## RESEARCH ARTICLE

# Novel Mutation in the KCNQ4 Gene in a Large Kindred With Dominant Progressive Hearing Loss

Zohreh Talebizadeh,[1]* Philip M. Kelley,[1] James W. Askew,[1] Kirk W. Beisel,[1] and Shelley D. Smith[2]

[1]Center for Hereditary Communication Disorders, Boys Town National Research Hospital, Omaha, Nebraska
[2]Center for Human Molecular Genetics, Munroe Meyer Institute, University of Nebraska Medical Center, Omaha, Nebraska

Analysis of genotyping of a five-generation American family with nonsyndromic dominant progressive hearing loss indicated linkage to the DFNA2 locus on chromosome 1p34. This kindred consists of 170 individuals, of which 51 are affected. Pure tone audiograms, medical records, and blood samples were obtained from 36 family members. Linkage analysis with five microsatellite markers spanning the region around DFNA2 produced a lod score of 6.6 for the marker MYCL1 at $\theta = 0.0$. Hearing loss in this family showed a very similar pattern as the first reported American family with the same linkage. High frequency hearing loss was detectable as early as 3 years of age, and progressed to severe to profound loss by the fourth decade. Using intronic primers, we screened the coding region of the KCNQ4 gene. Heteroduplex analysis followed by direct sequencing identified a T→C transition at position 842, which would produce an L281S amino acid substitution. The observed mutation was shown to segregate completely with affected status in this family. The L281 residue is significantly conserved among the other members of the voltage-gated $K^+$ channel genes superfamily. Hydrophobicity analysis indicated that L281S substitution would lower formation of the $\beta$ structure at the P region of this ion channel. Mutation analysis of KCNQ4 was also performed on 80 unrelated probands from families with recessive or dominant nonsyndromic hearing loss. None of these cases showed a truncated mutation in KCNQ4. Hum Mutat 14:493–501, 1999.    © 1999 Wiley-Liss, Inc.

KEY WORDS: DFNA2; KCNQ4; GJB3; hearing loss; mutation detection; potassium channel; phenotypic heterogeneity; hydrophobicity analysis

## INTRODUCTION

Deafness is one of the most common communication disorders. Approximately one out of every 1,000 children is born with a significant degree of hearing loss. Hearing impairment is both genetically and clinically heterogeneous. It is estimated that up to 60% of the cases of profound early onset hearing loss are caused by genetic factors and about 75–80% of the hereditary cases are nonsyndromic. Among nonsyndromic forms of hearing loss, almost 10–15% are transmitted as autosomal dominant traits [Van Camp et al., 1997a,b]. Loci responsible for this form of hereditary hearing loss are designated as DFNA. To date, at least 19 different DFNA loci have been localized by genetic linkage analysis (http://dnalab-www.uia.ac.be/dnalab/hhh/). However, only nine genes have been identified: HDLA1, GJB3, KCNQ4, GJB2 (Cx26), DFNA5, TECTA, COCH-5B2, MYO7A, and POU4F3 [Lynch et al., 1997;

Xia et al., 1998; Kubisch et al., 1999; Kelsell et al., 1997; Van Lear et al., 1998; Verhoeven et al., 1998; Robertson et al., 1998; Liu et al., 1997; Vahava et al., 1998]. Except for MYO7A, Cx26, and TECTA, mutations in these genes have been reported to cause solely a nonsyndromic dominant type of hearing impairment.

We studied an extended American family (2177) with autosomal dominant nonsyndromic progressive sensorineural hearing loss (DPHL). Affected individuals experienced an early-onset high frequency hearing loss that progressed with

age to include middle and lower frequencies. The hearing loss gene in family 2177 was linked to the DFNA2 locus which was identified on chromosome 1p34 on the basis of linkage analysis in two families with DPHL, originating from Indonesia and the USA [Coucke et al., 1994].

Recently, two different genes in the DFNA2 region have been reported with mutations causing hearing loss. Xia et al. [1998] reported a missense and a nonsense mutation in a novel connexin protein (Cx31) (GJB3, MIM# 603324) responsible for the nonsyndromic autosomal dominant hearing loss in two small Chinese families. This connexin gene was localized to chromosome 1p34 and consisted of two exons. Later, Kubisch et al. [1999] identified a novel, voltage-gated $K^+$ channel family (KCNQ4, MIM# 603537) in the same region, and a missense mutation was found causing autosomal dominant hearing loss in a small French family. The KCNQ4 gene consists of 14 exons which encodes a protein with 695 amino acid residues.

Mutation screening in family 2177 was performed for these two genes by heteroduplex analysis and confirmed by direct sequencing of PCR products. The results revealed a novel missense mutation in KCNQ4 that segregated with the hearing loss.

## MATERIALS AND METHODS
### Clinical Diagnosis

Diagnosis of progressive sensorineural hearing loss was based on pure tone audiometry, questionnaire information, and review of medical records. Pure tone audiometry was performed on every family member who participated in this study. Some family members were screened by portable audiometer and a certified audiologist verified any abnormal findings. Others provided copies of audiograms that were taken by an audiologist. Family members with unilateral or age-related hearing loss (onset over 60 years of age) were not included in the linkage analysis. Individuals with sensorineural hearing loss who may have been subjected to environmental factors such as noise exposure were also excluded from this study.

### DNA Analysis

Blood samples were obtained following informed consent from 36 family members, 20 affected and 16 unaffected. A panel of 96 samples from normal population screened as a control. Genomic DNA was extracted from the blood samples by standard phenol extraction. Microsatellite markers from ABI Prism panels were used for genotyping on an ABI 377 DNA Sequencer. Fine mapping was also performed using five additional markers linked to the DFNA2 gene (see Table 1). The LINKAGE program version 5.1 [Lathrop et al., 1984] was utilized to calculate two-point lod scores. Haplotypes were constructed to confirm the linkage and locate crossover points.

