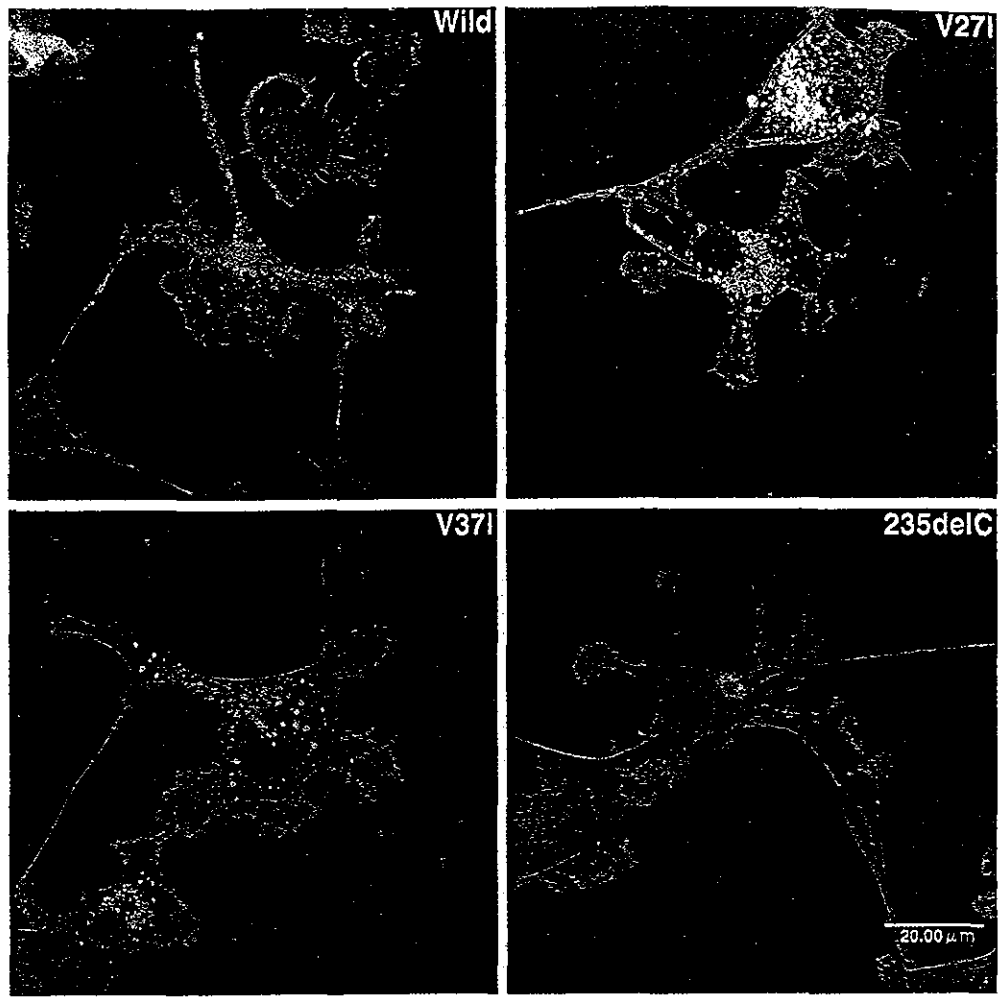

Fig. 3 Protein expression in transfected COS-7 cells. COS-7 cells transfected with GFP-Cx26 wt, GFP-Cx26 V27I, and GFP-Cx26 V37I, which were associated with normal–mild phenotypes, showed a characteristic puncta along the membrane. In contrast, only perinuclear staining was seen in GFP-Cx26 235delC. *Red* actin filament (TRITC- conjugated phalloidin): cell membrane, *Blue* DAPI: nucleus, *Green* Green fluorescent protein: chimeric protein

35delG exhibit severe-to-profound hearing impairment (Cohn et al. 1999; Cryns et al. 2004; Denoyelle et al. 1997, 1999; Green et al. 1999; Marlin et al. 2001; Wilcox et al. 2000). The status of the 235delC mutation, which seems to be a unique mutation in populations with Asian ancestry, is comparable to the 35delG mutation in Caucasoid populations. High prevalence of 35delG and 235delC mutations in the respective populations are due to a founder effect (Ohtsuka et al. 2003; Van Laer et al. 2001). Patients homozygous or compound heterozygous for the 235delC mutation exhibit a comparatively severer phenotype (Fig. 2), indicating that this frequent mutation should be the first to be considered when genetic screening for congenitally deaf patients is performed in Asian populations.

Several reports have indicated the existence of less-severe phenotypes correlated with certain specific mutations, especially in association with V37I (Bason et al. 2002; Cryns et al. 2004; Marlin et al. 2001; Rabionet et al. 2000; Wilcox et al. 2000). The exact phenotype has been rather difficult to prove because of the relatively small number of patients with V37I. The V37I mutation was originally reported as a polymorphism (Kelley et al. 1998), but the fact that valine 37 residue is highly conserved among different connexins, and that a series of reports identified homozygous or compound heterozygous V37I deafness patients (Abe et al. 2000; Bason et al. 2002; Marlin et al. 2001; Rabionet et al. 2000; Wilcox et al. 2000), indicate that it may be a disease-causing mutation. There seem to be ethnic differences in the allele frequency of V37I, as it was not detected in the control subjects from Italy, Spain, Germany, Greece, Israel, Ghana, or Austria (see Discussion in Bason et al. 2002) in spite of a high prevalence in the Japanese population (Abe et al. 2000; Kudo et al. 2000; Ohtsuka et al. 2003). The reported patients in whom the ethnic background was known were all of eastern-Asian origin (Abe et al. 2000; Bason et al. 2002; Kudo et al. 2000; Ohtsuka et al. 2003). In Japanese, V37I is the second most frequent mutated allele, and in this study, it was possible to collect a significant number of patients, and the present data confirmed a less severe phenotype caused by V37I. Due to such a mild phenotype, timing of presentation at clinics and diagnosis may be comparatively delayed. For patients with V37I/V37I, hearing impairment was noticed at the age of 20.6 (range 7–49, SD 17.08) years of age in contrast with 0.33 (range 0–3, SD 1.00) years for patients with 235delC/235delC. It

should therefore be noted that patients with *GJB2* mutations can also be found among less-severe hearing-impaired populations.

A recent multi-center-based genotype–phenotype correlation study clearly showed that severity of hearing impairment is correlated with some particular genotype and proposed a hypothetical general rule that inactivating mutations (stop or frameshift mutations) cause more severe phenotypes than those caused by noninactivating mutations (Cryns et al. 2004). Concerning the comparison between combinations of inactivating mutations and combinations of noninactivating mutations, the present study also showed that the former cause a severer phenotype than that caused by the latter. Therefore, our study supports the above hypothetical general rule.

Overlapped audiograms showed high-frequency-predominant sensorineural hearing loss regardless of genotype. Overall, there seemed to be certain rules regarding genotype and phenotype correlations. Particular genotypes tended to have similar audiograms with minor exceptions (Fig. 1). Therefore, genotype is a fundamental factor to predict phenotype. However, variations among the same phenotypes still exist (Fig. 1). These variations may be explained by the following factors involved in phenotypes: (1) alterations in promoter regions, (2) additional genes such as *GJB6* (del Castillo et al. 2002), (3) modifier genes (Abe et al. 2001), (4) environmental factors. Concerning patients with G45E/Y136X, there was great variability in their phenotypes, ranging from normal to profound. A segregation study indicated that either G45E or Y136X situated on the same allele or different alleles. Our subcloning experiments confirmed the existence of two types of allele: cis allele and trans allele (data not shown). When two mutations are on different alleles (compound heterozygous state), the patients may exhibit severe-to-profound hearing impairment.

The present study further investigated whether the differences in phenotype could be explained by protein-expression study. In contrast to transfected GFP-Cx26 wt, which were found to be localized as labeled puncta along the plasma membrane (Fig. 3), the localization of transfected GFP-Cx26 235delC was not seen on the cellular membrane but mainly cohered at or around the nucleus. Such abnormal subcellular localization of mutated Cx26 protein with 235delC is consistent with a previous study (Choung et al. 2002). From these results, truncated mutations at the transmembrane domain, such as 235delC, were considered to lead to loss of function, resulting in serious hearing impairment. In the case of V37I, which is categorized as a noninactivating mutation, transfected GFP-Cx26 V37I was found along the membrane as in the wild type, indicating that the V37I protein may retain its function and therefore results in a rather mild phenotype. As expected, V27I, a known polymorphism, showed a similar distribution pattern to the wild type and V37I. To summarize, in the present study, the results indicate that protein expression patterns are well correlated with clinical phenotypes. A series of in vitro studies, including protein expression study, cell-to-cell communication properties, or physiological conductance experiments, sometimes provided discrepant results when compared to the phenotypic results, and limitations have been suggested (see discussion in Cryns et al. 2004). In the case of V37I, a complete loss of junctional properties has been reported (Bruzzone et al. 2003) in spite of a rather mild phenotype shown in a series of studies. The protein expression experiments in the current study, however, were in line with the phenotype associated with this mutation.

In conclusion, the present genotype–phenotype correlation results supported the view that phenotypes caused by the truncating *GJB2* mutations are severer than those caused by missense mutations. Anticipating severity of hearing impairment is sometimes difficult, but if such general rules can be drawn with regard to genotype–phenotype correlation, determination of these correlations will facilitate the prediction of the course of hearing and help in making decisions regarding treatment/intervention.

Acknowledgements We thank all subjects who participated in the present project. This work was 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.).

