Objective: To determine the spectrum of connexin 26 (Cx26) mutations and their phenotypes in children with sensorineural hearing loss (SNHL) or mixed hearing loss (MHL).

Design: Children with SNHL or MHL were prospectively tested for mutations in the entire coding region of the Cx26 gene.

Patients: Children with SNHL or MHL with no obvious etiology for the hearing loss.

Results: Between December 1, 1998, and July 1, 2000, 107 patients with SNHL or MHL from 99 families underwent Cx26 testing. Most patients were aged 1 week to 16 years (61 boys and 46 girls). Thirty (30%) of 99 probands had Cx26 mutations: biallelic mutations were detected in 18 (9 homozygous and 9 compound heterozygous) and single mutations were detected in 12. Twelve previously reported mutations (35delG, 167delT, E47X, L90P, M34T, G12V, V37I, R143W, V84L, V153I, V271, and 310del14) and 3 novel mutations (E129K, T8M, and N206S) were found. Hearing loss in patients with biallelic Cx26 mutations ranged from unilateral high frequency to bilateral profound. Four children, 2 with biallelic mutations, had temporal bone abnormalities.

Conclusions: Connexin 26 mutations are common in children with SNHL, and it is likely that the homozygous and compound heterozygous mutations cause the SNHL. However, pathogenicity is less certain when only a single Cx26 mutation is present. Patients with biallelic Cx26 mutations had a slightly higher incidence of milder hearing loss than in previous studies. Children with SNHL or MHL should be tested for Cx26 mutations early in their evaluation.


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PATIENTS AND METHODS

PATIENTS

All children with SNHL or mixed hearing loss (MHL) of unknown etiology aged newborn to 18 years and cared for in the outpatient clinics of the Department of Otolaryngology, Children's Hospital Boston, Boston, Mass, were eligible for inclusion. These children and their families were offered Cx26 testing as part of their SNHL evaluation.

GENETIC TESTING

All Cx26 testing was performed in the Genetics Diagnostic Laboratory at Children's Hospital Boston. This laboratory is a Clinical Laboratory Improvement Act–approved facility. Genomic DNA was extracted from patients, and 2 overlapping polymerase chain reactions were performed to amplify the entire coding region of the Cx26 gene (GJB2). The following primer sets (60°C annealing temperature) were used for polymerase chain reaction amplification: Cx1-F TCT TTT CCA GAG CAA ACC GCC and Cx1-R GAC ACG AAG ATC AGC TGC AG; Cx2-F CCA GGC TGC AAC AAG GTG TG and Cx2-R TGA GCA CGG GTT GCC TCA TC. Polymerase chain reaction products were purified and sequenced using a fluorescence automatic DNA sequencer (Applied Biosystems Division, Perkin-Elmer Corp, Foster City, Calif).

GENETIC COUNSELING

Genetic counseling through the Division of Genetics, Children's Hospital Boston, was offered to all patients before and after genetic testing.

AUDIOMETRIC EVALUATION

All audiometric testing was performed in the Department of Audiology, Children's Hospital. Hearing loss was confirmed using age-appropriate audiometric testing, including auditory brainstem evoked response testing in newborns, infants, and young children; otoacoustic emission testing to further confirm and characterize the hearing loss; and behavioral and frequency-specific testing in children who were old enough to participate. A combination of audiometric tests was often used to confirm the diagnosis of SNHL. Degree of hearing loss was classified by calculating a 3-frequency pure-tone average hearing level (500, 1000, and 2000 Hz). Hearing loss was categorized as mild (21-40 decibels hearing level [dBHL]), moderate (41-55 dBHL), moderately severe (56-70 dBHL), severe (71-90 dBHL), or profound (>90 dBHL). Hearing loss was also classified as conductive, sensorineural, or mixed. The severity of loss in each ear was noted in cases of asymmetric hearing loss (eg, mild/severe).

The recent development of more accurate diagnostic techniques, including high-resolution computed tomography and magnetic resonance imaging of the temporal bone, has enabled an improved yield in the evaluation of children with SNHL. In addition, with the identification of many genetic loci involved in syndromic and nonsyndromic deafness and the subsequent discovery of some of the genes responsible for deafness at these loci, genetic testing is beginning to emerge as a valuable tool in the clinical assessment of deafness.