### Mutation Screening

Mutation analysis was performed on genomic DNA. PCR was conducted using primers flanking the exons for Cx31 and KCNQ4 genes. Primer sequences were obtained from Xia et al. [1998] for Cx31, and were kindly provided by Dr. T. Jentsch for KCNQ4. All PCRs were done in a total volume of 50 µl containing 1x PCR buffer, 200 µM dNTPs, 0.5 µM (each) primer, and one unit of Taq polymerase enzyme. Amplified fragments of each primer set were investigated for mutation by heteroduplex analysis using MDE gels (FMC Bioproducts, Rockland, ME). Ethidium bromide at a concentration of 1 µg/ml was used to stain the MDE gels. For direct sequencing, PCR products were purified by Microcon ultrafiltration (Amicon, Beverly, MA). DNA sequencing was performed on fragments having a heteroduplex band to determine the cause(s) and nature of the extra band using ABI Prism Big Dye Terminator Kit. For cloning of the GATA-P18584 alleles, a TA cloning kit (Invitrogen, Carlsbad, CA) was utilized.

TABLE 1. **Two-Point Lod Scores for Linkage Between Hearing Loss and Microsatellite Markers in Family 2177\***

| Marker/θ | 0.0 | 0.05 | 0.10 | 0.15 | 0.20 | 0.25 | 0.30 | 0.35 | 0.40 | 0.45 |
|---|---|---|---|---|---|---|---|---|---|---|
| D1S255 | 2.81 | 2.50 | 2.19 | 1.87 | 1.56 | 1.24 | 0.94 | 0.66 | 0.40 | 0.18 |
| D1S432 | –99.99 | 3.37 | 3.17 | 2.85 | 2.46 | 2.05 | 1.62 | 1.19 | 0.78 | 0.37 |
| AFMB338WG5 | 3.67 | 3.01 | 2.92 | 2.52 | 2.11 | 1.70 | 1.30 | 0.92 | 0.57 | 0.25 |
| MYCL1 | 6.60 | 5.99 | 5.35 | 4.70 | 4.03 | 3.35 | 2.67 | 1.98 | 1.30 | 0.62 |
| GATA-P18584 | 6.18 | 5.59 | 4.98 | 4.36 | 3.73 | 3.09 | 2.45 | 1.83 | 1.20 | 0.52 |
| D1S193 | 6.39 | 5.78 | 5.14 | 4.49 | 3.82 | 3.14 | 2.46 | 1.77 | 1.10 | 0.46 |
| D1S197 | 0.35 | 2.90 | 2.74 | 2.44 | 2.09 | 1.69 | 1.28 | 0.86 | 0.44 | 0.10 |

\*Two-point lod scores at different recombination fraction (θ) between the disease gene and polymorphic markers on chromosome 1p34. Lod score for the GATA-P18684 was calculated with excluding individual IV-12.

## Protein Structure Analysis

The PEPTIDSTRUCTURE program from the graphical user interface of the Wisconsin package (Seqlab) was used for protein analysis (http://www.gcg.com/). Two methods, SSP (segment oriented secondary structure prediction) and SSPAL (nearest neighbor with local alignments secondary structure prediction) from the Sanger web site (http://genomic.sanger.ac.uk/) were utilized for secondary structure prediction.

## RESULTS

A five-generation American family (2177) with nonsyndromic dominant progressive hearing loss was studied. This family was of European descent and traced its ancestry to Austria. Hearing loss in family 2177 has been reported as early as age 3 beginning with the high frequencies and affecting the middle and low frequencies later in life. In general, the hearing-impaired individuals showed an audiogram with sloping configuration beginning after 1000 HZ. A high frequency hearing loss of at least 50 dB was diagnosed in several of the affected members by the age of 6. Tinnitus was reported by most of the affected members. No other clinical symptoms were diagnosed.

In the initial genome screen, lod scores of 2.81 at $\theta = 0.0$ was obtained for D1S255 and 2.90 at $\theta = 0.05$ for D1S197. These markers are known to span the DFNA2 locus. Therefore, for fine mapping five additional markers were typed from this region (see Table 1). These markers were consistent with linkage and one of these, MYCL1, produced a maximum lod score of 6.6 at $\theta = 0.0$. These data clearly indicated linkage of the hearing loss to the DFNA2 locus in this family.