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Deafness Due to A1555G Mitochondrial Mutation Without Use of Aminoglycoside

Tatsuo Matsunaga, MD, PhD; Hiroshi Kumanomido, MD; Masae Shiroma, PhD; Akihiro Ohtsuka, MD; Kenji Asamura, MD; Shin-ichi Usami, MD, PhD

Objectives/Hypothesis: **The objective was to clarify the characteristics of deafness associated with the A1555G mutation within mitochondrial 12S ribosomal RNA gene in the absence of aminoglycoside exposure.** ***Study Design:*** **Clinical and genetic studies in family members with the A1555G mitochondrial mutation were performed.** ***Methods:*** **The subjects were 123 maternally related members of a large Japanese family with the A1555G mutation. All subjects had no previous history of exposure to aminoglycosides. Hearing disability and handicap, tinnitus, and medical histories were analyzed by interviews in all of the subjects, genetic testing was performed in 41 subjects, and pure-tone audiometry was conducted in 26 subjects with hearing disability and handicap.** ***Results:*** **The A1555G mutation was detected in a homoplasmic form (meaning that all the mitochondrial DNA carries the mutation) in all 41 subjects who were screened. The risk for developing postlingual hearing loss was likely to be much higher in the present subjects than in the general population. Both the severity and age at onset of the phenotype were similar in affected subjects within the same sibling group. Pure-tone averages were significantly worse in subjects who developed hearing loss before age 10 years than in those who developed hearing loss later.** ***Conclusion:*** **The present study demonstrated that the prevalence of deafness in individuals with the A1555G mitochondrial mutation was likely to be high even in the absence of aminoglycoside exposure and clearly showed the association of severe to profound hearing loss with the onset of hearing loss before age 10 years.**

Key Words: **Hereditary hearing loss, nonsyndromic, cochlea, mitochondria.**

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INTRODUCTION

The A1555G mutation (i.e., A to G substitution at position 1555) in the mitochondrial 12S ribosomal RNA (rRNA) gene contributes to nonsyndromic sensorineural hearing loss and is prevalent in Japanese[1] and other populations.[2] This mutation is homoplasmic, indicating that all the mitochondrial DNA in different cells and tissues of the subject harbors the mutation. The A1555G mutation was first identified in family members who exhibited aminoglycoside-induced deafness,[3,4] and this ototoxic susceptibility has its basis in the increased binding affinity of aminoglycosides for A1555G rRNA.[5] In addition, this mutation may also induce hearing loss in the absence of aminoglycosides.[3,6–12] The expressivity of the A1555G mutation varies even within the same family and is not strictly associated with aminoglycoside exposure. Prevalence, onset, and severity of the A1555G mutation–associated hearing loss in the absence of aminoglycoside exposure have not been characterized. Furthermore, what modulates the expressivity of this mutation is not clear.

We identified a large Japanese family in which the A1555G mutation was prevalent. All the family members were not previously exposed to aminoglycosides. We conducted genetic tests, interviews, and pure-tone audiometry in maternally related members of this family and elucidated the prevalence, characteristics, and intrafamilial patterns of the auditory dysfunction associated with the A1555G mutation in the absence of aminoglycoside exposure.

PATIENTS AND METHODS

Subjects

Subjects were maternally related members of a large Japanese family in which hearing loss was common in a pattern that was compatible with maternal inheritance (Fig. 1). Because of the inheritance pattern, mutations in the mitochondrial DNA was suspected and the following genetic analysis in the proband (V-10) revealed homoplasmic A1555G mutation in the mitochondrial 12S rRNA gene. There were 124 maternally related family members in all (57 male and 67 female family members) comprising

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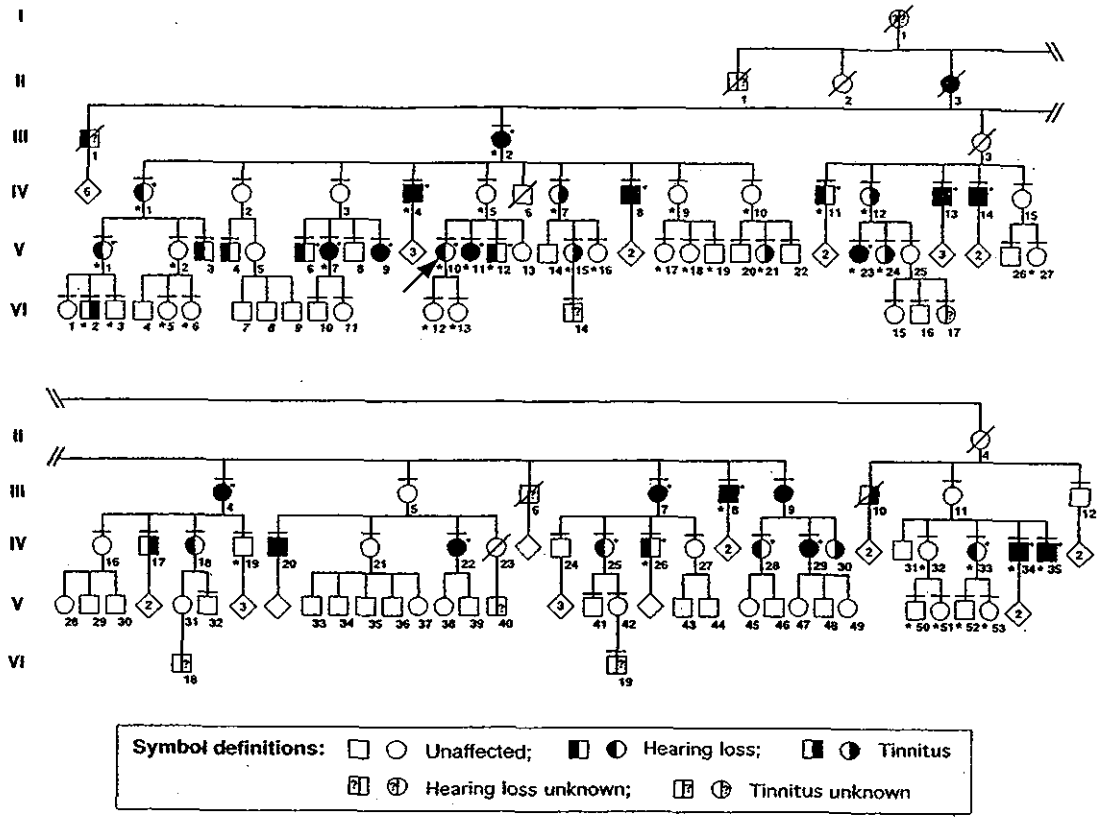

Fig. 1. Pedigree of the family. Generations are indicated by Roman numerals on the left side, and the numbers under the symbols identify the maternally related family members in each generation. Horizontal lines above the symbols indicate individuals who were personally interviewed, dots indicate individuals in whom pure-tone audiometry was conducted, and asterisks indicate individuals from whom DNA samples were obtained. The arrow indicates the proband of the family.

six generations in this family. None of the family members had a history of aminoglycoside exposure. The medical histories of the family members indicated no other significant defects related to mitochondrial mutations apart from hearing loss.

Genetic Analysis

Genetic analysis was performed in 41 maternally related family members (Fig. 1). DNA was extracted from blood samples and screened for the A1555G mutation by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) using endonuclease *Bsm*AI and was confirmed by sequencing as previously described.[8] By the PCR-RFLP analysis, only a single slower-migrating band of a DNA fragment can be detected in the case of the homoplasmic A1555G mutation, whereas two faster-moving fragments can be seen in the case of a normal control without the mutation. In addition, connexin 26 gene mutations were screened by sequencing a PCR-amplified DNA fragment containing the entire coding region as previously described.[13]

Analysis of Hearing Disability and Handicap, Tinnitus, and Medical Histories

Information about hearing disability and handicap, tinnitus, and medical histories were available in total of 123 maternally related family members, (i.e., all but I-1). Using a formatted questionnaire, 81 family members were directly questioned about hearing disability and handicap, tinnitus, aminoglycoside usage, general medical history, and other hearing-related incidents. The *information of three other infant members* was obtained from their mothers, and these infants were also examined for hearing-related behavior and reflexes. One (VI-14) of the three infants exhibited normal response in screening test for hearing loss using an ALGO Newborn Hearing Screener (Natus Medical, Foster City, CA). The information for the other 39 family members was obtained from *family members who were in close contact with the* subjects (usually living with the subjects) by using the same formatted questionnaire. First-degree relatives always confirmed the information provided by affected members, whereas that of unaffected members was confirmed either by first-degree relatives or, in a few cases, by second-degree relatives. Most subjects had been previously screened for *hearing loss during their school* years or for employment purposes. Five subjects (III-8, IV-1, IV-4, IV-25, and IV-26) had a previous history of noise exposure of at least 8 years' duration.

The degree of hearing disability and handicap was classified into the following categories: 1) no problem; 2) mild problem (difficulty in hearing during specific situations such as conversation over background noise, generally not requiring hearing aids); 3) moderate problem (need for hearing aids for daily conversation, and able to communicate by telephone with hearing aids); 4) severe problem (may carry on a conversation only with hearing aids under quiet, face-to-face situations, and inability to communicate by telephone even with hearing aids); and 5) profound problem (need for visual information or signing in addition to hearing aids for conversation in any situation). Tinnitus was considered pathologically significant if it continued for longer than 5 minutes and occurred more than once a week with a duration of 1 month or longer for intermittent tinnitus or 3 days or longer for continuous tinnitus.

Analysis of Hearing Impairment

The degree of hearing impairment was analyzed by pure-tone audiometry following otoscopic examination in 26 subjects with hearing disability and handicap (Fig. 1). The majority of the subjects were tested for pure-tone audiometry using an AA75 audiometer (Rion, Tokyo, Japan) in a soundproof room. For a minority of subjects, the test was conducted with an AA72B audiometer (Rion) using circumaural earphones in quiet rooms where background noise was less than 40 dB sound pressure level (SPL) (measured using a RION NA29 sound level meter with A-weighting). Both air-conducted and bone-conducted thresholds were examined.

The study protocol was approved by the Ethical Committee of the National Tokyo Medical Center (Tokyo, Japan) and was conducted in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained for all subjects participating in the study.

RESULTS

Genetic Analysis

The A1555G mutation was detected in a homoplasmic form (i.e., only a single slower-migrating band of a PCR-amplified DNA fragment could be detected) in all 41 maternally related family members who were screened. Forty of these 41 subjects were also screened for mutations in the nuclear connexin 26 gene (a technical problem prevented the analysis of one subject) that are related to hearing loss, and one individual (IV-19) exhibited such a mutation. This subject had a heterozygous 299–300delAT frameshift mutation (i.e., deletions of A and T at position 299 and 300) in the connexin 26 gene that altered the amino acid sequence from codon 100 and generated a stop at codon 113. The subject exhibited neither hearing loss nor tinnitus.

Hearing Disability and Handicap

In 123 subjects evaluated for hearing disability and handicap, 90 subjects exhibited no problem, and those with any problems were distributed as follows: 20 with mild problem, 8 with moderate problem, 3 with severe problem, and 2 with profound problem (Fig. 2). Overall, hearing disability and handicap were identified in 26.8% (33 of 123) of the subjects and were more frequent in older members than in younger members. There was no significant difference in occurrence between male (26.3%) and female (27.3%) subjects. It is important that affected subjects within the same sibling group tended to exhibit a similar degree of hearing disability and handicap, whereas the severity between sibling groups varied. For example, severe or profound hearing disability and handicap were aggregated in two sibling groups (V-6 to V-9 and V-10 to V-13). The age at onset varied, and neither congenital nor prelingual onset was identified. As was seen in the degree of hearing disability and handicap, the age at onset was similar in affected subjects within the same sibling group, whereas this parameter varied between distinct sibling groups.

