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Nonsyndromic Hearing Loss DFNA10 and a Novel Mutation of *EYA4*: Evidence for Correlation of Normal Cardiac Phenotype With Truncating Mutations of the Eya Domain

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Dominant, truncating mutations of eyes absent 4 (*EYA4*) on chromosome 6q23 can cause either nonsyndromic hearing loss DFNA10 or hearing loss with dilated cardiomyopathy (DCM). It has been proposed that truncations of the C-terminal Eya domain cause DFNA10 whereas upstream truncations of the N-terminal variable region cause hearing loss with DCM. Here we report an extended family co-segregating autosomal dominant, postlingual-onset, progressive, sensorineural hearing loss (SNHL) with a novel frameshift mutation, 1490insAA, of *EYA4*. The 1490insAA allele is predicted to encode a truncated protein with an intact N-terminal variable region, but lacking the entire C-terminal Eya domain. Clinical studies including electrocardiography, echocardiography, and magnetic resonance imaging (MRI) of the heart in nine affected family members revealed no DCM or associated abnormalities and confirmed their nonsyndromic phenotype. These are the first definitive cardiac evaluations of DFNA10 hearing loss to support a correlation of *EYA4* mutation position with the presence or absence of DCM. These results will facilitate the counseling of patients with these phenotypes and *EYA4* mutations.
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INTRODUCTION

Nonsyndromic autosomal dominant sensorineural hearing loss (SNHL) is genetically heterogeneous, with more than 40 distinct loci that have been mapped. Many of these loci, termed DFNA loci, have been positionally cloned [Friedman and Griffith, 2003]. For example, mutations in the eyes absent 4 (*EYA4*) gene cause hearing loss at the DFNA10 locus on chromosome 6q23 [Wayne et al., 2001]. The function of *EYA4* is unknown, although it has sequence similarity to the *EYA1* gene on chromosome 8q13.3, which has been implicated in transcriptional regulation of inner ear development [Zhang et al., 2004]. Dominant mutations of *EYA1* cause ear malformations and hearing loss as part of the branchio-oto-renal (BOR) syndrome [Abdelhak et al., 1997].

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The *EYA4* gene product has two distinct domains: an amino-terminal variable region followed by a carboxy-terminal Eya domain. Two different truncating *EYA4* mutations have been identified in American and Belgian families segregating DFNA10 hearing loss without other reported clinical features [Wayne et al., 2001]. A third truncating mutation of *EYA4* co-segregates with hearing loss in a Hungarian family for which the presence or absence of syndromic associations was not mentioned [Pfister et al., 2002]. These three mutations of *EYA4* are 1468insAA (American family), 2200C > T (a nonsense mutation in the Belgian family), and 1558insTTTG (Hungarian family), all of which partially or completely delete the Eya domain. The only other reported *EYA4* mutation (E193) is a 4,846-bp genomic deletion that results in loss of the Eya domain as well as part of the variable region [Schonberger et al., 2005]. E193 co-segregates with hearing loss and dilated cardiomyopathy (DCM) in a single large family, suggesting that truncations affecting only the Eya domain cause SNHL alone whereas truncations affecting the variable region lead to SNHL and DCM [Schonberger et al., 2005]. In vitro studies of mutant EYA4 proteins provide functional evidence for this correlation of mutation position with cardiac phenotype [Schonberger et al., 2005].

However, cardiomyopathy displays age-related penetrance that becomes symptomatic later than SNHL in the family segregating E193 as well as in a second family segregating SNHL and cardiomyopathy associated with a *MYO6* mutation [Mohiddin et al., 2004]. Therefore a cardiomyopathy phenotype may have been overlooked in the DFNA10 families [O'Neill et al., 1996; Verhoeven et al., 2000; Pfister et al., 2002] since they were described before the association of *EYA4* with DCM had been established [Schonberger et al., 2005]. Here we report a family co-segregating dominant hearing loss and a novel truncating mutation of *EYA4* that deletes the Eya domain. Comprehensive clinical evaluations confirm a nonsyndromic DFNA10 phenotype and support the correlation of cardiac phenotype with *EYA4* mutation position.