After gene localization, haplotyping was performed to detect recombination within this chromosomal segment (Fig. 1). Occurrence of two informative recombinations in family members III-5 and IV-13 localized the gene between the markers AFMB338WG5 and D1S193. All of the affected individuals inherited the disease haplotype, and no cases of phenocopy were found. However, haplotyping detected an inconsistency in individual IV-12 for marker GATA-P18584. Single alleles from this marker were cloned. Sequencing of these alleles revealed that the marker consisted of a segment with the tandem GATA repeats. An expansion in the allele, which was transmitted from the father, was noticed, explaining the observed allele mismatch. The allele, which segregated with the disease haplotype in this family, had 12 repeats of GATA while individual IV-12 had 13 repeats of GATA in this polymorphic marker (data not shown). All other markers were consistent with the stated paternity and the affected haplotype.
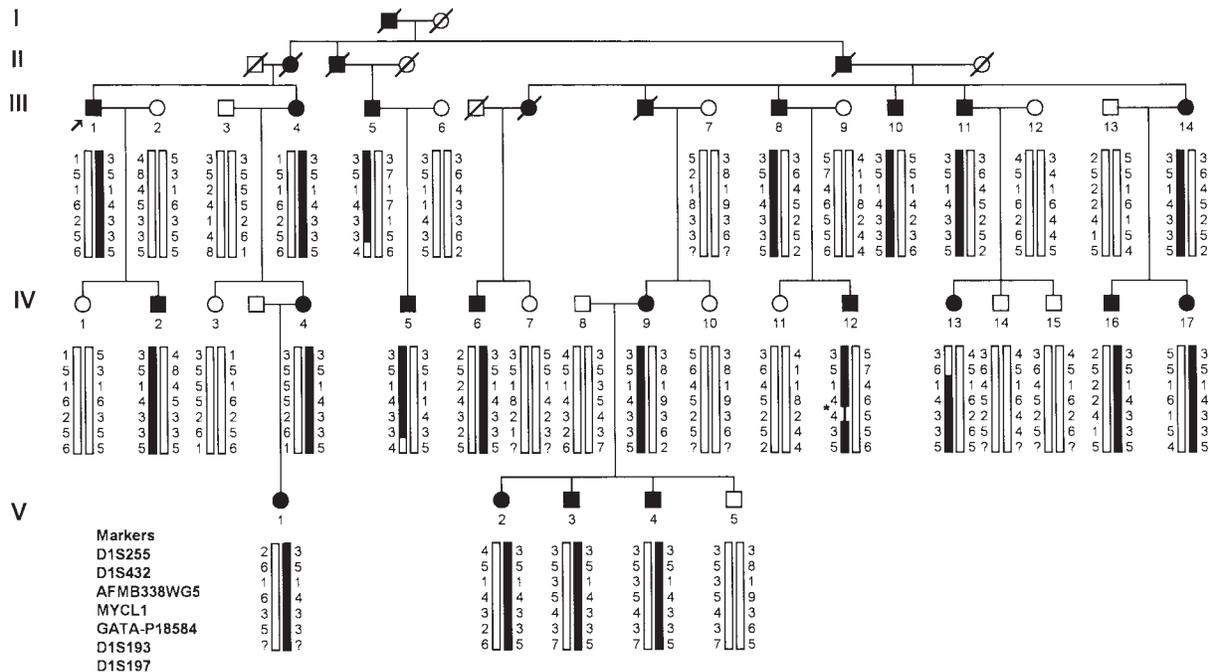


**FIGURE 1.   Pedigree of family 2177 with the haplotyping. All affected members in the family share the disease haplotype (black bar). An asterisk shows incompatibility for the marker GATA-P18684. Recombinations observed between markers are indicated with change in the bar color.**

In order to identify the responsible gene for hearing loss in family 2177, connexin 31 and KCNQ4 were each considered as a candidate gene. To look for mutation in these genes, their coding regions were amplified from genomic DNA samples. The coding sequence of the Cx31 gene is transcribed from a single exon. Therefore, two primers, cx31dg2 and cx31af, were used to amplify the coding region of this gap junction protein [Xia et al., 1998]. No mutation was detected for the Cx31 gene by heteroduplex analysis.

To detect a possible mutation in the KCNQ4 gene, exon-flanking primers were used to amplify all 14 coding exons. Heteroduplex analysis of the PCR products, which amplified exon 6, produced heteroduplex bands in affected members of the family (Fig. 2A). Sequencing of these amplimers revealed a missense mutation (842 T→C) which would produce an amino acid substitution (L281S) (Fig. 2B). In addition, two silent nucleotide substitutions, P291P and T293T, linked to the mutation were also noticed. Genomic DNA from 36
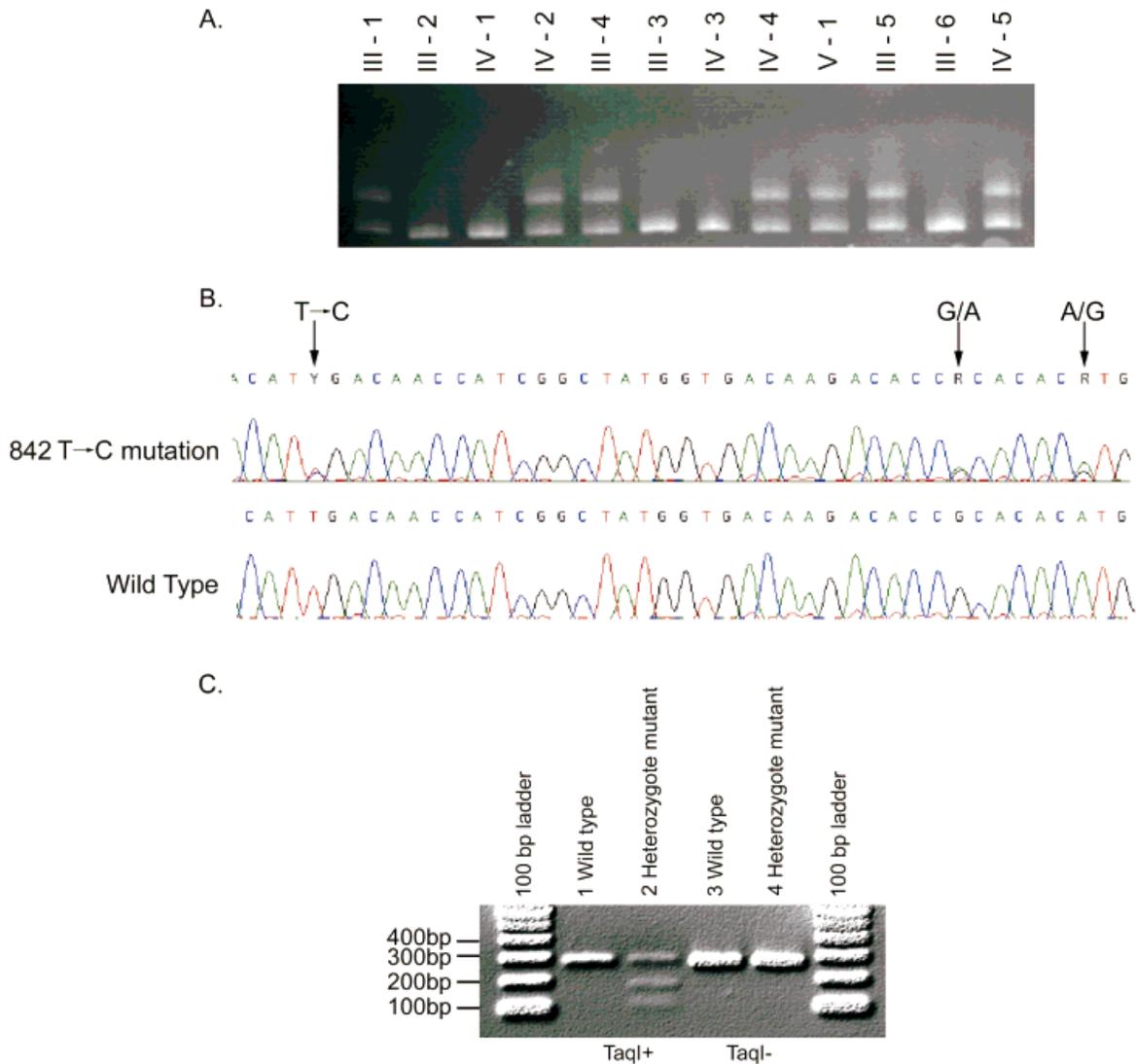