Tinnitus

One hundred fifteen subjects could be evaluated for tinnitus. Tinnitus that met our criteria was noted in 24.3% (28 of 115) of the family members (Fig. 2). Tinnitus was continuous (24 h a day) in 15 subjects, intermittent in 11 subjects, and present but with unknown persistence in 2 subjects. The age at onset varied. The disappearance of tinnitus or a significant improvement was noted within 1 month after onset in three subjects, between 1 month and 1 year in one subject, between 1 year and 10 years in three subjects, and after 10 years in one subject. As was noted for hearing loss, both the age at onset and duration of tinnitus were similar among affected subjects within the same sibling group but varied among different sibling groups.

With designation of hearing loss based on reported hearing disability and handicap, both hearing loss and tinnitus were noted in 19 subjects. Fourteen subjects had hearing loss only, and nine subjects had tinnitus only. The onset and duration of hearing loss and tinnitus for all subjects are presented in Figure 3. Of 19 subjects who initially noted tinnitus not associated with hearing loss, 10 subjects later developed hearing loss.

Hearing Impairment

Pure-tone audiometry in 26 subjects with hearing disability and handicap revealed sloping or sharp-sloping audiograms for all but one subject (subject IV-1) who had a previous history of noise exposure and exhibited an audiogram typical for noise-induced hearing loss (i.e., increased bone-conducted thresholds at 4 kHz). In 18 of the 26 subjects, the pure-tone average (PTA) over the frequencies of 0.5, 1, 2, and 4 kHz in the better ear was 25 dB HL or worse, which meets the common lower-threshold definition of hearing impairment.[14] The remaining eight subjects also exhibited significantly elevated pure-tone thresholds at least at 8 kHz (>95th percentile or >50 dB HL), compared with the normal range for their respective ages and sexes.[15] Hearing impairment was sensorineural in type and mostly symmetrical when detected.

The relationship between the degree of hearing impairment and the age at onset of hearing loss in the 26 subjects is shown in Figure 4. The association of severe to profound hearing loss with an onset of hearing loss before age 10 years was evident. In six subjects (three men and three women) with a mean age of 36 years (age range, 31–40 y), who developed hearing loss before 10 years of age, the binaural PTA at the three middle frequencies (binaural PTA at 0.5–2 kHz) ranged from 75 to 103 dB HL (mean value, 89.2 dB HL [SD = 11.7 dB HL]) and the binaural PTA at the three higher frequencies (binaural PTA at 2–8 kHz) ranged from 103 to 112 dB HL (mean value, 108.3 dB HL [SD = 3.7 dB HL]). In 20 subjects (8 men and 12 women) with a mean age of 55 years (age range, 33–87 y), who developed hearing loss at age 10 years or later, the binaural PTA at 0.5 to 2 kHz ranged from 1.5 to 61.5 dB HL (mean value, 29.5 dB HL [SD = 19.6 dB HL]) and binaural PTA at 2 to 8 kHz ranged from 27 to 91.5 dB HL (mean value, 56.6 dB HL [SD = 22.7 dB HL]). Statistical analysis performed using Mann-Whitney *U* test on the Statview statistical software program (SAS Institute Inc., Cary, NC) exhibited a significant difference between these two groups in both the binaural PTA at 0.5

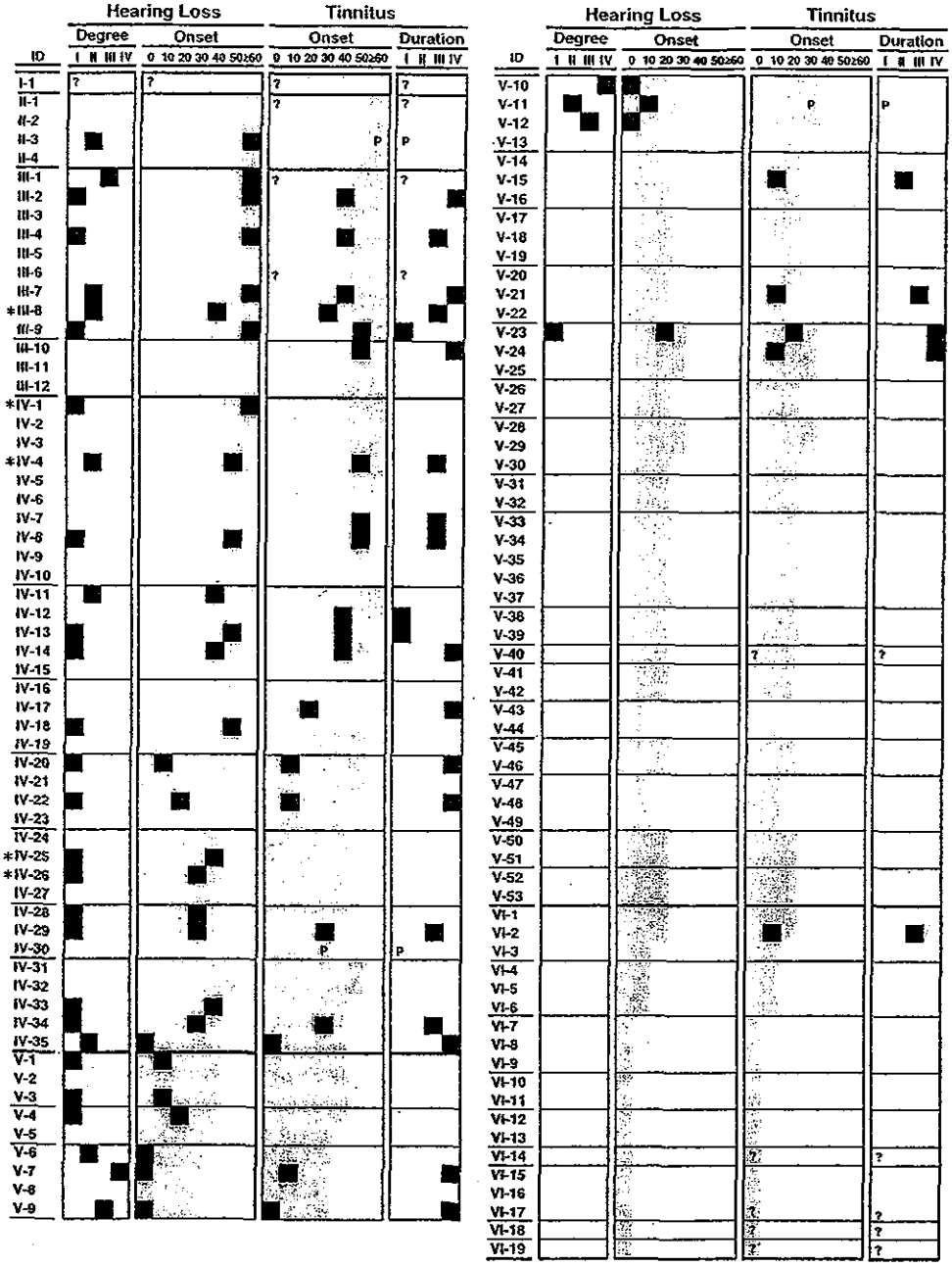

Fig. 2. Summary of phenotypic expression in all maternally related family members ordered by generation and identification number (ID) as designated in the pedigree. Horizontal lines divide different sibling groups. Asterisks indicate individuals with a long history of noise exposure. Each subject is categorized by age in 10-year increments as indicated by horizontal gray bars. Black squares represent degree and onset of hearing loss based on interviews about hearing disability and handicap and onset and duration of tinnitus as classified at the top of the columns. The degree of hearing loss is classified as follows: I, mild; II, moderate; III, severe; and IV, profound (for details, see "Materials and Methods" section). Ages are indicated in 10-year increments as follows: 0, 0–9 years; 10, 10–19 years; 20, 20–29 years; 30, 30–39 years; 40, 40–49 years; 50, 50–59 years; ≥60, 60 years or older. Duration of tinnitus is classified as follows: I, less than 1 month; II, 1–11 months; III, 1–9 years; IV, 10 years or more. The symbol 'p' indicates that the individual presented with tinnitus but the age at onset and the duration of the tinnitus was undefined. The absence of a black square indicates that the individual did not exhibit the corresponding phenotype. Question marks denote undefined phenotypes.

to 2 kHz ($P = .0003$) and the binaural PTA at 2 to 8 kHz ($P = .0003$).

DISCUSSION

Homoplasmic A1555G Mitochondrial Mutation in Maternally Related Family Members

In the proband of the family in the present study, the A1555G mutation was detected in a homoplasmic pattern by the PCR-RFLP analysis. This result indicates that all the mitochondrial genomes in different cells and tissues of the proband harbor the mutation. Besides, all the maternally related family members of the present family should carry the A1555G mutation also in a homoplasmic form because the mitochondrial DNA exhibits exclusively maternal inheritance.[16] This was substantiated by the detection of this homoplasmic mutation in all of the 41 subjects who were screened.

Prevalence of Hearing Loss

Based on the interviews about hearing disability and handicap in the present study, 26.8% of the maternally related members had hearing loss. The prevalence of hearing loss according to age was as follows: 0%, less than 18 years of age; 21.7%, 18 to 44 years of age; 44.4%, 45 to 64 years of age; and 47.1%, 65 years of age or older. None of the present subjects had congenital or prelingual hearing loss; however, 10% of adult subjects exhibited postlingual hearing loss before the age of 20 years. Because the prev-