MATERIALS AND METHODS

Subjects

The study subjects were 8 male and 11 female members of LMG265, a North American Caucasian family of mixed European ancestry (Fig. 1). This study was approved by an Institutional Review Board at the National Institutes of Health (National Institute

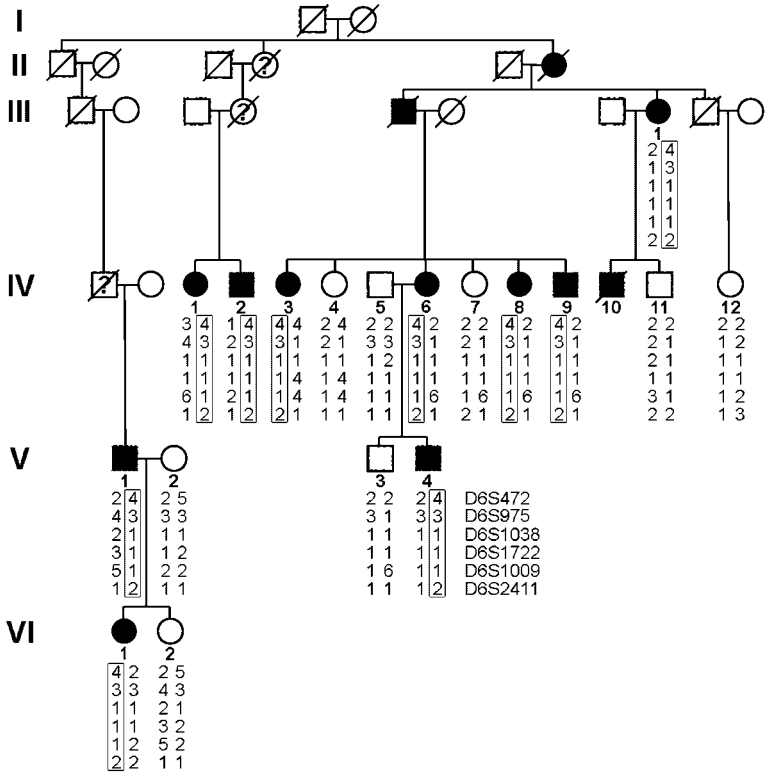

FIG. 1. North American Caucasian family LMG265. LMG265 co-segregates autosomal dominant sensorineural hearing loss (filled symbols) with a haplotype (boxed) of short tandem repeat marker genotypes at the DFNA10 locus on chromosome 6q23. The 19 participating family members are identified with bold Arabic numerals. Individual IV-10 was anamnestically reported to be affected but did not undergo genotype analysis.

of Neurological Disorders and Stroke and the National Institute on Deafness and Other Communication Disorders). Written informed consent was obtained from all subjects and parents of minor subjects.

Genotype Analysis

Genomic DNA was extracted from peripheral blood samples using PureGene (Gentra Systems, Minneapolis, MN). Genotypes of microsatellite markers at known DFNA loci (see the online Supplementary Table I at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html) were analyzed and all 20 coding exons and flanking intronic regions of *EYA4* were PCR-amplified for bidirectional nucleotide sequence analysis as described [Bork et al., 2001]. Genomic DNA fragments were PCR-amplified with primer pairs shown in Supplementary Table II (see the online Supplementary Table II at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html), KOD Hot Start DNA Polymerase (Novagen, San Diego, CA), and the following cycling parameters: initial denaturation at 95°C for 2 min, 35 step-cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec, extension at 68°C for 1.5 min, with a final extension at 68°C for 5 min. Exon 12 amplification products were subcloned and sequenced in unaffected and affected subjects. Ninety-six genomic DNA samples from unrelated, ethnically matched (Caucasian) controls were obtained from Coriell Cell Repositories (Camden, NJ).

Phenotype Analysis

We defined affected auditory phenotype status as an air-conduction pure-tone (1, 2, and 4 kHz) threshold average greater than 30 dB HL on the subject's most recent audiogram. Nine subjects with the *EYA4* mutation, 1490insAA, underwent evaluations at the NIH Clinical Center including general medical and developmental history interviews and physical examinations, interviews and examinations by cardiology consultants, pure-tone and speech audiometry, middle ear immittance testing, videonystagmography including caloric testing, magnetic resonance imaging (MRI) of the inner ears and temporal bones, genetic counseling, electrocardiography, echocardiography, chest X-ray, and cardiac MRI.