FIGURE 2.    **Mutation detection by heteroduplex analysis, sequencing, and restriction enzyme digestion. A:** Heteroduplex analysis of exon 6 shows a hetero band in affected samples compared with unaffected. **B:** Sequences of the sense-strand in the wild-type and mutated alleles are shown. **C:** PCR amplification products from exon 6 of the KCNQ4 gene after digestion with the enzyme TaqI. This enzyme cuts DNA at T/CGA sequences and was used to confirm 842 T→C missense mutation. In wild-type, digestion yields fragment of 270 bp (lane 1). In the heterozygote mutant after TaqI digestion there are also fragments of 170 and ~100 bp (lane 2). These bands are not present in the normal allele (lane 1). This restriction enzyme digestion assay could be used as a fast technique to detect the 842 T→C missense mutation. The 3D feature was used to enhance the gel image.

members of the family was sequenced from exon 6 to study segregation of the 842 T→C mutation with the hearing loss. All 20 affected individuals had inherited this missense mutation and it was not found in unaffected family members, nor in 96 control samples.

Restriction enzyme mapping was done to identify whether a digestion assay would detect the 842 T→C mutation. A restriction site was created by this substitution and was detected by the TaqI enzyme. Digestion with this enzyme cut PCR products of exon 6 from the mutated allele and produced two additional bands on a 2% agarose gel. The wild-type allele was not cut with the TaqI enzyme and migrated as a single band on the agarose gel (Fig. 2C).

To investigate the impact of the L281S amino acid change, the effect of this substitution was examined for the secondary structure of the KCNQ4 peptide. A 40 residues segment of the protein representing the P region, including the mutated leucine in its middle, was analyzed with the PEPTIDSTRUCTURE program. Changing the L→S increased the hydrophilicity in at least three residues upstream and downstream of this position. It was expected that the observed change in the hydrophilicity would create a noticeable change in the secondary structure of the KCNQ4 protein. Results of using both SSPAL and SSP methods indicated that L281S substitution would truncate formation of the β structure at this part of the protein (Fig. 3).

## Mutation Screening of KCNQ4 Gene for Dominant and Recessive Nonsyndromic Hearing Loss

To investigate whether mutation in the KCNQ4 gene is involved in hearing loss in other families, DNA samples from 80 unrelated probands with inherited nonsyndromic hearing loss were screened for the KCNQ4 gene. Twenty of these samples belonged to families with a dominant pattern of inheritance and 60 samples to the families with a recessive form of disease. Mutation in KCNQ4 gene was not identified in any of these samples. However, 10 sequence variations were detected in the coding regions of the gene without changing the amino acid codon (Table 2). In addition, one nucleotide change (1365 G→T) was noticed in the coding region of exon 10, which would lead to a nonconservative substitution (Q455H). This

**A.**

| AA[a] | H | AA[b] | H |
|---|---|---|---|
| W | -0.657 | W | -0.657 |
| G | -0.671 | G | -0.671 |
| T | -0.671 | T | -0.014 |
| I | -0.700 | I | -0.043 |
| T | -0.729 | T | -0.071 |
| **L** | -1.429 | **S** | -0.771 |
| T | -1.471 | T | -0.814 |
| T | -0.643 | T | 0.014 |
| I | -0.686 | I | -0.029 |
| G | 0.357 | G | 0.357 |
| Y | 0.814 | Y | 0.814 |

H= Hydrophilicity according to the Kyte-Doolittle
[a]Amino acid segment of the P region from the wild type channel protein
[b]Amino acid segment of the P region from the mutated channel protein

**B.**

| | SSP[a] | SSPAL[b] |
|---|---|---|
| | P=2.9 | |
| | BBBBBBBBB | BBBBB |
| Wild type allele | ...DSLWWGTITLTTI... | ...DSLWWGTITLTTI... |
| | P=2.0 | |
| | BBBBBBB | BBB |
| Mutated allele | ...DSLWWGTITSTTI... | ...DSLWWGTITSTTI... |

[a]SSP= Segment oriented secondary structure prediction
[b]SSPAL= Nearest neighbor with local alignments secondary structure prediction

FIGURE 3. **Hydrophobicity effect and secondary structure prediction. A:** Hydrophilicity of a segment of the KCNQ4 that includes the L281 residue. There are differences in the hydrophilicity of this segment of the protein in the truncated versus the normal channel. The parts with this discrepancy are enclosed in the box. **B:** The probability for formation of the β structure is predicted to be 2.9 in the normal protein vs. 2.0 in the L281S mutation based on the SSP method. Reduction in strength of the β strand is also seen in the truncated protein by using the SSPAL method.