Genetic evaluation of a child with SNHL used to be limited to a dysmorphologic examination and a detailed study of the family. Although genetic counseling was frequently offered, there was so little specific information available that most patients (and many physicians) did not find it helpful. The uncertainty about diagnosis of genetic hearing loss is changing with the identification of many “deafness genes” for nonsyndromic and syndromic causes of SNHL.

For nonsyndromic cases, 28 genetic loci have been identified for recessive hearing loss, 33 for dominant, 3 for either dominant or recessive inheritance, 5 for X-linked, and 2 for mitochondrial. To date, 19 genes have been cloned for nonsyndromic deafness among these 71 loci. In addition to nonsyndromic deafness, more than 400 syndromic forms of deafness have been described, of which several have deafness as a prominent and common feature. These syndromes include Waardenburg, Usher, Alport, Jervell and Lange-Nielsen, Norrie, branchio-oto-renal, Stickler, Pendred, and Treacher Collins. Most of these syndromes have substantial genetic heterogeneity, with 20 genes identified at the 28 loci involved in these 9 syndromes.

The most significant breakthrough was made in 1997 with the discovery of the first nuclear gene to be implicated in nonsyndromic recessive SNHL, the gap junction beta-2 gene (GJB2). Now thought to be responsible for up to half of all recessive nonsyndromic SNHL, this gene encodes the connixin 26 (Cx26) protein and segregates at the DFNB1 locus on 13q12. More than 60 mutations have been described for the Cx26 gene; however, 1 mutation seems to be especially common, particularly in white populations: the 35delG mutation, which results in a frameshift and subsequent premature termination of the protein. A second mutation, 167delT, has a high frequency in the Ashkenazi Jewish population. In addition, there are many other Cx26 defects, including missense and nonsense mutations and small deletions and insertions. Although mutations in Cx26 were initially thought to be responsible only for recessive nonsyndromic SNHL, at least 6 mutations now seem to be associated with dominant SNHL and 3 with syndromic SNHL.

Connexins are a family of membrane proteins that combine to form intercellular or gap junction channels. Although the exact function of connexins still remains unclear, it seems that the intercellular connections that they form are important in electrolyte, second messenger, and metabolite exchange. Immunostaining of rat cochlea shows that Cx26 is located within 2 groups of cells in the cochlea. The first are the nonsensory epithelial cells, including inner sulcus cells, interdental cells of the spiral limbus, supporting cells of the organ of Corti, outer sulcus cells, and cells within the root process of the spiral limbus. The second group includes fibro-
cytes of the spiral limbus and spiral ligament, basal and intermediate cells of the stria vascularis, and mesenchymal cells lining the scala vestibuli. Kelsell and colleagues found Cx26 in the basement membrane, the spiral limbus, the stria vascularis, and the spiral prominence in humans. The location of Cx26 in these areas supports the hypothesis that it is involved in the recycling of potassium ions during the transduction process. It is proposed that functional communication between the supporting cells of the organ of Corti provides an intercellular pathway for the transport and release of potassium back to the endolymph.

In December 1998 we set up a genetic assay to identify mutations throughout the entire coding region of the Cx26 gene. Since then, we studied 107 children with SNHL. Herein we report the findings from this study, including the spectrum of mutations present and the clinical and associated audiologic findings.

