Figure 2**. They demonstrated bilateral mild to moderate, symmetrical, and stable sensorineural hearing impairment in the midfrequencies. The mean±SD level of hearing impairment at 250, 500, 1000, 2000, and 4000 Hz was 42.2±3.7 dB (range, 38-47dB) in the right ear and 42.3±4.5 dB (range, 36-47dB) in the left ear. The mean±SD level at each frequency was 22.6±5.5 dB (250 Hz), 32.5±14.4 dB (500 Hz), 51.3±14.3 dB (1000 Hz), 66.3±12.5dB (2000 Hz), and 35±20.0 dB (4000 Hz) in the right ear and 26.3±7.5 dB (250 Hz), 36.3±8.5 dB (500 Hz), 48.8±7.5 dB (1000 Hz), 57.5±15.5 dB (2000 Hz), and 42.5±17.0 dB (4000 Hz) in the left ear. The history of the progression of hearing impairment charted by audiometry over 15 years (from age 6-21 years) in patient III:2 is shown in **Figure 3**. The mean level of maximum speech discrimination was 95% at the stimulus level of 70 dB. The responses on the distortion-product otoacoustic emissions and the transient evoked otoacoustic emissions were decreased, which indicated that the current hearing impairment was caused by inner ear dysfunction. All the affected members had normal vestibular function. Abnormality of the inner ear was not found with computed tomography.

The G→A missense mutation at nucleotide 6063 in exon 20 in the *TECTA* gene was detected in all 4 affected members (**Figure 4**). This mutation replaces arginine at residue 2021 with histidine (R2021H). All 4 affected members were heterozygous for this mutation. The present mutation was not found in any of the samples from Japanese controls.

COMMENT

A Japanese family with nonsyndromic autosomal dominant hearing loss was investigated. The hearing loss was bilateral and symmetrical, and there was inner ear dysfunction. Stable, moderate, and midfrequency hearing loss was detected on auditory examinations of all 4 affected members at a mean age of 5 years. Histories of delayed speech development and distortion of utterance suggested a prelingual onset of hearing impairment. The

©2002 American Medical Association. All rights reserved.