TABLE 2. **Sequence Variations and Polymorphisms in the KCNQ4 Gene Found in This Study***

| Exon | Nucleotide change | Amino acid change | Region | Frequency |
|---|---|---|---|---|
| 5 | 777 C→T | A259A | S5 | 34/66 T/C |
| 6 | 841 T→C | L281L | Pore | 10/90 C/T |
| 6 | 873 G→A | P291P | Pore | 15/86 A/G |
| 6 | 879 A→G | T293T | P/S6 | 15/85 G/A |
| 7 | 972 G→A | L324L | S6 | 2/98 A/G |
| 9 | 1188 C→T | P396P | Distal to S6 | 2/98 T/C |
| 10 | 1347 G→A | T449T | Distal to S6 | 2/98 A/G |
| 10 | 1365 G→T | Q455H | Distal to S6 | 25/75 T/G |
| 10 | 1473 C→T | F491F | Distal to S6 | 2/98 T/C |
| 12 | 1657 C→T | L553L | Distal to S6 | 10/90 T/C |
| 12 | 1665 G→A | P555P | Distal to S6 | 10/90 A/G |

**\*The KCNQ4 potassium channel protein consists of six transmembrane domains (S1-S6) and a pore (P) region.**

glutamine is not a conserved residue among other potassium channel genes. Thus, it was not expected that the observed substitution would have any influence on function of the channel. This change was seen in the heterozygous state in some of these cases and in the controls. Nevertheless, one sample from a consanguineous family with recessive inheritance of hearing impairment showed this change in a homozygous state. To assess segregation of the Q455H substitution with the disease gene in this family, DNA samples from one affected and one unaffected sib of this proband were screened. The results did not show segregation of the Q455H with the recessive form of hearing loss in that family (data not shown).

## DISCUSSION

In family 2177, a gene responsible for hearing loss showed linkage to the DFNA2 (1p34) locus with maximum lod score of 6.6 for the marker MYCL1 at $\theta = 0.0$. This is the second American DPHL family reported with linkage to the DFNA2 gene. We excluded Cx31 as the causative gene for the hearing loss in this family. Recently, Kubisch et al. [1999] identified a mutation (G285S) in exon 6 of a novel $K^+$ channel (KCNQ4) in a small French family with autosomal dominant hearing loss. We have found another missense mutation (L281S) in this exon as the etiology of the hearing loss in our DFNA2 family. Simultaneously, Coucke et al. [1999] reported mutations in the KCNQ4 gene in four DFNA2 families.

As in other voltage-gated $K^+$ channels (Kv channel), the KCNQ4 protein is created from six transmembrane alpha helices (S1–S6). The functioning channel is thought to consist of four separate subunits that come together to form a pore (P region) between them. The conducting region of the open channel is formed by a stretch of amino acids, the P region that connects the putative S5 and S6 helices. Hydrophobicity analysis of the primary sequence of the voltage gated $K^+$ channels suggests that the amino acids which form the P region are intercalated into the membrane and form two antiparallel $\beta$ strands. Since a Kv channel is formed from four $\alpha$ subunits, the $\beta$ strand is repeated by four of these motifs and creates the pore [Catterall, 1988; Stevens, 1991].

The L281S substitution is a nonconservative change. Leucine is a more hydrophobic amino acid, while serine has a hydrophilic characteristic. The more hydrophobic amino acids are usually found buried within the interior of the protein structure. X-ray analysis on the $K^+$ channel from *Streptomyces lividans* (KcsA $K^+$ channel) provided evidence that the extracellular part of the pore is mainly lined by hydrophobic amino acids [Doyle et al., 1998]. Thus, the L→S substitution is expected to yield a significant interruption in the pore structure of the KCNQ4 channel. Hydrophobicity analysis suggested that observed substitution would increase the hydrophilicity and would influence the secondary structure by decreasing the formation of the $\beta$ strands. Changes in the secondary structure may result in a significant alteration of folding and function of the protein. To further investigate influence of this mutation at the protein structure level, the 3D structure needs to be studied.

KCNQ4 is the fourth member of the KCNQ gene subfamily to be identified in humans. The first gene from this subfamily, known as KVLQT1, is associated with the inherited long QT syndromes and is characterized by a prolonged QT interval in the electrocardiogram. Mutations in this gene also cause a recessive long QT syndrome known as Jervell and Lange-Nielsen (JLN) syndrome [Neyroud et al., 1997]. Affected individuals in addition to cardiac abnormalities have congenital deafness. The KCNE1 gene, which encodes an accessory subunit for KVLQT and modulates the gating kinetics of the active channel, also has been found to be involved in JLN syndrome [Tyson et al., 1997]. Recently, two other members of this family, KCNQ2 and KCNQ3, were cloned and shown to cause a form of hereditary epilepsy [Singh et al., 1998; Charlier et al., 1998].

Most mutations have been found in the P region of these KCNQ channels, suggesting that this region is highly sensitive to the changes. Comparison of the amino acid residues among different members of the Kv superfamily channels indicates existence of conserved regions. In Figure 4, the stretch