Statistical Analysis

We estimated age-related progression of hearing loss as the slope of a simple linear regression of hearing threshold and age using statistiXL version 1.6 (downloaded from http://www.statistixl.com/) with the Windows™ version of Microsoft Excel™. The same software was used to compare regression slopes and intercepts among genders and families by analysis of variance (ANOVA).

RESULTS

LMG265 Auditory Phenotype

LMG265 is a North American Caucasian family of mixed European ancestry segregating autosomal dominant SNHL (Fig. 1). There are no extra-auditory phenotypes co-segregating with the SNHL. The SNHL was anamnestically reported to start during the second to fourth decade of life, primarily beginning in the middle and high frequencies, and progressing to moderate to severe levels affecting the entire frequency range (Fig. 2). The SNHL at 0.5, 1, 2, and 4 kHz was more severe in males compared to females ($P < 0.05$) but there were no differences ($P > 0.05$) in the rates of progression (Fig. 4A). Word recognition scores (not shown) are within expected ranges for the degree of hearing loss when the hearing loss is of cochlear origin [Yellin et al., 1989]. Normal tympanometry and acoustic reflex test results are indicative of normal middle ear function (not shown).

In comparison with the Belgian DFNA10 carriers of 2200C > T [Verstreken et al., 2000], the 1490insAA carriers had slower progression of hearing loss ($P < 0.05$) (Fig. 4B). We were unable to extract accurate pure-tone threshold average data for the other published families segregating *EYA4* mutations [Schonberger et al., 2000; Verstreken et al., 2000; De Leenheer et al., 2001].

We performed videonystagmography on six affected individuals (IV-1, IV-2, IV-3, IV-6, V-1, V-4) who reported episodes of dizziness and one affected individual (VI-1) who denied any history of dizziness. Subjects IV-1, IV-3, and V-1 had normal findings on videonystagmography. Subject IV-2 reported two remote episodes of brief, self-limiting vertigo while supine. He had a positive response to the Dix-Hallpike maneuver with the right ear down, indicative of benign paroxysmal positional vertigo affecting the right ear. Caloric testing of IV-6 elicited a borderline reduced left-sided response to caloric irrigations and a right directional preponderance suggestive of a left-sided peripheral vestibular weakness. Both V-4 and VI-1 had borderline reduced left-sided responses to caloric irrigations suggestive of a potential left peripheral vestibular pathology.

Magnetic resonance images of the temporal bones of four affected individuals (IV-1, IV-3, IV-6, V-4) revealed no structural malformations (not shown).

LMG265 Genotype

Genotype analysis of microsatellite markers at known autosomal dominant loci (see the online