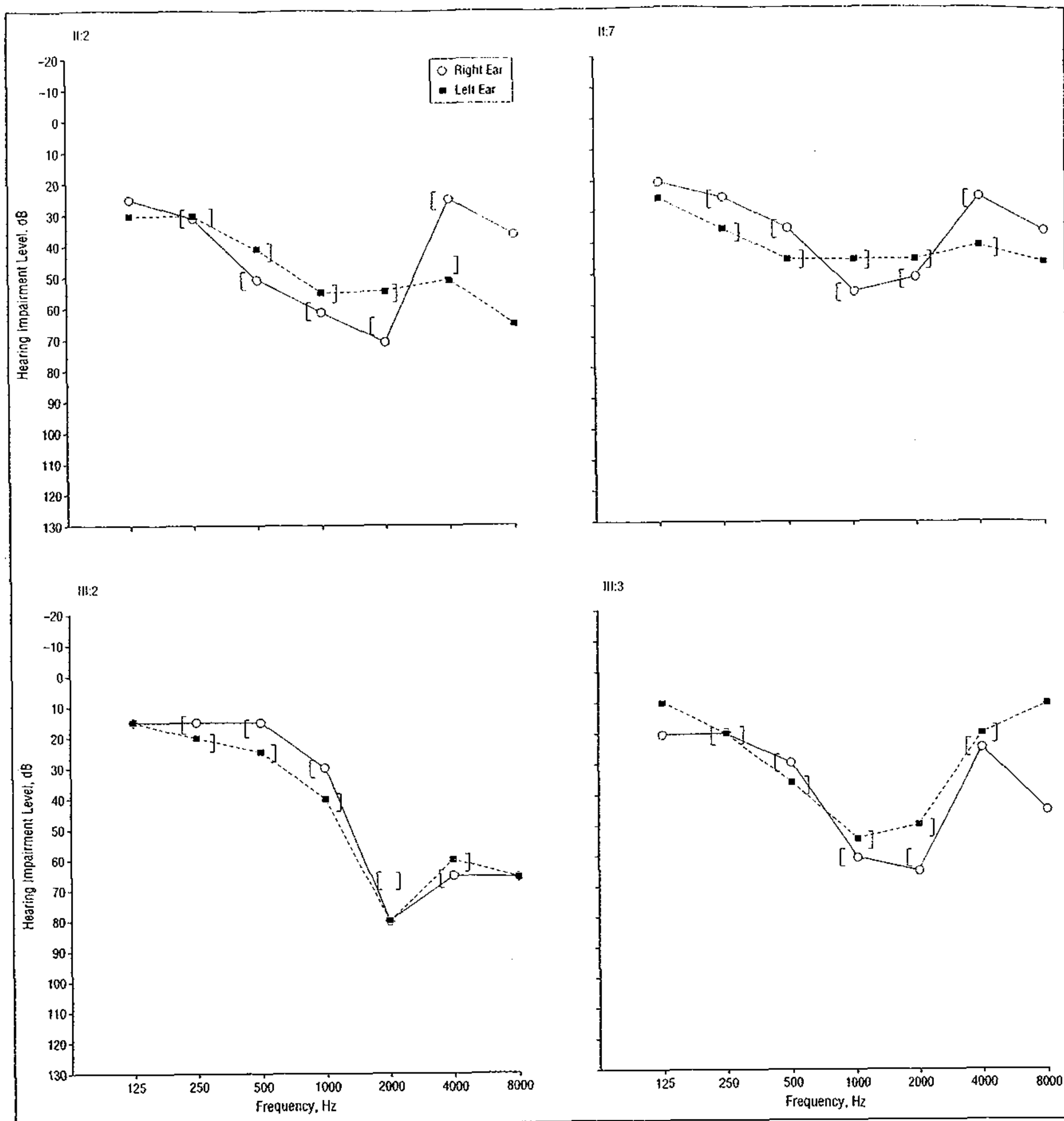

Figure 2. Representation of audiograms for the 4 affected family members (II:2, II:7, III:2, and III:3) at the age of 7 years (III:3), 18 years (III:2), 33 years (II:7), and 42 years (II:2). The mean±SD level of hearing loss at 5 frequencies was 42.2±3.7 dB (right ear) and 42.3±4.5 dB (left ear), and the maximum level of hearing loss was 2000 Hz (57.5±15.5 dB).

mean±SD level of hearing impairment was 42.2±3.7 dB (right ear) and 42.3±4.5dB (left ear). The mean threshold level of 2000 Hz was the worst among the 5 frequencies (250-8000 Hz). We were able to follow the history of hearing impairment with audiometry for 15 years (from age 6-21 years) in 1 affected member. The hearing impairment did not change during that period. The head trauma of 1 affected member did not induce a progression of hearing impairment.

Mutation of the *TECTA* gene has been identified in Belgian,[3] Austrian,[3] French,[13] Swedish,[12] and Lebanese[10] families with nonsyndromic hearing loss. The characteristics of nonsyndromic hearing loss in these families were classified into 2 phenotypes. Nonsyndromic autosomal dominant hearing loss was found in the Belgian,[3,9,14] Austrian,[3,15] French,[13] and Swedish,[12,16] families in addition to the current family. Nonsyndromic autosomal recessive hearing loss was found in the Lebanese family.[10] Mild to severe and progressive hearing loss in the high frequencies was reported in the French and Swedish autosomal dominant families. The onset of hearing impairment was different between the French and Swedish families. Although the hearing impairment in the Swedish family was postlingual, with a mean age at on-

©2002 American Medical Association. All rights reserved.

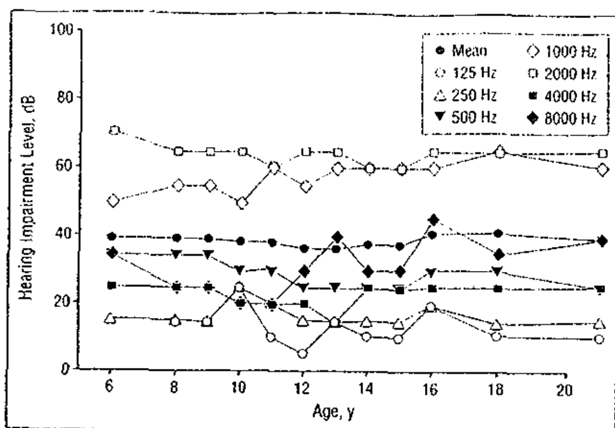

Figure 3. Long-term follow-up on audiometric tests in an affected member (III:2). The history over 15 years, from the age of 6 years to 21 years, of hearing impairment at 7 frequencies and the mean level of hearing loss in the right ear are shown.