|  |  | Pore | | | Organism | Reference |
|---|---|---|---|---|---|---|
| KCNQ4 | 270 Y A D S L W W G T I T | L | T T I G Y G D K T P | 291 | Human | gi\|4262523 |
| KCNQ2 | 264 Y A D A L W W G L I T | L | T T I G Y G D K Y P | 285 | Human | gb\|AAD16988 |
| KCNQ3 | 303 Y A D A L W W G L I T | L | A T I G Y G D K T P | 324 | Human | gi\|4028015 |
| KVLQT1 | 299 Y A D A L W W G V V T | V | T T I G Y G D K V P | 320 | Human | gi\|2465531 |
| nKQT1 | 296 F A D A L W W G V I T | L | S T V G Y G D K T P | 317 | Caenorhabditis elegans | gi\|1945495 |
| KvEBN2 | 256 Y A D A L W W G L I T | L | A T I G Y G D K T P | 277 | Human | gi\|2801450 |
| mKv1.1 | 359 I P D A F W W A V V S | M | T T V G Y G D M Y P | 380 | Mouse | sp\|P16388 |
| hKv2.1 | 360 I P A S F W W A T I T | M | T T V G Y G D I Y P | 381 | Human | sp\|Q14721 |
| rKv3.1 | 424 I P I G F W W A V V T | M | T T L G Y G D M Y P | 445 | Rat | pir\|S17150 |
| hKv3.4 | 423 I P I G F W W A V V T | M | T T L G Y G D M Y P | 444 | Human | ref\|NP004969 |
| mKv4.1 | 359 I P A A F W Y T I V T | M | T T L G Y G D M V P | 380 | Mouse | pir\|A39372 |
| aKv5.1 | 409 I P M G F W W S I I T | M | T T V G Y G D V Y P | 430 | Aplysia californica | gi\|688438 |
| Kv8.1 | 383 V P C A W W W A T T S | M | T T V G Y G D I R P | 404 | Mesocricetusauratus | gi\|1438971 |
| mKv1.9 | 234 Y A D A L W W G V V T | V | T T I G Y Y D K V P | 255 | Mouse | sp\|P97414 |

FIGURE 4.   **Multiple alignment of KCNQ4 and homologous genes in the P region. The L281 residue, which is mutated in family 2177, is indicated in bold. This leucine is significantly conserved among other gene family, and would replace methionine in mKv1.1, hKv2.1, rKv3.1, hKv3.4, mKv4.1, aKv5.1, and Kv8.1, or valine in KVLQT1 and mKv1.9. Both of these amino acids are nonpolar, which indicates the essential role of a hydrophobic residue at this position. Residues homologous to L281 in the KCNQ4 are boxed.**

of amino acids forming the P region of the KCNQ4 molecule is compared with its homologous region from the other voltage-gated K$^+$ channels. The L281 residue is semiconserved in these channels, which implies that the nonconservative L281S substitution is the disease-causing mutation.

In the report of the identification of KCNQ4 and its role in hereditary deafness, Kubisch et al. [1999] presented evidence that this gene is active only in the outer hair cells of the cochlea. However, there are reasons to question this restrictive cellular localization. RT-PCR analyses for expression of the four isoforms of the KCNQ subfamily were presented which demonstrated that all but KCNQ2 were expressed in the vestibular and cochlear end-organs. The authors reconciled the RT-PCR results, which indicated that mouse KCNQ4 was expressed in vestibular tissue, by suggesting the vestibular RNA preparation was contaminated by cochlear cells (i.e., OHCs). However, another possibility is that mKCNQ4 is expressed elsewhere in the inner ear. In situ hybridization of inner ear tissue could have resolved this issue, but only cochlear tissue was examined. In addition, the majority of the signal in the OHCs were at the apical ends, while typically in situ hybridization studies of the cochlea have demonstrated a uniform perinuclear cytoplasmic staining in hair cells [Lewis et al., 1998; Safieddine and Wenthold, 1997]. Only

the first two rows of OHCs were stained, while the third row showed no signal. A faint signal appears to be present in IHCs, thereby suggesting that there may be low levels of expression in these cells. Prolonging the alkaline phosphatase staining reaction may have revealed the presence of low abundance KCNQ4 message in IHCs. In the cochlea there are at least three major cell types which display a variety of ion channels. These are the neurosensory epithelium, marginal cells of the stria vascularis, and the neurons of the spiral ganglion [Lin, 1997; Moore et al., 1996; Santos-Sacchi, 1993; Sanguinetti et al., 1996; Mammano et al., 1995; Kros et al., 1998; Eatock and Rüsch, 1997; Sunose et al., 1994; Shen and Marcus, 1998; Wangemann et al., 1995]. Of these three cell types, ganglion cells were not represented in the microphotography. The innervating vestibular ganglion and cochlear spiral ganglion cell, which are present within the inner ear, are likely cell types which could also express the KCNQ-associated channels. Therefore, the expression pattern of the KCNQ4 needs to be further studied in the cochlea.

The function of KCNQ4 is not yet known. However, the impact of disease-causing mutations could be investigated by considering their phenotypic expression. Despite the complete penetrance of the L281S mutation in family 2177, the resulting phenotypes were not exactly the same among affected

family members. Review of the audiograms revealed significant differences in age of onset and level of hearing loss, at least in some affected members. Individual V-1 is a 13-year-old without any previous report of hearing impairment. Surprisingly, her first pure tone audiometry indicated moderate bilateral hearing loss of 40–50 dB at high frequencies. A second audiogram was taken 2 months later to confirm the result of original screening. The hearing loss was confirmed in this individual, and later mutation screening proved her affected status. By contrast, all three affected offspring of individual IV-9 had documented hearing loss as early as 3 years old. They are severe to profoundly hearing-impaired in early childhood with a 60–80 dB loss at high frequencies. This substantial difference in the manifestation of the mutated gene suggests involvement of another factor, such as mtDNA [Fischel-Ghodsian, 1999], other nuclear gene(s), or environmental factors as a secondary cause that together produce the phenotypic variability. Environmental factors such as noise exposure and ototoxic agents have been shown to have a deleterious effect on the auditory system. However, considering the magnitude of this dissimilarity and age of subjects, it is more likely that other genetic factors contribute, rather than solely environmental influences. All three affected offspring of individual IV-9 share almost the same mitochondrial DNA. Thus, the first possible explanation would be accumulation of mtDNA mutations that genetically predispose these individuals to hearing loss.