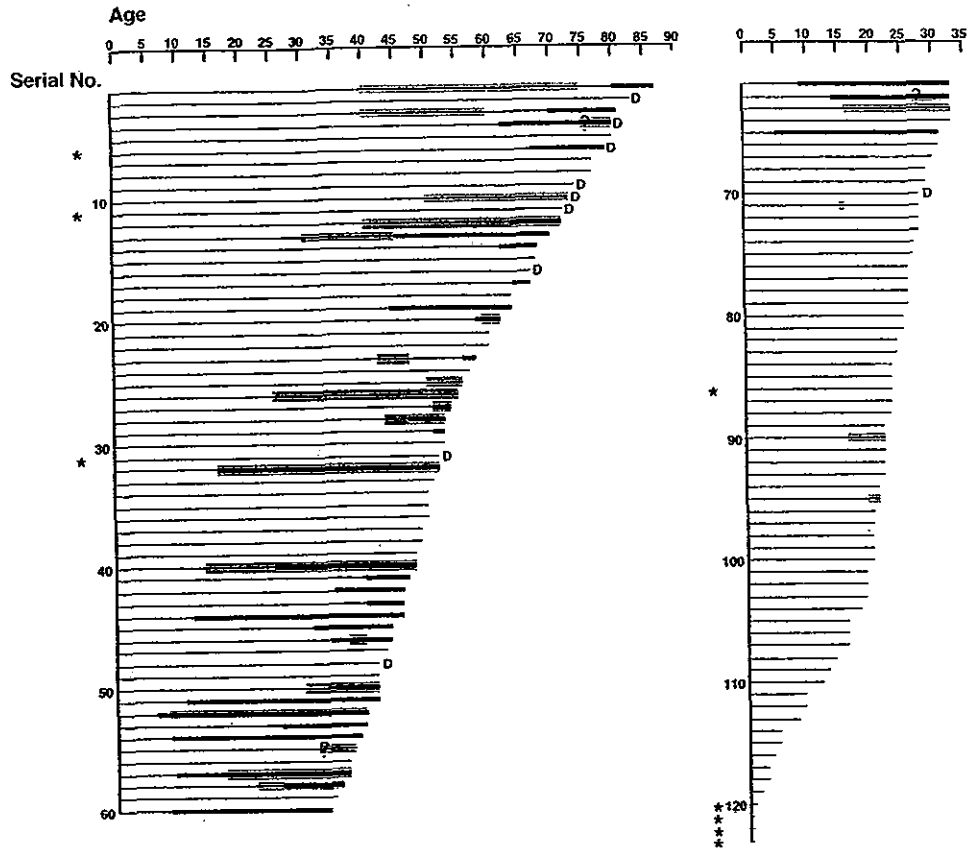

Fig. 3. Age at onset and duration of hearing loss and tinnitus based on interviews in all maternally related family members except I-1, ordered by age. The thin horizontal lines represent the age of each subject. Thick black and gray horizontal lines delineate the presence of hearing loss and tinnitus, respectively. Question marks to the left of the thick gray lines indicate cases in which the age at onset of tinnitus was unknown. Asterisks to the left of the thin lines denote subjects for whom tinnitus information was not obtained. Individuals who were deceased (D) at the time of the present study are also indicated. Age = years.

alence of hearing loss has not been studied by the method similar to the present study in Japan, comparison of the prevalence of hearing loss in the present subjects and that in general Japanese population could not be conducted. In a similar, previous study employing interviews about hearing handicaps in the United States, the prevalence of hearing loss in the general population according to age was as follows: 1.6%, less than 18 years of age; 4.8%, 18–44 years of age; 12.8%, 45–64 years of age; and 30.0%, 65 years of age or older.[17] Although there is a difference in the population examined, comparison of the report just cited with the present study suggests that maternally related members of the family with the A1555G mitochondrial mutation may have much higher risk for developing postlingual hearing loss than the general population even in the absence of aminoglycoside exposure.

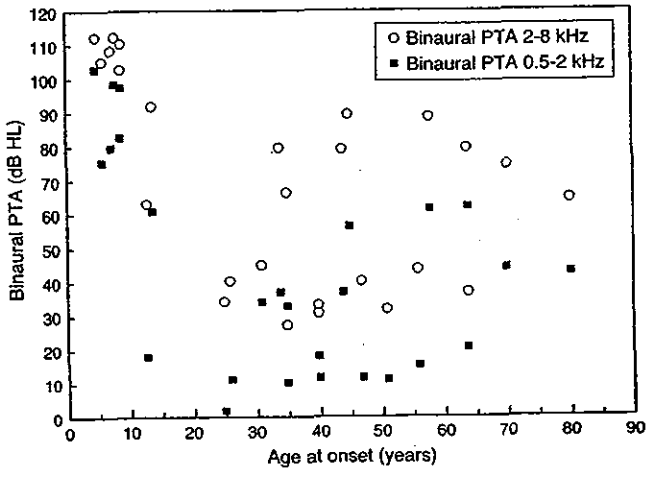

Fig. 4. Binaural pure-tone average (PTA) as a function of age at onset of hearing loss in 26 subjects with reported hearing disability and handicap. Binaural PTA of 2 to 8 kHz represents an average of pure-tone thresholds at 2, 4, and 8 kHz in both ears, and binaural PTA of 0.5 to 2 kHz represents an average of pure-tone thresholds at 0.5, 1, and 2 kHz in both ears.

Similar Phenotypic Expression Patterns Within Same Sibling Group

The degree and onset of hearing loss based on interviews about hearing disability and handicap were similar in affected subjects within the same sibling group but were distinct in different sibling groups. In addition, both affected and nonaffected subjects were found within the same sibling group. These phenotypic expression patterns were also observed in the onset and duration of tinnitus. The subjects in the present study were maternally related, therefore sharing the same mitochondrial DNA including the A1555G mutation, and had no history of aminoglycoside exposure. Thus, these phenotypic expression patterns suggest that nuclear background may be involved in modulating the phenotypic expression of the mutation, as was previously demonstrated in an Arab-Israeli kindred.[18–20]

The connexin 26 gene was recently reported as a candidate aggravating factor in the phenotypic expression

of the A1555G mutation.[21] Mutations in the connexin 26 gene are the most prevalent genetic cause of recessive sensorineural hearing loss in many populations,[22] including the Japanese.[13] One subject in the present study was heterozygous for a 299–300delAT mutation in the connexin 26 gene. However, this subject did not present with significant hearing loss. This observation is inconsistent with a previous study that reported synergy between a 235delC mutation (i.e., a deletion of C at position 235) in the connexin 26 gene and the A1555G mutation with regard to hearing loss.[21] This discrepancy may be due to the different functional properties of the two connexin 26 mutations or differences in the genetic backgrounds of the two families. Linkage analysis in this family, such as that conducted in the aforementioned Arab-Israeli kindred,[23] will be required to ascertain the unknown modifier genes.

Clinical Significance of Tinnitus

Tinnitus is a disorder that manifests as an abnormal sensation perceived in the head or ear without any external sound stimulus.[24] In the present study, 24.3% of the subjects had tinnitus that met our criteria at some time in their lives. It is important that approximately 50% of the family members who initially experienced tinnitus alone developed hearing loss at a later time, indicating that tinnitus is a clinically significant indication of future hearing loss associated with the A1555G mutation. Thus, it is important that individuals who carry the A1555G mutation and experience tinnitus take proper precautions against environmentally induced hearing loss (i.e., by avoiding loud noises and undergoing periodic hearing tests).

Association of Severe to Profound Hearing Loss With Early Onset

The association of severe to profound hearing loss with an onset of hearing loss before age 10 years was clearly shown by the pure-tone audiometry in subjects with hearing disability and handicap. This association suggests that the defensive or reparative system of the cochlea may be immature during early childhood. It has been reported that developing mammals, including humans, are more sensitive to noise, as well as chemical and drug-induced ototoxicity, than adults, although the molecular mechanisms of this hypersensitivity have not been delineated.[25,26] This association also illustrates the importance of careful audiological evaluation and follow-up in maternally related family members who carry the A1555G mutation and who are suspected of having hearing loss during early childhood.

CONCLUSION

The present study elucidated the characteristics, prevalence, and intrafamilial patterns of the auditory dysfunction in maternally related members of a family with the A1555G mitochondrial mutation in the absence of aminoglycoside exposure. The risk for developing postlingual hearing loss was likely to be much higher in the subjects than in the general population. The phenotypic expression patterns in the subjects suggested the involvement of nuclear backgrounds, but the connexin 26 gene was not the nuclear modifier gene in the present family. Tinnitus was identified as a clinically significant warning sign for future hearing loss. Association of severe to profound hearing loss with early onset highlighted the importance of careful audiological evaluation in individuals with the A1555G mutation in case hearing loss is suspected during early childhood.

Acknowledgments

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Identification of *CRYM* as a Candidate Responsible for Nonsyndromic Deafness, through cDNA Microarray Analysis of Human Cochlear and Vestibular Tissues

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Through cDNA microarray analysis of gene expression in human cochlea and vestibule, we detected strong expression of μ-crystallin (CRYM; also known as "NADP-regulated thyroid hormone-binding protein") only in these inner-ear tissues. In a subsequent search for mutations of CRYM, among 192 patients with nonsyndromic deafness, we identified two mutations at the C-terminus; one was a de novo change (X315Y) in a patient with unaffected parents, and the other was a missense mutation (K314T) that segregated dominantly in the proband's family. When the mutated proteins were expressed in COS-7 cells, their subcellular localizations were different from that of the normal protein: the X315Y mutant showed vacuolated distribution in the cytoplasm, and the K314T mutant localized in perinuclear areas, whereas normal protein was distributed homogeneously in the cytoplasm. Aberrant intracellular localization of the mutated proteins might cause dysfunction of the CRYM product and result in hearing impairment. In situ hybridization analysis using mouse tissues indicated its expression in the lateral region of the spiral ligament and the fibrocytes of the spiral limbus, implying its possible involvement in the potassium-ion recycling system. Our results strongly implicate CRYM in normal auditory function and identify it as one of the genes that can be responsible for nonsyndromic deafness.

Introduction

Hearing loss that disturbs normal communication is a common sensory disorder worldwide. The incidence of congenital deafness is ~1 in 1,000 newborns, and half of those cases are thought to result from genetic factors (Marazita et al. 1993). Most congenital or childhood-onset hearing impairments are nonsyndromic. So far, >70 genetic loci linked to nonsyndromic deafness have been described, and 26 genes whose mutations can cause deafness have been cloned (Hereditary Hearing Loss Homepage). Those data indicate that deafness is a highly heterogeneous disorder, and that genes responsible for deafness encode a large diversity of molecules. However, little is known of the molecular basis of inner-ear function, because the tissues in question are too small to be investigated in detail. The classical genetic approach through linkage analysis has limitations because the causes of deafness are so heterogeneous, and because linkage analysis requires DNA from a relatively large number of affected and unaffected members in a single family. Hence, we need to establish an effective alternative approach to searching for as-yet-unidentified genes that may be involved in hearing loss.

Obviously, genes that are expressed specifically in auditory tissues are likely to be good candidates to screen for genetic alterations in patients with deafness. In fact, several genes associated with deafness have been efficiently identified by way of organ-specific approaches involving, for example, subtractive human and mouse cDNA cochlear libraries (Robertson et al. 1994; Yasunaga et al. 1999; Simmler et al. 2000; Verpy et al. 2000). Several databases are now available which contain information about genes expressed in cochlea or in the developing ear (Morton Cochlear EST Database, Table of Gene Expression in the Developing Ear Web site, and Corey Lab Inner Ear Gene Expression Database).