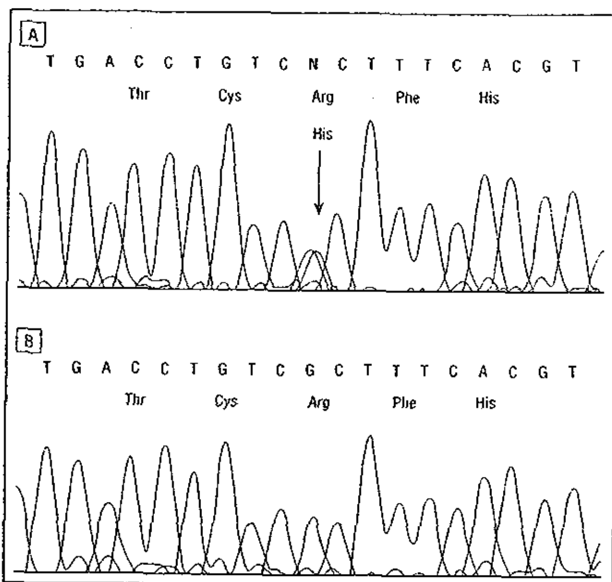

Figure 4. Sequence electropherograms. A, Affected member (III:3) with heterozygous G→A missense mutation at nucleotide 6063 in the α-tectorin exon 20 (arrow): an arginine (Arg) to histidine (His) substitution (R2021H). The same mutation was found in all 4 affected members (II:2, II:7, III:2, and III:3). B, Unaffected normal control. Thr indicates threonine; Cys, cysteine; and Phe, phenylalanine.

set of 14 years old, hearing impairment in the French family was detected before the age of 6 years, and a prelingual onset has been suggested because of a history of delayed speech development. Mild to moderately severe, prelingual, and stable midfrequency hearing loss was reported in the Belgian and Austrian autosomal dominant families. The current audiological features are similar to those in the Austrian and Belgian families (**Table**). The autosomal recessive hearing loss in the Lebanese family was severe to profound (70-110 dB) and prelingual in all frequencies. The hearing loss in an autosomal recessive family is always characterized by profound, prelingual onset and a stationary pattern. In contrast, most autosomal dominant families have postlingual and progressive hearing loss.[8] However, the human tectorial membrane is formed between the 12th and 20th weeks of embryonic development.[3] α-Tectorin and β-tectorin, which are major components of the noncollagenous matrix of the tectorial membrane, are only expressed transiently during cochlear development in the mice.[3] Referring to these features, the characteristics of nonsyndromic hearing loss caused by mutation of *TECTA* seem to be prelingual onset and a stationary pattern.

There was no history of vertigo or dizziness in the present family or in the previously described families. A caloric test as a vestibular examination also revealed normal function. However, some affected members of the French family were late to start walking, which can be explained by a deficit of α-tectorin in the vestibular organ. α-Tectorin is expressed in the 2-day mouse utricle and saccule.[7]

The *TECTA* gene is composed of 3 distinct modules: entactin G1 domain, zonadhesin domain (with von Willebrand factor type D repeat), and zona pellucida domain.[12] The mutations found in the Belgian, Austrian, and present families were localized in the zona pellucida domain. A missense mutation at nucleotide 5876 in exon 18 replaced tyrosine at residue 1870 with cysteine (Y1870C) in the Austrian family.[3] The Belgian family demonstrated 2 mutations in exon 17, which replaced leucine at residue 1820 with phenylalanine (L1820F) and aspartic acid at residue 1824 with glycine (G1824D).[3] The present missense mutation at nucleotide 6063 in exon 20 leads to a substitution of arginine for histidine at resi-