Other genomic mutations might also be taken into consideration. It has been proven that mutations in Cx26 could account for 50% of childhood nonsyndromic recessive hearing loss, with a carrier rate of 2.8% [Kelley et al., 1998]. Abe et al. [1999] found a significantly higher ratio of Cx26 mutations in families with the A1555G mtDNA mutation compared to a control population. Individuals with both mutations had more severe hearing loss, suggesting that the connexin 26 mutation might be an aggravating factor in the phenotypic expression of the aminoglycoside-induced hearing loss due to the A1555G mtDNA mutation. Thus, it is reasonable to hypothesize that Cx26 mutations or other nuclear genes play similar roles in causing the clinical variation in family 2177. A third factor could be the presence of different isoforms or splice-variants of potassium channel subunits such as those observed for KCNQ2 [Nakamara et al., 1998]. KCNQ4 can also form a heteromeric channel with KCNQ3 subunits. There is some evidence that mutated KCNQ4 produces a dominant negative effect on expression of wild-type subunits [Kros et al., 1998]. If indeed there were more than one active isoforms of any of these subunits, some might be more resistant to this negative effect, and lead to less reduction in channel current activity.

Finally, several studies have indicated that single nucleotide polymorphisms (SNPs) in the coding regions may play a role in disease susceptibility. These apparently neutral polymorphisms would not alter the protein structure, but may have an effect on the stability or translation rate of the relevant mRNA. Inheritance of a specific haplotype for a set of SNPs in a gene may produce susceptibility to a genetic disease.

In the present study, we identified a novel mutation in the P region of the KCNQ4 potassium channel which yielded dominant nonsyndromic hearing loss. In addition, 11 sequence variations in the coding regions were found. Highly heterogeneous ones (SNPs) could be considered as a marker in population studies [Kruglyak, 1997].

## REFERENCES

Abe S, Usami S, Shinkawa H, Kelley PM, Kimberling WJ. 1999. Connexin 26 mutations are aggravating factors in phenotypic expression in the non-syndromic deafness associated with the A1555G mitochondrial mutation. ARO meeting, Abstract #42.

Catterall WA. 1988. Structure and function of voltage-sensitive ion channels. Science 242:50–61.

Charlier C, Singh NA, Ryan SG, Lewis TB, Reus BE, Leach RJ, Leppert M. 1998. A pore mutation in a novel KQT-like potassium channel gene in an idiopathic epilepsy family. Nat Genet 18:53–55

Coucke PJ, Van Camp G, Djoyodiharjo B, Smith SD, Frants RR, Padberg GW, Darby JK, Huizing EH, Cremers CW, Kimberling WJ, Oostra BA, Van De Heyning PH, Willems PJ. 1994. Linkage of autosomal dominant hearing loss to the short arm of chromosome 1 in two families. N Engl J Med 331:425–431.

Coucke PJ, Van Hauwe P, Kelley PM, Kunst H, Schatteman I, Van Velzen D, Meyers J, Ensink RJ, Verstreken M, Declau F, Marres H, Kastury K, Bhasin S, McGuirt WT, Smith RJH, Cremers CWRJ, Van de Heyning P, Willems PJ, Smith SD, Van Camp G. 1999. Mutations in the KCNQ4 gene are responsible for autosomal dominant deafness in four DFNA2 families. Hum Mol Gent 8:1321–1328.

Doyle DA, Cabral JM, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT, MacKinnon R. 1998. The structure of the potassium channel: molecular basis of $K^+$ conduction and selectivity. Science 280:69–77.

Eatock RA, Rüsch A. 1997. Developmental changes in the physiology of hair cells. Semin Cell Dev Biol 8:265–275.

Fischel-Ghodsian N. 1999. Mitochondrial deafness mutations reviewed. Hum Mutat 13:261–270.

Kelley PM, Harris DJ, Comer BC, Askew JW, Fowler T, Smith SD, Kimberling WJ. 1998. Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss. Am J Hum Genet 62:792–799.

Kelsell DP, Dunlop J, Stevens HP, Lench NJ, Liang JN, Parry G, Mueller RF, Leigh IM. 1997. Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. Nature 387:80–83.

Kros CJ, Ruppersberg JP, Rüsch A. 1998. Expression of a potassium current in inner hair cells during development of hearing in mice. Nature 394:281–284.

Kruglyak L. 1997. The use of a genetic map of biallelic markers in linkage studies. Nat Genet 17:21–24.

Kubisch C, Schroeder BC, Friedrich T, Lutjohann B, El-Amraoui A, Marlin S, Petit C, Jentsch TJ. 1999. KCNQ4, a novel potassium channel expressed in sensory outer hair cells, is mutated in dominant deafness. Cell 96:437–446.

Lathrop GM, Lalouel JM, Julier C, Ott J. 1984. Strategies for multilocus linkage analysis in humans. Proc Natl Acad Sci USA 3443–3446.

Lewis AK, Frantz GD, Carpenter DA, de Sauvage FJ, Gao WQ. 1998. Distinct expression patterns of notch family receptors and ligands during development of the mammalian inner ear. Mech Dev 78:159–163.

Lin X. 1997. Action potentials and underlying voltage-dependent currents studied in cultured spiral ganglion neurons of the postnatal gerbil. Hear Res 108:157–179.

Liu XZ, Walsh J, Mburu P, Kendrick-Jones J, Cope MJ, Steel KP, Brown SD. 1997. Mutations in the myosin VIIA gene cause non-syndromic recessive deafness. Nat Genet 16:188–190.

Lynch ED, Lee MK, Morrow JE, Welsch PL, Leon PE, King MC. 1997. Nonsyndromic deafness DFNA1 associated with mutation of a human homolog of the Drosophila gene diaphanous. Science 278:1315–1318.

Mammano F, Kros CJ, Ashmore JF. 1995. Patch clamped responses from outer hair cells in the intact adult organ of Corti. Pflugers Arch 430:745–750.