It follows that identification of transcripts specific to the inner ear should also be helpful for studying hearing disorders. Therefore, in the work reported here we ap-

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Nucleotide sequence data reported herein are available in the DDBJ/EMBL/GenBank databases; for details, see the Electronic-Database Information section of this article.

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Table 1

Genes Preferentially Expressed in the Inner Ear with a Cy3/Cy5 Ratio of >10

GenBank Accession No.	Cy3/Cy5 Ratio in		Description	Locus	Correspondence with Deafness Locus
	Cochlea	Vestibule			
AA669336	156.51	136.15	Coagulation factor C homology (*cochlin, COCH*)	14q12-q13	
L02950	119.55	59.74	μ-crystallin (CRYM)	16p13.11-p12.3	
M61901	114.30	70.40	Prostaglandin D synthase	9q34.2-34.3	DFNB33 (9q34.3)
NM_020157	79.87	27.51	Otoraplin	20p12.1-p11.23	Cochlear gene*
NM_003460	77.36	185.57	Zona pellucida glycoprotein 2 (sperm receptor)	16p12	
M64722	69.52	104.29	clusterin (TRPM-2, apolipoprotein J)	8p21-p12	
J02611	68.59	108.76	Apolipoprotein D	3q26.2-qter	
XM_051860	56.54	22.55	*KIAA1199* protein	15q24	
W39428	55.10	67.96	F-box only protein 2	1p36.23	
AA972852	43.41	45.94	Retinol-binding protein 1, cellular	3q23	DFNB15 (3q21-25/19p13)
J05096	41.98	55.99	ATPase, Na+/K+ transporting, alpha 2 (+) polypeptide	1q21-q23	DFNA7 (1q21-q23)
AA252389	38.77	54.70	Lipoma HMGIC fusion partner	13q12	
AA292179	37.41	16.65	Ubiquitin A-52 residue ribosomal protein fusion product 1	19p13.1-p12	DFNB15 (3q21-25/19p13)
M62402	30.46	44.97	Insulin-like growth factor binding protein 6	12q13	
AF039699	30.22	22.61	*USH1C*	11p14.3	
X99920	26.20	70.30	S100 calcium-binding protein A13	1q21	DFNA7 (1q21-q23)
U59832	26.04	13.68	Forkhead box D1	5q12-q13	
X53331	23.79	16.19	Matrix Gla protein	12p13.1-p12.3	
J02984	21.92	10.26	Ribosomal protein S15	19p-tel	
X06617	20.19	17.83	Ribosomal protein S11	19q13.3	DFNA4 (19q13)
X75450	18.48	19.39	Melanoma inhibitory activity (SH3 domain+)	19q13.32-q13.33	DFNA4 (19q13)
AA308743	17.91	11.85	Ribosomal protein L35	9q34.1	
AA526377	17.90	10.60	Ribosomal protein L15	19q13.3	DFNA4 (19q13)
AA496786	17.64	33.33	Collagen, type IX, alpha 3	20q13.3	
X96484	16.42	11.71	DiGeorge syndrome critical region gene 6	22q11.21	
AA058578	16.00	46.30	Homo sapiens cDNA FLJ10158 fis	3q12.3-21.3	DFNB15 (3q21-25/19p13)
U14970	15.76	12.25	Ribosomal protein S5	19q13.4	DFNA4 (19q13)
AF052685	15.27	10.71	Protocadherin gamma subfamily C, 3	5q32	
F22593	15.27	34.13	Vesicle-associated membrane protein 5 (myobrevin)	2p11.2	
W73992	15.15	11.10	Serologically defined colon cancer antigen 43	9q22.2	
AA633908	15.00	11.10	ESTs	20p12	
X03342	14.89	15.31	Ribosomal protein L32	3q13.3-q21	
AA205528	14.79	14.90	Carbonic anhydrase XIV	1q21	DFNA7 (1q21-q23)
AI344213	14.28	21.21	Copper chaperone for superoxide dismutase	11q13	
AA057243	13.00	10.90	PH domain containing protein in retina 1	11q13.5-q14.1	
AA434038	12.96	11.83	Gap junction protein, beta 2, 26kD (connexin 26)	13q11-q12	
AA148265	12.60	12.00	Ribosomal protein L21	13q12.13	
W84565	12.50	10.60	Secreted protein of unknown function	1q32.3	
AI268685	12.33	11.38	HSPC023 protein	19p13.13	DFNB15 (3q21-25/19p13)
AY043487	12.22	29.28	Selenoprotein SelM (SELM)	22q12	
S72043	11.95	13.10	Metallothionein 3 (growth inhibitory factor [neurotrophic])	16q13	
AF284751	11.90	12.00	Hypothetical protein HT036	1q34.1	
X54412	11.88	35.98	Collagen, type IX, alpha 1	6q12-q14	
T55019	11.83	17.47	Ribosomal protein L28	19q13.4	DFNA4 (19q13)
AB003184	11.60	30.36	Immunoglobulin superfamily containing leucine-rich repeat	15q23-q24	
X89401	11.43	20.16	Ribosomal protein L21	13q12.13	
AA625532	11.37	14.36	Discoidin domain receptor family, member 2	1q12-q23	DFNA7 (1q21-q23)
M68864	10.88	16.00	ORF	11cen-q12.1	
AI240945	10.60	13.80	Ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome	Yq11	
AA446913	10.51	13.71	Ubiquitin specific protease 11	Xp21.2-p11.2	
N71750	10.46	26.54	Zinc-finger protein 288(BC015587)	3q13	
X13916	10.31	17.43	Low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)	12q13-q14	

* Otoraplin (OTOR [MIM 606067]) has been identified from a human fetal cochlear. So far, there is no report for human deafness-causing mutation.
Abbreviations: DFNA, autosomal dominant loci; DFNB, autosomal recessive loci.

plied a genome-wide cDNA microarray analysis to investigate gene-expression profiles in human cochlea and vestibule, and focused on one of the genes that was expressed at high levels in both of those tissues. Mutant alleles of this gene were responsible for nonsyndromic deafness in two individuals among the group of probands we studied.

Family, Material, and Methods

Preparation of Tissues and RNA

Tissues from one cochlea and seven vestibules were obtained with written informed consent from different adult patients undergoing labyrinthectomy; each patient had been diagnosed with a nonlabyrinthine disorder, such as temporal-bone tumor or acoustic tumor.

Total RNA was extracted from each inner-ear sample using Trizol (Life Technologies) according to the manufacturer's instructions. After treatment with DNase I, T7-based RNA amplification was performed as described elsewhere (Luo et al. 1999), with some modifications. Using an estimated 3 ng of total RNA from the cochlear tissue, we performed three rounds of amplification. For vestibular tissues, we performed two rounds of RNA amplification, using 3 μg of total RNA. We obtained 70–80 μg of each amplified RNA (aRNA) sample. As a control, we mixed PolyA(+) RNAs derived from 29 normal human tissues (bone marrow, brain, heart, kidney, liver, lung, lymph node, mammary gland, pancreas, placenta, prostate, salivary gland, skeletal muscle, small intestine, spinal cord, spleen, stomach, testis, thymus, thyroid, trachea, uterus, fetal brain, fetal

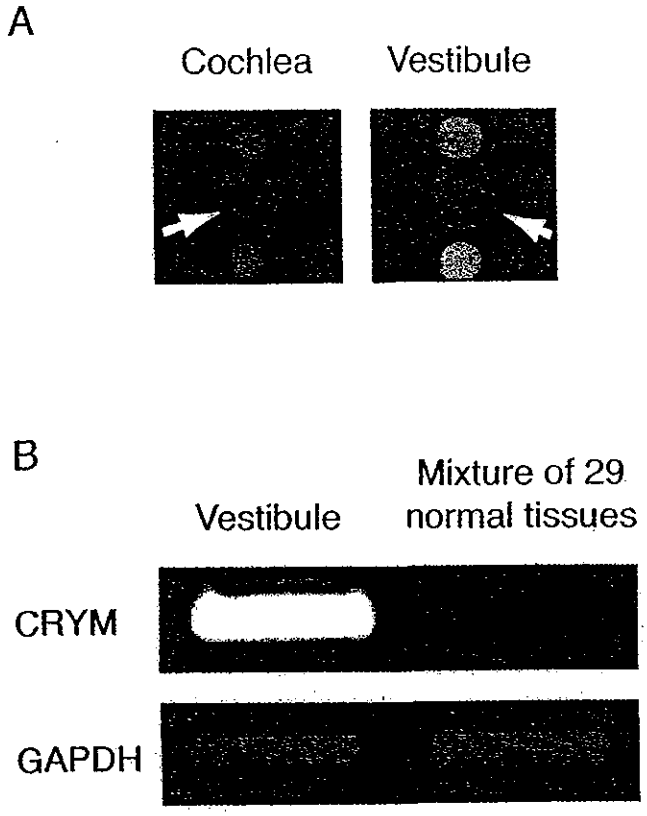


Figure 1 Analysis and confirmation of cDNA microarray results. *A*, Signals of CRYM in the microarray (*white arrows*). aRNA from the human inner ear (cochlear and vestibular tissues) was labeled with Cy3-dCTP (*red*); mixed aRNA from 29 other tissues yielded green signals from Cy5-dCTP (*green*). *B*, Confirmation of microarray data by semiquantitative RT-PCR of CRYM with one-round amplified RNAs.