Association of Clinical Features and Genotype With *TECTA* Mutations*

Family	Mutation	Protein	Inheritance	Hearing Loss	Time of Onset	Vertigo
Belgian	5725C→T (exon 17) 5738G→A (exon 17)	G1824D, ZP domain	AD	Mild to moderately severe (21-80 dB; mean, 51 dB); stable, midfrequency	Prelingual (before 6 y)	Unknown
Austrian	5876A→G (exon 18)	Y1870C, ZP domain	AD	Moderate to severe (60-80 dB); stable, midfrequency	Prelingual	Unknown
Swedish	3170T→A (exon 10)	C1057S, ZD domain	AD	Mild to severe; progressive high frequency	Postlingual (9 or 19 y)	Unknown
French	4857G→C (exon 14)	C1619S, ZD-like domain (D4 vWf type D repeat)	AD	Mild to moderate; progressive high frequency	Prelingual (before 6 y)	(–) Late start walking
Lebanese	*Intron 9 donor site G→A (exon 9)*	*Amino acid 972 stop codon*	AR	*Moderately severe to profound; all frequencies*	*Prelingual*	*Unknown*
Present case	6063G→A (exon 20)	R2021H, ZP domain	AD	Moderate (36-47 dB; mean, 42 dB); stable, midfrequency	Prelingual (before 6 y)	(–)

*ZP indicates zona pellucida; ZD, zonadhesin; AD, autosomal dominant; AR, autosomal recessive; vWf, von Willebrand factor; and minus sign, none.

©2002 American Medical Association. All rights reserved.

due 2021 (R2021H) and has been identified in 4 affected members. The mutation of *TECTA* in the zona pellucida domain (residues 1805-2057) may disrupt the interactions between the different polypeptides of tectorial membrane, and, as a consequence, improper assembly of the tectorial membrane might cause an inefficient mechanotransduction process. The mutations found in the French and Swedish families were identified in the zonadhesin domain of *TECTA*. This mutation abolishes the first of the vicinal cysteine present in the D4 von Willebrand factor type D repeat[12] and may cause a change in the cross-linking of the polypeptide. A missense mutation at nucleotide 4857 in exon 14 replaced the cysteine at residue 1619 with serine (C1619S) in the French family.[13] The mutation found in the Swedish family resulted in the replacement of cysteine with serine at residue 1057 (C1057S) in exon 10.[12] Although these mutations were identified heterozygously in the affected members, the mutation found in the Lebanese family led to the identification of a G→A transition in the donor splice site of intron 9 homozygously in the affected members, skipping exon 9 and resulting in a stop codon at amino acid position 972.[10]

The mutations localized in the zona pellucida domain resulted in the prelingual and stable hearing loss in the midfrequencies in the autosomal dominant families. The progressive hearing loss in the high frequency was found in the autosomal dominant family in which the mutation was identified in the zonadhesin domain. These findings suggest that the localization of the mutation in the different modules of the protein may result in the different phenotypes.

Accepted for publication January 10, 2002.

This work was supported by a grant from the Acute Profound Deafness Research Committee of the Ministry of Health, Labour, and Welfare of Japan.

We thank all members of the family for their participation in this study.

Corresponding author and reprints: Satoshi Iwasaki, MD, Department of Otolaryngology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu City 431-3192, Japan (e-mail: iwasaki@hama-med.ac.jp).