Moore EJ, Hall DB, Narahashi T. 1996. Sodium and potassium currents of type I spiral ganglion cells from rat. Acta Otolaryngol (Stockh) 116:552–560.

Nakamura M, Watanabe H, Kubo Y, Yokoyama M, Matsumoto T, Sasai H, Nishi Y. 1998. KQT2, a new putative potassium channel family produced by alternative splicing. Isolation, genomic structure, and alternative splicing of the putative potassium channels. Receptors Channels 5:255–271.

Neyroud N, Tesson F, Denjoy I, Leibovici M, Donger C, Barhanin J, Faure S, Gary F, Coumel P, Petit C, Schwartz K, Guicheney P. 1997. A novel mutation in the potassium channel gene KVLQT1 causes the Jervell and Lange-Nielsen cardioauditory syndrome. Nat Genet 15:186–189.

Robertson NG, Lu L, Heller S, Merchant SN, Eavey RD, McKenna M, Nadol JB, Miyamoto RT, Linthicum FH, Lubianca Neto JF, Hudspeth AJ, Seidman CE, Morton CC, Seidman JG. 1998. Mutations in a novel cochlear gene COCH5B2 cause DFNA9, a human nonsyndromic sensorineural deafness with vestibular dysfunction. Nat Genet 20:299–303.

Safieddine S, Wenthold RJ. 1997. The glutamate receptor subunit delta1 is highly expressed in hair cells of the auditory and vestibular systems. J Neurosci 17:7523–7531.

Sanguinetti MC, Curran ME, Zou A, Shen J, Spector PS, Atkinson DL, Keating MT. 1996. Coassembly of K(V)LQT1 and minK (IsK) proteins to form cardiac IKs potassium channel. Nature 384:80–83.

Santos-Sacchi J. 1993. Voltage-dependent ionic conductances of type I spiral ganglion cells from the guinea pig inner ear. J Neurosci 13:3599–3611.

Shen Z, Marcus DC. 1998. Divalent cations inhibit IsK/KvLQT1 channels in excised membrane patches of strial marginal cells. Hear Res 123:157–167.

Singh NA, Charlier C, Stauffer D, DuPont BR, Leach RJ, Melis R, Ronen GM, Bjerre I, Quattlebaum T, Murphy JV, McHarg ML, Gagnon D, Rosales TO, Peiffer A, Anderson VE, Leppert M. 1998. A novel potassium channel gene, KCNQ2, is mutated in an inherited epilepsy of newborns. Nat Genet 18:25–29.

Stevens CF. 1991. Ion channels: making a submicroscopic hole in one. Nature 349:657–658.

Sunose H, Ikeda K, Suzuki M, Takasaka T. 1994. Voltage-activated K channel in luminal membrane of marginal cells of stria vascularis dissected from guinea pig. Hear Res 80:86–92.

Tyson J, Tranebjaerg L, Bellman S, Wren C, Taylor JFN, Bathen J, Aslaksen B, Sorland SJ, Lund O, Malcolm S, Pembrey M, Bhattacharya S, Bitner-Glindzicz M. 1997. Isk and KvLQT1: mutation in either of the two subunits of the slow component of the delayed rectifier potassium channel can cause Jervel and Lange-Nielsen syndrome. Hum Mol Genet 61:2179–2185.

Vahava O, Morell R, Lynch ED, Weiss S, Kagan ME, Ahituv N, Morrow JE, Lee MK, Skvorak AB, Morton CC, Blumenfeld A, Frydman M, Friedman TB, King MC, Avraham KB. 1998. Mutation in transcription factor POU4F3 associated with inherited progressive hearing loss in humans. Science 279:1950–1954.

Van Camp G, Coucke PJ, Kunst H, Schatteman I, Van Velzen D, Marres H, Van Ewijk M, Declau F, Van Hauwe P, Meyers J, Kenyon J, Smith SD, Smith RJH, Djelantik B, Cremers CWRJ, Van de Heyning PH, Willems PJ. 1997a. Linkage analysis of progressive hearing loss in five extended families maps the DFNA2 gene to a 1.25-Mb region on chromosome 1p. Genomics 41:70–74.

Van Camp G, Willems PJ, Smith RJH. 1997b. Nonsyndromic hearing impairment: unparalleled heterogeneity. Am J Hum Genet 60:758–764.

Van Lear L, Huizing EH, Verstreken M, Van Zuylen D, Wauters JG, Bossuyt PJ, Van de Heyning P, McGuirt W, Smith RJH, Willems PJ, Legan PK, Richardson GP, Van Camp G. 1998. Nonsyndromic hearing impairment is associated with a mutation in DFNA5. Nat Genet 20:194–197.

Verhoeven K, Van Laer L, Kirschhofer K, Legan PK, Hughes DC, Schatteman I, Verstreken M, Van Hauwe P, Coucke P, Chen A, Smith RJ, Somers T, Offeciers FH, Van de Heyning P, Richardson GP, Wachtler F, Kimberling WJ, Willems PJ, Govaerts PJ, Van Camp G. 1998. Mutations in the human α-tectorin gene cause autosomal dominant non-syndromic hearing impairment. Nat Genet 19:60–62.

Wangemann P, Liu J, Marcus DC. 1995. Ion transport mechanisms responsible for K+ secretion and the transepithelial voltage across marginal cells of stria vascularis in vitro. Hear Res 84:19–29.

Xia JH, Liu CY, Tang BS, Pan Q, Huang L, Dai HP, Zhang BR, Xie W, Hu DX, Zheng D, Shi XL, Wang DA, Xia K, Yu KP, Liao XD, Feng Y, Yang YF, Xiao JY, Xie DH, Huang JZ. 1998. Mutations in the gene encoding gap junction protein beta-3 associated with autosomal dominant impairment. Nat Genet 20:370–373.