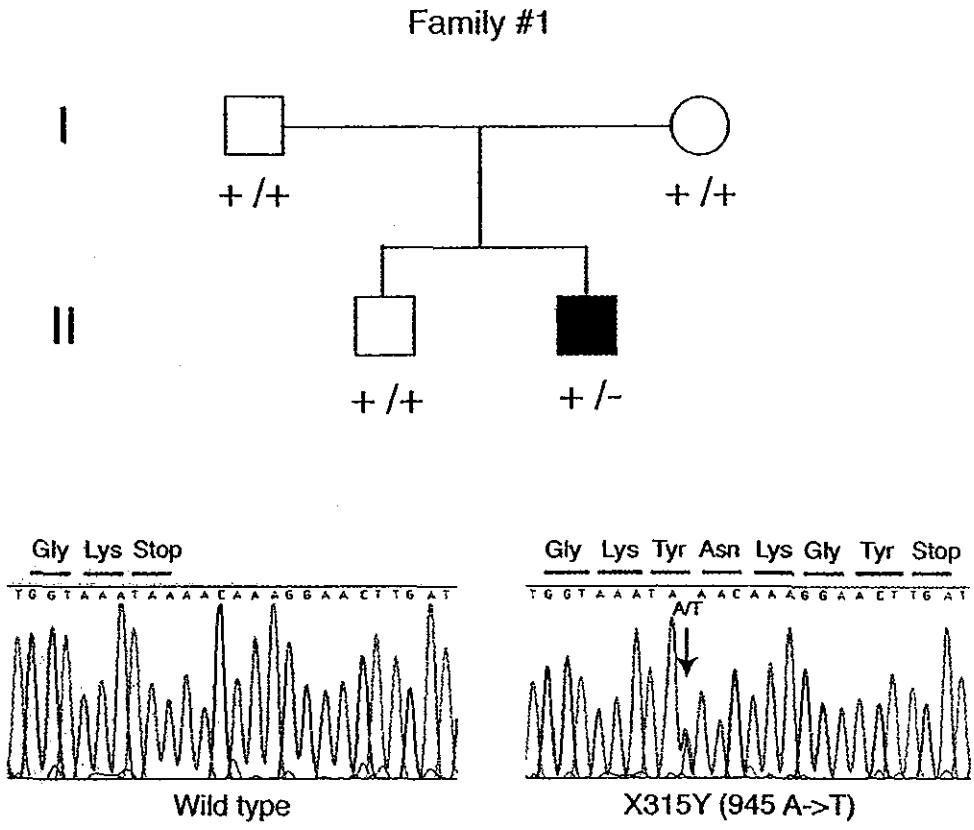
Table 2

Nonsyndromic Deafness Gene Expression in the Cochlea and Vestibule by cDNA Microarray Analysis

Gene	Cy3/Cy5 Ratio in		Association with Human Deafness
	Cochlea	Vestibule	
COCH	156.51	136.15	DFNA9
GJB2 (Cx26)	12.96	11.83	DFNB1/DFNA3/deafness with skin disorders
GJB6 (Cx30)	34.71	4.55	DFNB1/DFNA3
USH1C	30.22	22.61	DFNB18/Usher syndrome type 1C
MYO7A	6.20	3.63	DFNB2/Usher syndrome type 1B
MYH9	1.50	1.00	DFNA17/Epstein and Fechtner syndrome
MYO6	1.20	1.60	DFNA22
CLDN14	No signal	No signal	DFNB29
POU3F4	No signal	No signal	DFN3

Note.—MIM numbers are as follows: DFNA9 (MIM 601369), DFNB1 (MIM 220290), DFNA3 (MIM 601544), deafness with skin disorders (MIM 148350), DFNB18 (MIM 602092), Usher syndrome type 1C (MIM 605242), DFNB2 (MIM 600060), Usher syndrome type 1B (MIM 6276903), DFNA17 (MIM 03622), Epstein and Fechtner syndrome (MIM 153650), DFNA22 (MIM 606344), DFNB29 (MIM 60568), and DFN3 (MIM 304400).

A


Family #1
I
+/+
+/+
II
+/+
+/-
Gly Lys Stop
Wild type
Gly Lys Tyr Asn Lys Gly Tyr Stop
A/T
X315Y (945 A->T)

B

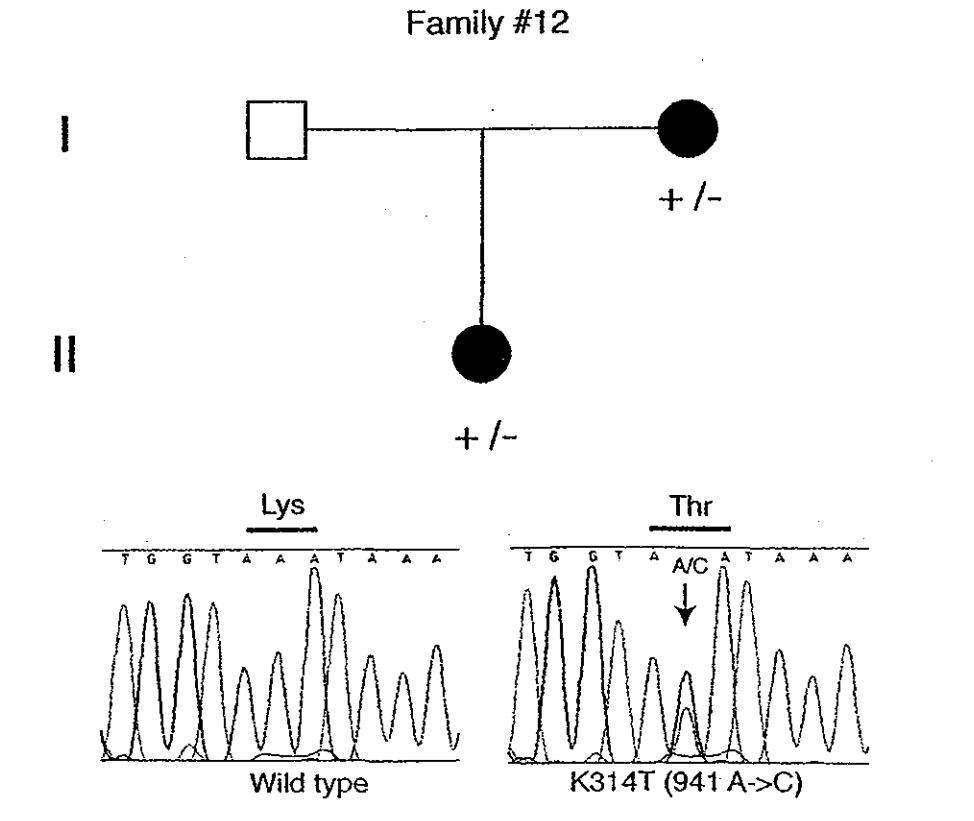

Family #12
I
+/-
II
+/-
Lys
Wild type
Thr
A/C
K314T (941 A->C)

Table 3

Nucleotide Changes Detected by Mutation Analyses in the *CRYM* Gene

Change	Exon/ Intron	Amino Acid	Family No.	Allele Frequency	
				in Patients	in Control Subjects
IVS1-116C→T	Intron1			90/364	Not done
IVS1-119G→A	Intron 1			3/364	Not done
IVS4+38C→G	Intron 4			84/336	.12[a]
864G→C	Exon 7	Thr288Thr		94/330	Not done
941A→C	Exon 8	Lys314Thr	12	1/384	0/192
945A→T	Exon 8	X315Tyr → extended protein	1	1/384	0/192

[a] See "A Database of Japanese Single Nucleotide Polymorphisms" Web site.

kidney, fetal liver, fetal lung [Clontech], colon, ovary [Biochain], and mesenteric adipose tissue).

cDNA Microarray

Microarray slides containing 23,040 cDNA spots selected from the UniGene database of the National Center for Biotechnology Information were utilized for our analysis of gene expression in the human inner ear. As described elsewhere (Ono et al. 2000; Saito-Hisaminato et al. 2002), aRNAs from each tissue sample (2.5–5 μg) were labeled with Cy3-dCTP (Amersham Pharmacia Biotech), and an equal amount of aRNAs from a mixture of 29 human tissues was labeled with Cy5-dCTP (Amersham Pharmacia Biotech). Labeling and hybridization were performed according to protocols noted elsewhere (Ono et al. 2000). The intensity of each hybridized spot was measured and analyzed with the ArrayVision computer program (Amersham Bioscience), and background signals were subtracted. Each Cy3- and Cy5-fluorescence intensity was normalized by use of averaged signals from 52 housekeeping genes. We calculated a cut-off value for each gene's expression to dissolve background fluctuation, according to parameters established previously (Saito-Hisaminato et al. 2002).

RT-PCR

To confirm our cDNA microarray data we performed semiquantitative RT-PCR experiments using cDNAs derived from the vestibule and from the 29 normal tissues, using single-round aRNAs. GAPDH served as an internal control. The amount of cDNA, as judged by the intensity of the GAPDH signal, was optimized in both samples. Primers used to amplify specific CRYM gene products were F: TCTGGAGATGTCCTGCTGTC and R: GGCTACCTAGCTTTGCTTTC; the PCR proceeded for 25 cycles of 95°C for denaturing, 55°C for annealing, and 72°C for extension. The PCR products were electrophoresed on a 2% agarose gel and were visualized by ultraviolet light.

Family Selection and Mutational Search

We screened DNA from 192 Japanese families in which probands were found to have congenital or childhood-onset bilateral nonsyndromic sensorineural hearing loss and no history of drug toxicity, infections or injury. Of these families, 41 showed dominant inheritance of deafness and 21 showed recessive inheritance; the other 130 were represented by only one affected individual (simplex cases). Each patient was clinically well characterized by a series of auditory examinations (pure-tone audiometry) that indicated sensorineural hearing loss ranging from mild to profound in severity. All participants in the project had provided written informed consent and had already been examined for mutations in the GJB2 gene [MIM 121011] and the mitochondrial gene encoding 12S rRNA [MIM 561000], but no mutations were detected in either of those genes although both had been described as common causes of deafness (Abe et al. 2000; Usami et al. 2000). On the basis of our microarray results, we analyzed genomic DNA isolated from peripheral blood leukocytes of one affected member of each family for mutations in the CRYM gene (MIM 123740).

We determined the genomic structure of the CRYM gene by comparison with a BAC sequence (GenBank accession number AF001550) on chromosome 16. We amplified each of its eight exons and their flanking intronic sequences using the following oligonucleotide primers: F1, AGGCTGGGCTGTGACCAGCA; R1, AGCTGTTAGCAA CGGTTAGG; F2, TGTCTAAGGGAAGGGCAGAG; R2, TGTTGCTGGTATCCAG-

Figure 2 Pedigrees and electrophoregrams showing mutations in the *CRYM* gene in two families. Corresponding normal sequences are shown in the left-hand panels. *A*, In family 1, only patient II-2 shows a heterozygous A→T substitution, changing the stop codon to a Tyr residue and bringing about an extension of the protein by five amino acids at the C-terminal. *B*, In family 12, the patient (II-1) and her affected mother (I-2) are both heterozygous for a Lys314Thr mutation.