REFERENCES

1. Morton NE. Genetic epidemiology of hearing impairment. *Ann N Y Acad Sci.* 1991; 630:16-31.
2. Steel KP, Kros C. A genetic approach to understanding auditory function. *Nat Genet.* 2001;27:143-149.
3. Verhoeven K, Van Laer L, Kirschhofer K, et al. Mutations in the human α-tectorin gene cause autosomal dominant non-syndromic hearing impairment. *Nat Genet.* 1998;19:60-62.
4. McGuirt WT, Prasad SD, Griffith AJ, et al. Mutations in COL11A2 cause non-syndromic hearing loss (DFNA13). *Nat Genet.* 1999;23:413-419.
5. Simmler MC, Cohen-Salmon M, El-Amraoui A, et al. Targeted disruption of Otog results in deafness and severe imbalance. *Nat Genet.* 2000;24:139-143.
6. Steel KP. A take on the tectorial membrane. *Nat Genet.* 2000;24:104.
7. Legan PK, Rau A, Keen JN, Richardson GP. The mouse tectorins: modular matrix proteins of the inner ear homologous to components of the sperm-egg adhesion system. *J Biol Chem.* 1997;272:8791-8801.
8. Van Camp G, Willems PJ, Smith RJH. Nonsyndromic hearing impairment: unparalleled heterogeneity. *Am J Hum Genet.* 1997;60:758-764.
9. Verhoeven K, Van Camp G, Govaerts PJ, et al. A gene for autosomal dominant nonsyndromic hearing loss (DFNA12) maps to chromosome 11q22-24. *Am J Hum Genet.* 1997;60:1168-1173.
10. Mustapha M, Weil D, Chardenoux S, et al. An α-tectorin gene defect causes a newly identified autosomal recessive form of sensorineural pre-lingual non-syndromic deafness, DFNB21. *Hum Mol Genet.* 1999;8:409-412.
11. Hughes DC, Legan PK, Steel KP, Richardson GP. Mapping of the α-tectorin gene (*TECTA*) to mouse chromosome 9 and human chromosome 11: a candidate for human autosomal dominant nonsyndromic deafness. *Genomics.* 1998;48:46-51.
12. Balciuniene J, Dahl N, Jalonen P, et al. Alpha-tectorin involvement in hearing disabilities: one gene—two phenotypes. *Hum Genet.* 1999;105:211-216.
13. Alloisio N, Morle L, Bozon M, et al. Mutation in the zonadhesin-like domain of α-tectorin associated with autosomal dominant non-syndromic hearing loss. *Eur J Hum Genet.* 1999;7:255-258.
14. Govaerts P, Ceulaer GD, Daemers K, et al. A new autosomal-dominant locus (DFNA12) is responsible for a nonsyndromic, midfrequency, prelingual and non-progressive sensorineural hearing loss. *Am J Otol.* 1998;19:718-723.
15. Kirschhofer K, Kenyon JB, Hoover DM, et al. Autosomal-dominant, prelingual, nonprogressive sensorineural hearing loss: localization of the gene (DFNA8) to chromosome 11q by linkage in an Austrian family. *Cytogenet Cell Genet.* 1998; 82:126-130.
16. Balciuniene J, Dahl N, Borg E, et al. Evidence for digenic inheritance of nonsyndromic hereditary hearing loss in a Swedish family. *Am J Hum Genet.* 1998;63: 786-793.

©2002 American Medical Association. All rights reserved.

Cremers Cor WRJ, Smith RJH (eds): Genetic Hearing Impairment.
Adv Otorhinolaryngol. Basel, Karger, 2002, vol 61, pp 41–46

DFNA2/KCNQ4 and Its Manifestations

Els M.R. De Leenheer[a], *Robbert J.H. Ensink*[a], *Henricus P.M. Kunst*[a], *Henri A.M. Marres*[a], *Zohreh Talebizadeh*[b], *Frank Declau*[c], *Shelley D. Smith*[d], *Shin-ichi Usami*[e], *Paul H. Van de Heyning*[c], *Guy Van Camp*[f], *Patrick L.M. Huygen*[a], *Cor W.R.J. Cremers*[a]

[a] Department of Otorhinolaryngology, University Medical Centre Nijmegen, The Netherlands;
[b] Section of Medical Genetics and Molecular Medicine, Children's Mercy Hospital, Kansas City, Mo., USA;
[c] University Department of Otorhinolaryngology, University of Antwerp, Belgium;
[d] Center for Human Molecular Genetics, Munroe Meyer Institute, University of Nebraska Medical Center, Omaha, Nebr., USA;
[e] Department of Otorhinolaryngology, Shinshu University School of Medicine, Matsumoto, Japan, and
[f] Department of Medical Genetics, University of Antwerp, Belgium