### RESULTS

Between December 1, 1998, and July 1, 2000, 107 patients with SNHL or MHL of unknown etiology were tested for mutations in the Cx26 gene. The 107 children were from 99 families. Most children were aged 1 week to 16 years; 61 were boys and 46 were girls. Mutations in Cx26 were found in 30 (30%) of the 99 probands: biallelic mutations were detected in 18 (9 were homozygous and 9 were compound heterozygous) and single mutations were detected in 12. Of these 30 probands, 81% were white, 13% were Hispanic, 3% were African American, and 3% were Asian. In all, 15 different mutations were found: 12 that have been reported previously (35delG, 167delT, E47X, L90P, M34T, G12V, V37I, R143W, V84L, V153I, V27I, and 310del14) and 3 novel mutations (E129K, T8M, and N206S).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Connexin 26 Genotype</th>
<th>Inheritance</th>
<th>Age at Diagnosis</th>
<th>Hearing Loss†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P</td>
<td>35delG/167delT</td>
<td>Pseudodominant</td>
<td>Birth</td>
<td>Profound SNHL</td>
</tr>
<tr>
<td>2P</td>
<td>35delG/167delT</td>
<td>Singleton</td>
<td>2 y</td>
<td>Profound SNHL</td>
</tr>
<tr>
<td>3P</td>
<td>35delG/167delT</td>
<td>Some family history</td>
<td>1 y</td>
<td>Profound SNHL</td>
</tr>
<tr>
<td>4P</td>
<td>35delG/167delT</td>
<td>Singleton</td>
<td>Birth</td>
<td>Profound SNHL</td>
</tr>
<tr>
<td>5P</td>
<td>167delT/167delT</td>
<td>Singleton</td>
<td>6 mo</td>
<td>Profound SNHL</td>
</tr>
<tr>
<td>6P</td>
<td>35delG/143W</td>
<td>Singleton</td>
<td>2 y</td>
<td>Profound SNHL</td>
</tr>
<tr>
<td>7P</td>
<td>35delG/47X</td>
<td>Some family history</td>
<td>Birth</td>
<td>Profound SNHL</td>
</tr>
<tr>
<td>8P</td>
<td>V84LV84L</td>
<td>Singleton</td>
<td>4 y</td>
<td>Profound SNHL</td>
</tr>
<tr>
<td>9P</td>
<td>35delG/47X</td>
<td>Recessive</td>
<td>Birth</td>
<td>Severe/profound SNHL</td>
</tr>
<tr>
<td>10P</td>
<td>35delG/35delG</td>
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</tr>
<tr>
<td>11P</td>
<td>35delG/35delG</td>
<td>Pseudodominant?</td>
<td>1 y</td>
<td>Severe SNHL</td>
</tr>
<tr>
<td>12P</td>
<td>35delG/G12V</td>
<td>Recessive</td>
<td>5 y</td>
<td>Mild/moderate SNHL</td>
</tr>
<tr>
<td>13P</td>
<td>35delG/G12V</td>
<td>Recessive</td>
<td>5 y</td>
<td>Moderate SNHL</td>
</tr>
<tr>
<td>14P</td>
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<td>7 y</td>
<td>Mild/severe SNHL</td>
</tr>
<tr>
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<td>Recessive</td>
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<td>Mild/severe MHL</td>
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<td>V37I/V37I</td>
<td>Recessive</td>
<td>6 y</td>
<td>Mild SNHL</td>
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<td>17P</td>
<td>35delGN206S</td>
<td>Recessive</td>
<td>5 y</td>
<td>Unilateral mild SNHL</td>
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<tr>
<td>18P</td>
<td>T8M/V153I</td>
<td>Some family history</td>
<td>7 y</td>
<td>MHL with moderate 3- to 4-kHz notch</td>
</tr>
<tr>
<td>19P</td>
<td>E129K/NMD</td>
<td>Dominant?</td>
<td>8 y</td>
<td>Unilateral HF loss</td>
</tr>
<tr>
<td>20P</td>
<td>167delT/NMD</td>
<td>Singleton</td>
<td>Birth</td>
<td>Profound SNHL</td>
</tr>
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<td>L90P/NMD</td>
<td>Singleton</td>
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<td>Profound SNHL</td>
</tr>
<tr>
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<td>E47X/NMD</td>
<td>Recessive</td>
<td>3.5 y</td>
<td>Mild CHL</td>
</tr>
<tr>
<td>23P</td>
<td>E47X/NMD</td>
<td>Some family history</td>
<td>2.5 y</td>
<td>Profound SNHL</td>
</tr>
<tr>
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<td>Singleton</td>
<td>1 y</td>
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</tr>
<tr>
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<td>2 y</td>
<td>Profound SNHL</td>
</tr>
<tr>
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<td>35delGM34T</td>
<td>Singleton</td>
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<td>Severe SNHL</td>
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<td>35delGM34T</td>
<td>Some family history</td>
<td>3.5 y</td>
<td>Severe SNHL</td>
</tr>
<tr>
<td>28P</td>
<td>310del14/NMD</td>
<td>Dominant?</td>
<td>Unknown</td>
<td>HF loss</td>
</tr>
<tr>
<td>29P</td>
<td>M34T/NMD</td>
<td>Singleton</td>
<td>8 y</td>
<td>HF loss</td>
</tr>
<tr>
<td>30P</td>
<td>V37I/NMD</td>
<td>Singleton</td>
<td>12 y</td>
<td>Moderate SNHL</td>
</tr>
</tbody>
</table>

*SNHL indicates sensorineural hearing loss; MHL, mixed hearing loss; HF, high frequency; CHL, conductive hearing loss; and question marks, uncertainty about the inheritance pattern, ie, there are an insufficient number of reported families and patients to be sure at this time.