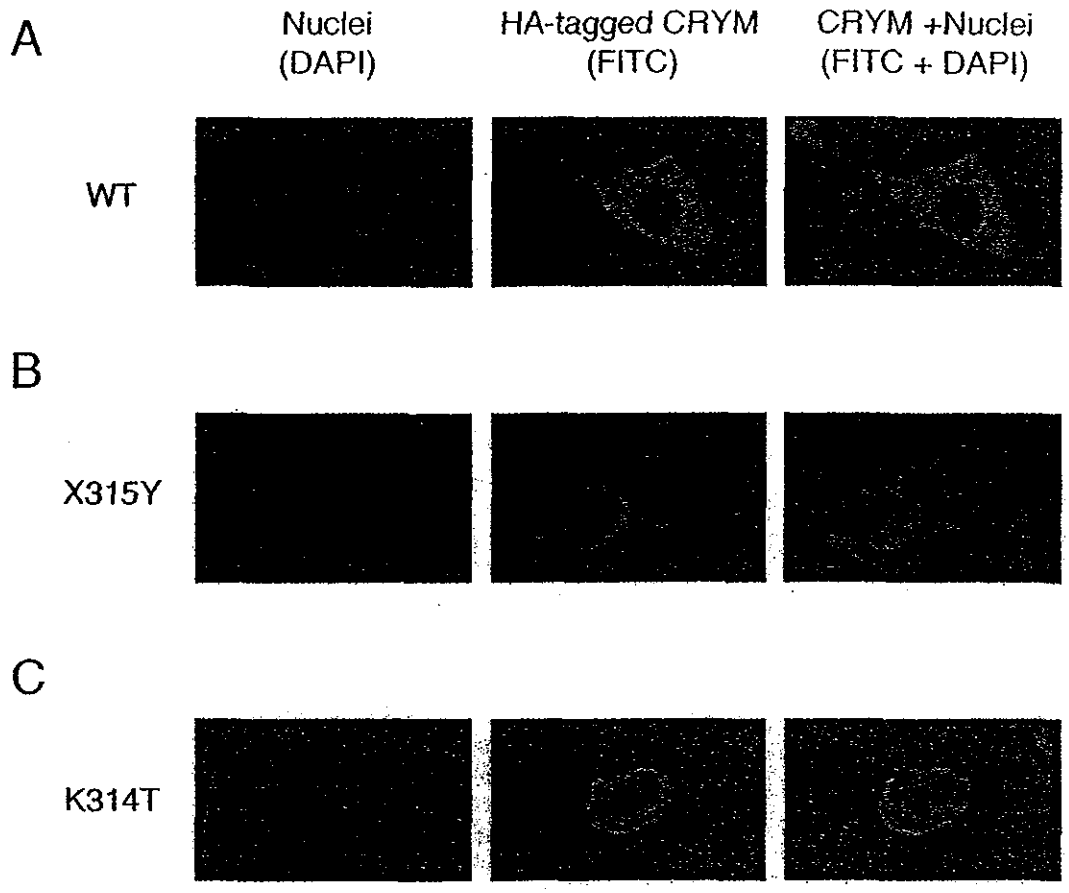


Figure 3 Fluorescent images of COS-7 cells expressing HA-tagged CRYM proteins. Nuclei are counterstained with DAPI (*blue*). Cells representative of six independent experiments are shown at a magnification of 60×. Subsequently, the numbers of cells were counted in 8–10 randomly selected fields (60×) using fluorescence microscopy (Nikon) at 24 h, 48 h, 72 h, and 96 h, respectively. *A*, Wild-type (WT) CRYM protein (*green*) localizes to cytoplasm diffusely throughout the time course of the experiments. *B*, The X315Y mutant with an extended C-terminal tail leads to vacuolated cytoplasmic distribution. The K314T mutant protein is localized predominantly in the perinuclear area.

TCAC; F3, AGGAATCGGATCCAGGTCTGA; R3, TCTGGAGTTCCAGCTATGTC; F4, ATTGCCTGCAAGCTCTTGAG; R4, CCTGACTCTTATCCTCCATC; F5, CGGTCTCATCAAGTTGAAAGG; R5, CTGCACCCAGCCAAATATTG; F6, GGGAATGAGGGGGTATTTTG; R6, GCCCATATTTTTCTGGAATGG; F7, CAGTGTACAAAGGATCTCTC; R7, TGACCTGAATGATGGAGCAC; F8, TAGGCATTGGCAACATGGAC; and R8, GGTAGAACAGAAGAAATGGC.

Each genomic DNA (5–10 ng) was amplified by the PCR, using Ex-*Taq* polymerase (Takara), for 2 min at 95°C, followed by 37 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 50 s, with a final extension of 5 min. After the products were purified by Multiscreen PCR (MILLIPORE), we performed standard cycle-sequencing reactions in an ABI 3700 autosequencer. DNA samples from 96 unrelated Japanese volunteers with normal hearing were used as controls.

Epitope Tagging of CRYM

To obtain constructs expressing the wild-type CRYM protein, as well as the X315Y and K314T variants, we PCR amplified the corresponding parts of the CRYM cDNA sequence (GenBank accession number NM_001888), using KOD-Plus-DNApolymerase (Toyobo) and the following primer sequences: (1) a W-Tag-F forward primer common to the wild type and both mutants, containing sequence corresponding to HA-tag (5′-CGTGAATTCCAGACCGTGCATCATGTACCCATACGACGTCCCAGACTACGCTAGCCGGGTACCAGCGTTCC-3′); and (2) reverse primers W-R (5′-AAGCTCGAGTTATTTACCAGATGACCAGGAATCA-3′) for the wild type, M(1)-R (5′-AAGCTCGAGTCAAGT TCCTTTGTTATATTTACCAG-3′) for the X315Y mutant, and M(2)-R (5′-AAGCTCGAGTTATGTACCAGATGACCAGGAATC-3′) for the K314T mutant. PCR products were cloned into the pcDNA 3.1(+) vector (Invitogen).

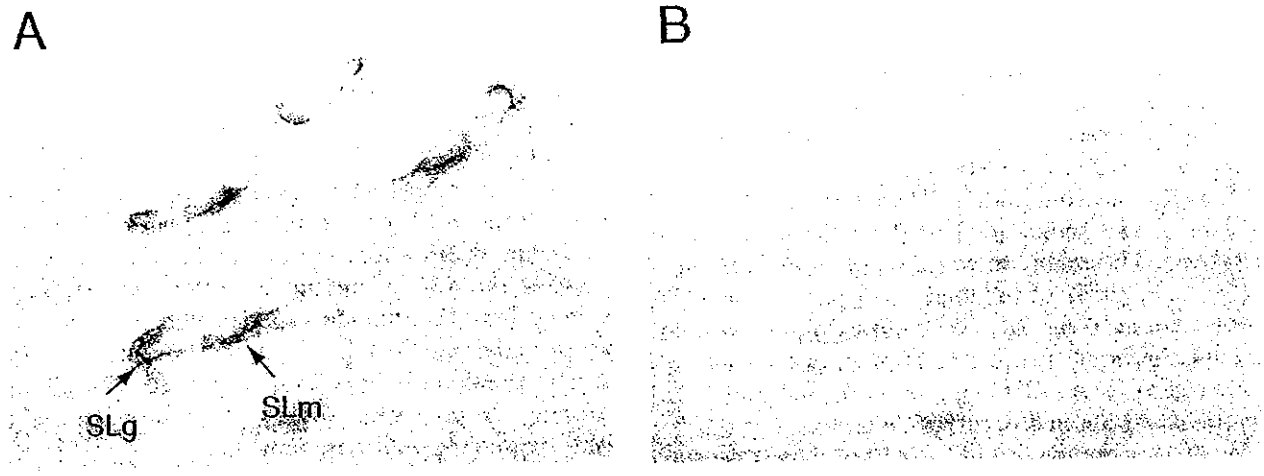


Figure 4 In situ hybridization analysis of *Crym* expression. The mouse cochlea reveals distinct labeling by the *Crym* antisense probe (*arrows*). *Crym* mRNA expression in the fibrocytes of spiral ligaments (SLg) and the fibrocytes of spiral limbus (SLm) in all turns of the mouse cochlea. Control hybridization was performed with a sense probe on consecutive tissue sections.

Microscopic Analysis of Immunofluorescence

For transfection of COS-7 cells, we used 1 μg of each construct and 3 μl of FuGENE6 (Roche Molecular Biochemicals) diluted in OPTIMEM medium (Gibco BRL) according to the manufacturer's instructions. In two-well glass chamber slides, 1×10^4 to 5×10^4 cells were seeded. Cell samples were fixed 24 h, 48 h, 72 h, and 96 h after transfection and were treated as described elsewhere (Tsujikawa et al. 1999). They were first incubated overnight at 4°C with mouse anti-HA tag antibody (1:1,500) (Santa Cruz) and then for 60 min at 25°C with FITC-conjugated goat anti-mouse IgG (1:3,000) (ICN/Cappel). Images were viewed by means of fluorescence microscopy. We examined a total of six independent experiments.

In Situ Hybridization

The mouse cochlea at postnatal days (Pn) P0 was dissected, was fixed by 10% neutral formalin, and was embedded in paraffin. Tissue sections (4 um) were dewaxed and hybridized as described elsewhere (Hoshino et al. 1999), with some modifications. A 701-bp DNA fragment corresponding to the nucleotide positions 260–960 of mouse Crym cDNA (GenBank accession number NM_016669) was subcloned into pBlueScript SK(−) vector (Stratagene) and was used for generation of sense or antisense RNA probes. We carried out hybridization with digoxigenin-labeled RNA probes at 42°C for 18 h. The bound label was detected using NBT-BCIP, an alkaline phosphatase color substrate, and tissue slides were stained with Kernechtrot stain solution.

Results

Verification of cDNA Microarray Data

Through analysis of expression profiles of human inner-ear tissues on a cDNA microarray containing 23,040 genes, we found 52 genes whose signal intensities were more than 10-fold higher in cochlea and vestibule than in a mixture of 29 other tissues (table 1). Of the 52 genes expressed preferentially in the inner ear, 14 were located in one of five chromosomal regions known to contain loci linked to human deafness (*DFNA4* [MIM 600652], *DFNA7* [MIM 601412], *DFNB15* [MIM 601869], and *DFNB33* [MIM 607239]). Among nine genes known to be responsible for nonsyndromic deafness (*COCH* [MIM 603196], *GJB2, GJB6* [MIM 604418], *MYO7A* [MIM 276903], *USH1C* [MIM 605242], *MYH9* [MIM 160775], *MYO6* [MIM 600970], *CLDN14* [MIM 605608], and *POU3F4* [MIM 300039]) that were present in our array, we confirmed expression in the inner ear of all except *CLDN14* and *POU3F*. Five of these seven genes showed relatively high levels of expression (ratio >5 compared with the control) in the cochlea and of those, four (*COCH, GJB2, GJB6*, and *USH1C*) were considered to be expressed specifically or preferentially (ratio >10) (table 2). Among the genes specifically or preferentially expressed in the inner ear, we focused on *CRYM* because it represented the second-highest Cy3/Cy5 ratio, after *COCH*: 119.55 in the cochlea and 59.74 in the vestibule (table 1; fig. 1*A*). We confirmed predominant expression of *CRYM* in the inner ear by semiquantitative RT-PCR (fig. 1*B*) and considered this gene to be a candidate for playing an important role in auditory function.