Over ten families have been linked to the DFNA2 locus [1–7]. This makes it one of the most frequently encountered loci implemented in autosomal dominant nonsyndromic hearing impairment. The DFNA2 region is located on chromosome 1p34 and has been shown to include *GJB3* and *KCNQ4* [1–4]. *GJB3* or *connexin 31* encodes a gap junction [3], whereas *KCNQ4* is responsible for the production of *KCNQ4* subunits, tetrameres of which constitute a voltage-gated potassium channel [4]. Both genes are believed to be involved in the K^+ recycling pathway of the inner ear [5]. Most families harbor a mutation in the *KCNQ4* gene [4–8] and, interestingly, there is still one family without any detectable mutation, suggesting a third, as yet unidentified, DFNA2-linked gene [9]. Cochlear expression studies in rat demonstrated a basal to apical increasing *kcnq4* gradient in inner hair cells and the spiral ganglion cells, whereas a reciprocal gradient was found in outer hair cells [10].

We briefly describe previously reported phenotypic data of all DFNA2 families with a known mutation [1, 3–6, 9, 11–15]. For the sake of comparison, we statistically analyzed eight of these families using similar methods and present typical audiometric curves for each of them.

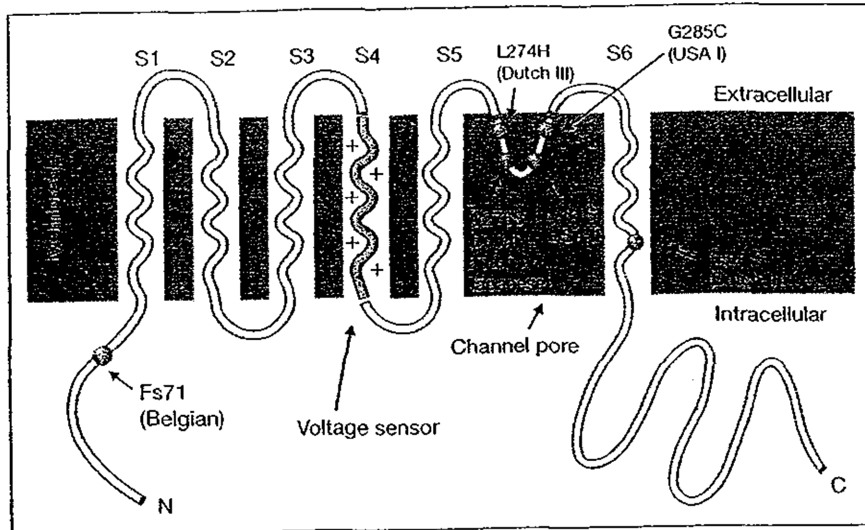

Fig. 1. Graphical representation of the *KCNQ4* voltage-gated K^+ channel. The six transmembrane domains (S1–S6) as well as the pore region are indicated. The specific mutations of all families represented in figure 2 are marked.

Patients and Methods

We collected audiograms from mutation carriers of one Belgian, one Japanese, two American and four Dutch DFNA2/*KCNQ4* families. They all carried a missense mutation, except for the Belgian subjects where an inactivating deletion in the *KCNQ4* gene was involved [6–9]. The Japanese, Dutch I and IV family harbor exactly the same mutation (W276S) and are probably unrelated [8]. W276S is therefore regarded as a potential hotspot for mutation. The specific mutations present in each family are shown in figure 1. Persons thought to have other nonhereditary causes of hearing impairment were excluded from the analyses.

Pure-tone hearing thresholds (binaural mean) were analyzed in relation to age (linear regression analysis) to construct age-related typical audiograms pertaining to age 10, 20, 30, 40, 50, 60 and 70 years for the separate families. A previous study on the Dutch IV family showed inconsistencies between the cross-sectional and the longitudinal analyses [14]. It seemed that the former one was unreliable and therefore longitudinal analysis was preferred.

Review and Results

Previously, *GJB3* mutations were detected in two small Chinese families [3]. They demonstrated progressive, high-frequency sensorineural hearing impairment and tinnitus, present from about age 30 years onwards. Later, the *KCNQ4* gene was cloned and shown to be mutated in a small French family with progressive, high-frequency hearing impairment [4].