†Each patient has a number representing his or her family and a letter indicating proband (P) or his or her relationship to the proband, ie, brother (B) or sister (S).

‡Hearing loss is bilateral unless otherwise noted.

A variety of forms of inheritance were observed in Cx26-positive families. Some displayed recessive inheritance (indicated by the presence of an affected sibling and normal-hearing parents). One family demonstrated...
OTOACOUSTIC EMISSIONS TESTING was too small to make a lesion was thought to be cochlear. Of these children, the heterozygous for Cx26 mutations, the presumed site of the patient 21P), and unilateral partial deficiency of the left enlarged vestibular aqueducts and Mondini deformities (patient of the right modiolus (patient 12P), bilaterally en-

Tm 2 Moderate SNHL 13
Hearing loss in the 21 children (18 probands and 3 sib-


ting (or behavioral testing) suggested a retrocochlear site of lesion, further evaluation would always be undertaken, even if the patient was Cx26 positive.

Three new mutations were found in this study: N206S, T8M, and E129K. In addition, 2 reportedly normal variants, V27I and V153I, were also observed in deaf probands. N206S seems to be a recessive mutation, although it may cause a slightly milder phenotype. Both siblings with the 35delG/N206S genotype had less severe audiologic characteristics, including moderate SNHL in one child and unilateral mild SNHL in the other.

The E129K mutation was found in 1 proband with a unilateral high-frequency SNHL. It is possible that E129K represents a dominant Cx26 mutation because the father had almost identical hearing loss. Indeed, this type of high-frequency hearing loss has been observed in patients with dominant Cx26 mutations.18,19 However, it is also possible that the E129K mutation is not related to the deafness and represents either a recessive mutation or a normal variant of the Cx26 gene.

The significance of the T8M mutation is also unclear. It was found in heterozygosity with the V153I missense change. The V153I mutation has been reported to be a normal variant found in 4 of 367 normal-hearing controls11; however, it is not clear if it has ever been found in a homozygous or compound heterozygous state. Therefore, it could have acted in concert with the T8M missense change to cause the hearing loss observed in this patient. If that were the case, these mutations would both represent mild recessive mutations.

The V27I variation was observed as the only detectable Cx26 mutation in 1 deaf patient. Despite this, there is substantial evidence that this missense change represents a normal variant because it has been found in many normal-hearing individuals in the heterozygous state and in some normal-hearing individuals in the homozygous or compound heterozygous state.20,21 Therefore, it is likely that the presence of the V27I variant in this proband is unrelated to her deafness.

There are a few recessive mutations that seem to cause mild SNHL, including the M34T and V37I mutations.
tions and the novel N206S mutation described earlier. Although results of an initial study indicated that the V37I mutation may have been a normal variant, several recent studies have clearly demonstrated its pathogenicity. We confirm the results of these studies but suggest that the phenotype due to this mutation may be relatively mild, as evidenced by the mild SNHL seen in our proband with a homozygous genotype. The audiologic severity of this mutation is not discussed in other reported cases, so the significance of this finding awaits further confirmation.

The M34T mutation has been the subject of debate since it was initially described as a dominant mutation. Numerous studies since then have suggested that it is a recessive mutation because of its occurrence in the heterozygous state in the general population and its occurrence together with the other mutant Cx26 alleles in many deaf individuals. Our results confirm the findings from these studies but also suggest that the M34T may be a more mild mutation. Four children in our study had a 35delG/M34T genotype, including 3 unrelated probands and a sibling. All of these children had only mild hearing loss in at least 1 ear (the other ear ranged from mild to severe loss). In addition, another study also described 2 individuals with compound heterozygous genotypes involving the M34T mutation. Those 2 children also had mild hearing loss. Although making generalizations about the severity of Cx26 mutations is difficult because of the known variability with the 35delG homozygous state, it is likely that there will be some mutations that will consistently show a milder phenotype, and the M34T mutation may be one such mutation.