Mutational Analyses

We analyzed the *CRYM* gene for mutations in 192 patients with nonsyndromic hearing loss. Direct DNA sequencing identified four genetic polymorphisms in this panel: one mutation in exon 8, causing an amino acid substitution, and one mutation affecting the stop codon (table 3). The latter mutation (X315Y; 945A→T) caused addition of five amino acids at the C-terminal end of the protein. This mutation was detected only in the proband, not in either of his unaffected parents or in his brother (family 1; fig. 2*A*). We confirmed paternity and maternity in family 1, using 12 highly polymorphic microsatellite markers (data not shown), and concluded that this mutation had occurred de novo.

The other mutation, K314T (941A→C), was detected in the proband and her affected mother in family 12 (fig. 2*B*). Although the unaffected father was not available for testing, the mutation appeared to cosegregate with hearing loss. To exclude a possibility that this alteration represented a polymorphism, we examined 192 control chromosomes but did not find the substitution in any of them.

Clinical Analyses

Affected individuals with the X315Y and the K314T mutations have normal stature and intelligence. There is no vestibular, visual, renal, or muscular involvement for all affected individuals with the *CRYM* mutations. Hearing impairment has been identified in the individual with mutation X315Y at age 19 mo. Auditory brainstem responses (ABR), conditioned orientation reflex audiometry, and pure tone audiometry examinations showed bilateral moderate sensorineural hearing loss (average 50–60 dB) affecting all frequencies by a down-sloping shaped audiogram pattern. Hearing loss progressed from moderate to severe (70 dB), bilaterally at age 13 years, and subsequent Carolic test has shown normal vestibular function. The age at diagnosis of hearing loss in affected members with the other K314T missense mutation has been documented as being 1 year. The K314T case showed bilateral severe (80–90 dB) sensorineural hearing loss affecting all frequencies with no progression. Temporal bone CT scan was performed for all affected individuals carrying a *CRYM* mutation, but no abnormal findings were observed.

Subcellular Localization of Mutant Proteins

To examine the consequences of *CRYM* mutations, we expressed HA-tagged wild-type CRYM protein, as well as variants corresponding to each of the two mutations in COS-7 cells. Expression of all three proteins was confirmed by western blotting of protein extracts from transfected cells with mouse anti-HA antibodies. Fluorescent microscopy revealed diffuse cytoplasmic staining in cells transfected with the wild-type expression construct, and, 24 h and 48 h after transfection, the subcellular localizations of both mutant proteins were similar to the distribution of wild-type protein. However, 72 h and 96 h after transfection, we observed different patterns in significant proportions of transfected cells (fig. 3); the wild-type protein still revealed diffuse cytoplasmic distribution, but nearly 20% of the cells expressing the X315Y mutant revealed a vacuolation pattern in the cytoplasm, and approximately one-fourth of those expressing the K314T mutant showed strong staining, predominantly in perinuclear regions. The aberrant staining patterns were observed in almost none of the cells expressing wild-type protein.

Expression Analysis of Crym in the Mouse Cochlea

To study a possible biological function of *CRYM*, we cloned mouse *Crym* cDNA and performed an in situ hybridization experiment using mouse tissue. As shown in fig. 4*A*, hybridization signals with the *Crym* probe were observed in the lateral fibrocytes of the spiral ligaments (SLg) and the fibrocytes of the spiral limbus (SLm), whereas no in situ hybridization signal was detected with the control sense RNA probe (fig. 4*B*). *Crym* expression was not detected in neurosensory epithelium such as inner and outer hair cells.

Discussion

To explore molecules essential for auditory function and to isolate genes responsible for deafness, we analyzed expression profiles of human inner-ear tissues on a cDNA microarray. The cochlea (for sound detection) and vestibule (for balance) have the same origin in the developmental process and possess similar properties both structurally and physiologically. We identified a set of 52 genes that were specifically or preferentially expressed in the inner ear and are investigating their possible roles in patients with nonsyndromic deafness. Among nine known nonsyndromic deafness genes that were present on our microarray, seven were confirmed to be expressed in the inner ear by our microarray analysis. Four of them, *COCH*, *GJB2*, *GJB6*, and *USH1C*, showed high Cy3/Cy5 ratios (>10) as compared with other tissues, indicating specific expression in the inner ear; one additional gene, *MYO7A*, revealed a relatively high Cy3/Cy5 ratio (6.20) in the cochlea. These results indicated that screening genes that are highly expressed in the inner ear might be an efficient approach to identifying aberrant molecules involved in deafness.

By screening DNA from a panel of patients with nonsyndromic deafness for mutations in *CRYM*, a gene that was especially abundant in the inner ear, we found pos-

sible disease-causing mutations in two cases. In one family, only the proband carried a mutation (X315Y), indicating that this change had arisen de novo in the affected individual, adding five amino acids at the C-terminal end of the CRYM protein. Mutations of a similar type have been reported as causes of some other hereditary conditions (Arlt et al. 1994; Marr et al. 2002). We underscored the deafness-causing potential of the X315Y mutation by demonstrating aberrant subcellular localization of the mutant protein.

The other mutation caused a nonconservative amino acid change of lysine to threonine at codon 314. Since the lysine residue at this position is conserved across mammalian species—including human, mouse, rat, and marsupial—according to alignments of human CRYM sequence with those of other mammals (human μ-crystallin [GenBank accession number Q14894], mouse μ-crystallin [GenBank accession number NP_057878], rat μ-crystallin [GenBank accession number NP_446407] and kangaroo μ-crystallin [GenBank accession number Q28488]), we assume that the K314T mutation would introduce a significant change in protein structure. Aberrant intracellular localization of the corresponding mutant protein supported the importance of this residue in human CRYM. Our findings imply that both mutations at the C-terminal end are likely to have deleterious effects on CRYM function. Although the precise mechanisms of these mutational effects remain to be determined, we consider that both of the heterozygous mutations we detected in the *CRYM* gene in deaf patients are likely to have affected auditory function, probably in a dominant-negative manner.

CRYM is one of the taxon-specific crystallins and is also called "μ-crystallin." It was first identified as a major structural protein of the ocular lens in some marsupials (Kim et al. 1992). CRYM mRNA expression in fetal cochlea has been also demonstrated in the Morton Cochlear EST Database (GenBank accession number N73414). Although expression of *CRYM* is not absolutely specific to the inner ear, our microarray results indicate that its product is extremely abundant in the cochlea and vestibule. Initially, the predicted amino acid sequence showed similarity to the ornithine cyclodeaminase (OCD) and to glutamyl-tRNA reductase (gluTR) of bacteria and plants; both of these proteins represent a diverse superfamily of enzyme. The similarity suggested to some that μ-crystallin might perform an enzymatic rather than a structural role in lens tissue (Kim et al. 1992; Segovia et al. 1997). However, now it is thought to be a cytosolic NADP-regulated thyroid hormone-binding protein (THBP), a member of a group of molecular entities responsible for most of the intracellular high-affinity binding of T3 and T4 (Vie et al. 1997). THBPs are involved in sequestration and release of intracellular thyroid hormones (homeostasis). Whereas the cytosolic binding sites for T3 and T4 are similar to those of thyroid hormone receptors (TRs), the binding activity of THBPs is 100 times greater than that of TRs (Vie et al. 1997). Therefore, we speculate that mutant CRYM could abrogate the affinity of thyroid hormone, an essential agent for development of the auditory system. Cochlear structures are extremely vulnerable to thyroid-hormone deficiency during critical developmental periods; such deficiency results in defective morphological differentiation and maturation in the organ of Corti (Uziel 1986).

In the mouse, *Crym* was expressed in the cochlea and utricle at P2 and P32 stages (Corey Lab Inner Ear Gene Expression Database). We detected localization of *Crym* mRNA in two distinct tissues of mouse cochlea; the lateral fibrocytes of the spiral ligaments and the spiral limbus fibrocytes (fig. 4*A*). The postulated roles for these cells are thought to be K^+ circulation/K^+ recycling (Spicer and Schulte 1996; Steel and Kros 2001), suggesting that CRYM dysfunction may be interfering with potassium ion recycling, thus disturbing its maintenance of K^+ rich endolymph and a positive electrical potential. We hypothesize that dysfunction of μ-crystallin may cause critical morphogenetic abnormalities of the labyrinth induced from thyroid-hormone deficiency and synergic auditory dysfunction involved in ionic homeostasis.

In conclusion, our results demonstrate that mutations of *CRYM* can be responsible for nonsyndromic deafness and that using the cDNA microarray approach to identify genes expressed specifically in the inner ear may be an efficient means of determining other good candidates for involvement in nonsyndromic deafness.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Corey Lab Inner Ear Gene Expression Database http://www.mgh.harvard.edu/depts/coreylab/index.html

Database of Japanese Single Nucleotide Polymorphisms, A, http://snp.ims.u-tokyo.ac.jp/

GenBank, http://www.ncbi.nlm.nih.gov/GenBank/ (for human μ-crystallin [accession number Q14894], mouse μ-crystallin [accession number NP_057878], rat μ-crystallin [accession number NP_446407], kangaroo μ-crystallin [accession number Q28488], human CRYM cDNA [accession number NM_001888], mouse Crym cDNA [accession number

NM_016669], BAC sequence [accession number AF001550], and a Morton fetal cochlea EST [accession number N73414])

Hereditary Hearing Loss Homepage, http://www.uia.ac.be/dnalab/hhh/

Morton Cochlear EST Database, http://hearing.bwh.harvard.edu/estinfo.htm

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for GJB2 [MIM 121011], 12S rRNA [MIM 561000], CRYM [MIM 123740], DFNA4 [MIM 600652], DFNA7 [MIM 601412], DFNB15 [MIM 601869], DFNB33 [MIM 607239], COCH [MIM 603196], GJB6 [MIM 604418], MYO7A [MIM 276903], USH1C [MIM 605242], MYH9 [MIM 160775], MYO6 [MIM 600970], CLDN14 [MIM 605608], POU3F4 [MIM 300039], OTOR [MIM 606067], DFNA9 [MIM 601369], DFNB1 [MIM 220290], DFNA3 [MIM 601544], deafness with skin disorders [MIM 148350], DFNB18 [MIM 602092], Usher syndrome type 1C [MIM 605242], DFNB2 [MIM 600060], Usher syndrome type 1B [MIM 276903], DFNA17 [MIM 603622], Epstein and Fechtner syndrome [MIM 153650], DFNA22 [MIM 606344], DFNB29 [MIM 60568], and DFN3 [MIM 304400])

Table of Gene Expression in the Developing Ear, http://www.ihr.mrc.ac.uk/hereditary/genetable/index.shtml

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