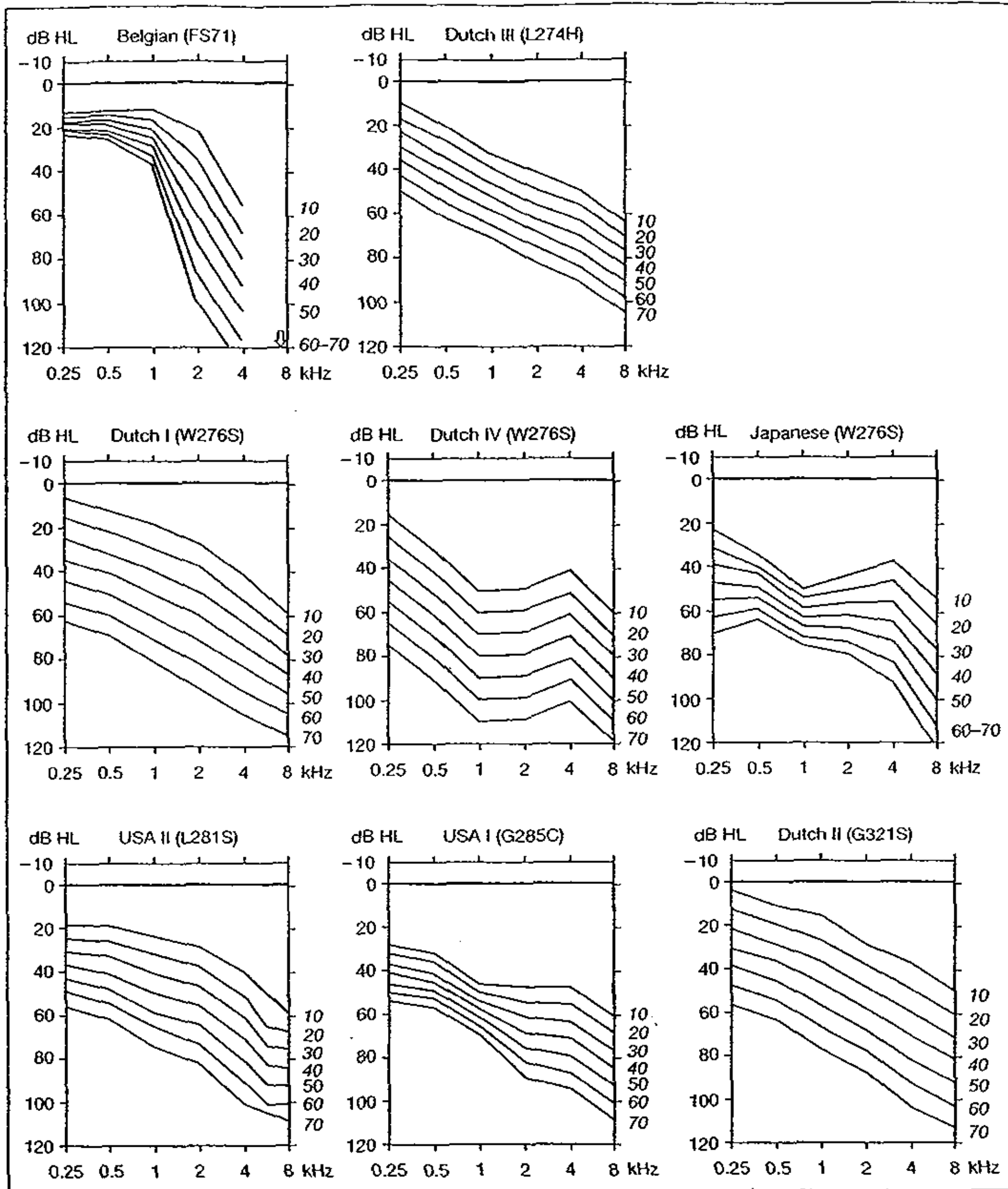

Fig. 2. Age-related typical audiograms of the DFNA2/*KCNQ4* families and the involved mutations. Italics indicate age in years.

The age-related typical audiograms of the remaining *KCNQ4* families are illustrated in figure 2. As previously described, all families have symmetrical, predominantly high-frequency sensorineural hearing impairment, progressive at all frequencies [1, 6, 9, 11–15]. The Belgian family however, has a rather atypical phenotype sparing the low frequencies, and more progressively and