The severity of hearing loss observed in this study varied but generally was similar in distribution to that reported in other large studies (Figure). The distribution in our study seems to be skewed toward milder hearing losses. This may be attributed to the increased incidence of genotypes with the M34T mutation and the presence of a few patients with V37I and N206S mutations. These missense mutations may have less severe consequences on gap junction function in the cochlea, leading to less severe hearing loss.

Two patients with only 1 detectable Cx26 mutation had temporal bone abnormalities. It is possible that the hearing loss and computed tomographic abnormalities in these patients were not associated with the Cx26 mutations. In contrast, 2 other patients with biallelic Cx26 mutations had temporal bone anomalies, indicating that some cases of Cx26 deafness are associated with temporal bone abnormalities. This is in contrast to a previous study noting a lack of temporal bone abnormalities in a Cx26-positive patient. More studies will be needed to address this issue.

Sensorineural hearing loss in children is relatively common and can be compared with the incidence of Down syndrome (1 per 600 to 800 births), cleft lip and cleft palate (1 per 750 births in the United States), and cystic fibrosis (1 per 3500 live white births and 1 per 17,000 live black births in the United States). Intervention includes early speech and language services and the use of assistive listening devices, including hearing aids and FM systems. Along with the development of digital and programmable hearing aids, and significant improvements in analog aids as well, cochlear implantation is now considered a standard option for the child with bilateral severe-to-profound SNHL who does not benefit significantly from hearing aids. Families want to know why their child has hearing loss because it may affect their educational planning, the type(s) of communication mode they choose, and the type of hearing aid or other device they purchase and because it may predict whether profoundly deaf children will benefit from a cochlear implant. Discovery of a genetic cause may impact family planning and raise questions about the availability of prenatal diagnosis. Because the ethical issues involved with genetic testing are complex, it is important to make an accurate and timely diagnosis and to provide genetic counseling services.

The high cost of medical tests used in the evaluation of children for hearing loss and the low yield of positive results from many such tests warrant careful consideration of the optimal protocol. Need is particularly urgent given the advent of newborn screening for hearing loss in many regions, which will require providing diagnostic and prognostic information to parents as quickly as possible.

In the present study, 30 (30%) of 99 children with SNHL or MHL had at least 1 mutation in the Cx26 gene. Although the relationship between the Cx26 genotype and hearing loss is unclear in a third of these patients because of the presence of only 1 detectable mutation, in the other two thirds a probable causal relationship exists between the genetic abnormalities and the hearing loss. The yield compares favorably with findings from high-resolution computed tomographic scanning of the temporal bones in children with an unknown etiology of SNHL (10%-20%). An algorithm in the evaluation of SNHL could therefore use Cx26 testing as one of the first studies rather than as one performed after all other test results are negative. Genetic counseling should be offered to all patients for whom genetic testing is being considered because the test results are often not straightforward and the patients need to understand the implication of either a “positive” or “negative” test result.

**CONCLUSIONS**

We studied children with SNHL or MHL who previously did not have a well-defined etiology for their hearing loss. Of 99 probands studied, 30 (30%) had mutations in their Cx26 gene: 9 were homozygous, 9 were compound heterozygous, and 12 were heterozygous. Three previously unreported mutations were identified. Hearing loss ranged from unilateral high-frequency hearing loss to bilateral profound SNHL. The severity of the hearing losses was similar to that in previous studies, although a slightly higher incidence of milder hearing loss was observed. Temporal bone abnormalities were present in 4 patients with Cx26 mutations, suggesting that loss of Cx26 function can cause abnormalities in the temporal bone. In patients with biallelic Cx26 mutations it is straightforward to conclude that the hearing loss is the result of the Cx26 mutations; however, it is difficult to
make this association when only a single Cx26 mutation is present (unless the mutation is dominant). Because of the high incidence of Cx26 mutations in children with SNHL, Cx26 testing should be performed early in the evaluation regardless of the severity of the hearing loss.

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