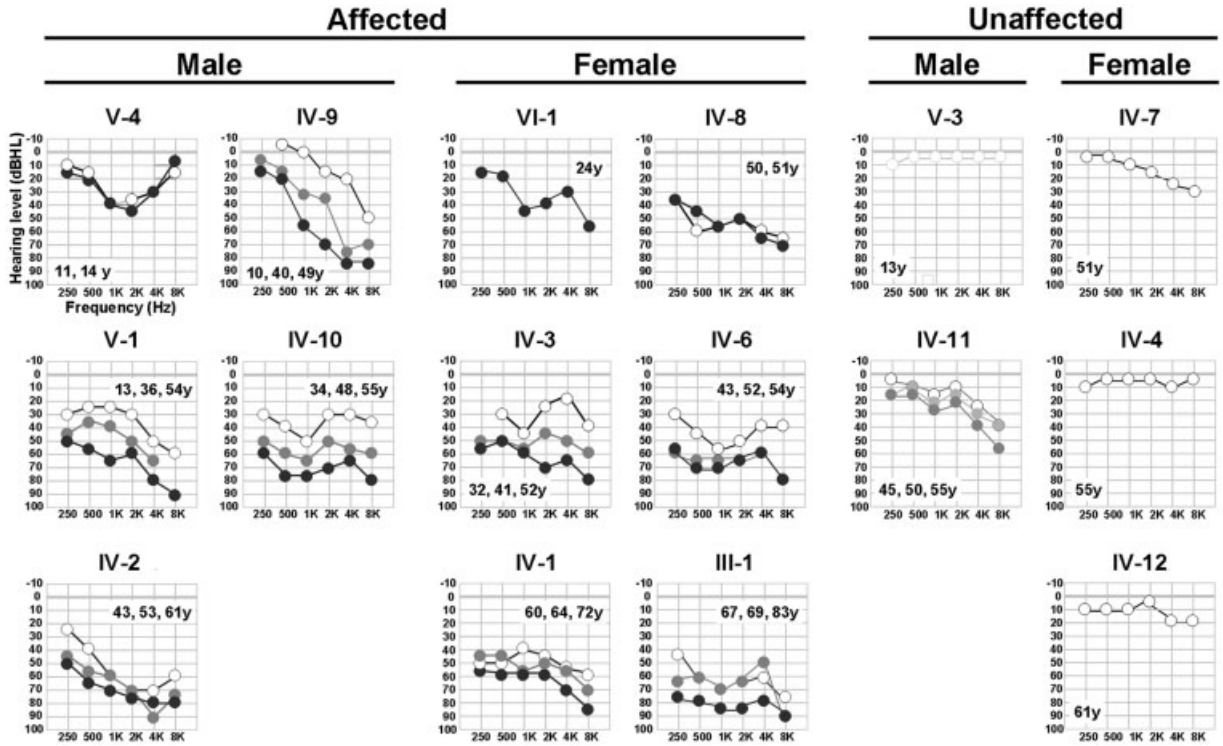

FIG. 2. LMG265 audiometric phenotype. Pure-tone air conduction thresholds are shown for the better-hearing ears of LMG265 family members. Three representative time points are shown when ≥3 audiograms were available. Open, gray, and black circles show thresholds for the indicated ages, from youngest to oldest, respectively. Bone conduction thresholds were consistent with sensorineural hearing loss. The hearing loss in individuals IV-7 and IV-11 is consistent with hearing levels associated with presbycusis in the general population.

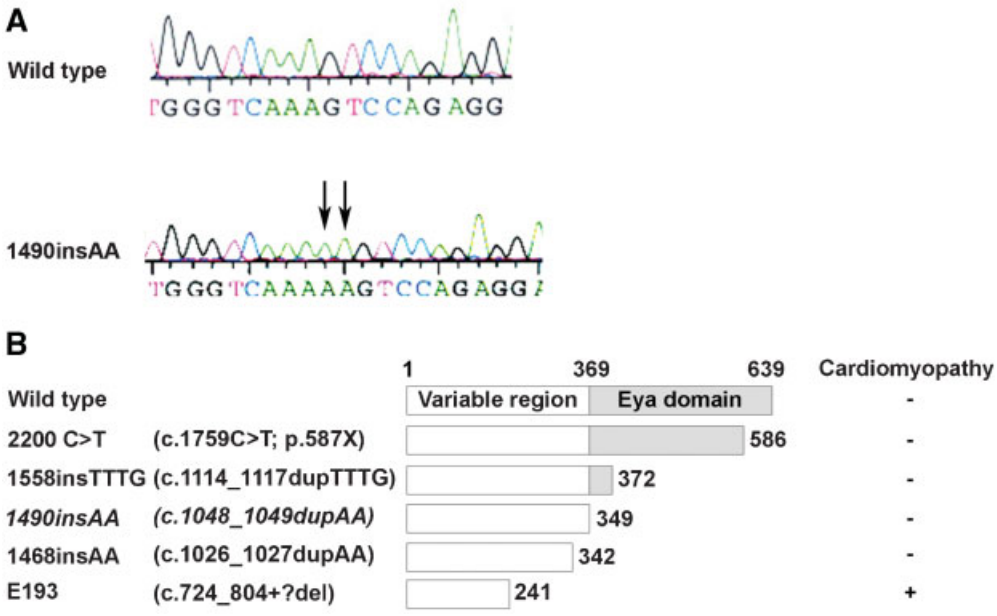

FIG. 3. *EYA4* genotype. **A**: Electropherograms show wild-type *EYA4* sequence from an unaffected family member and the 1490insAA mutation (arrows) in a subcloned genomic PCR amplification product from an affected LMG265 family member. All affected family members were heterozygous for 1490insAA. **B**: Effects of known *EYA4* mutations on EYA4 protein structure and the cardiac phenotype. The number of amino acids of each allele product is indicated. Mutations that truncate the C-terminal Eya domain are associated with DFNA10 hearing loss and a normal cardiac phenotype, whereas E193 truncates the N-terminal variable region and results in hearing loss plus dilated cardiomyopathy. Recommended mutation nomenclature (Human Genome Variation Society) is shown in parentheses.

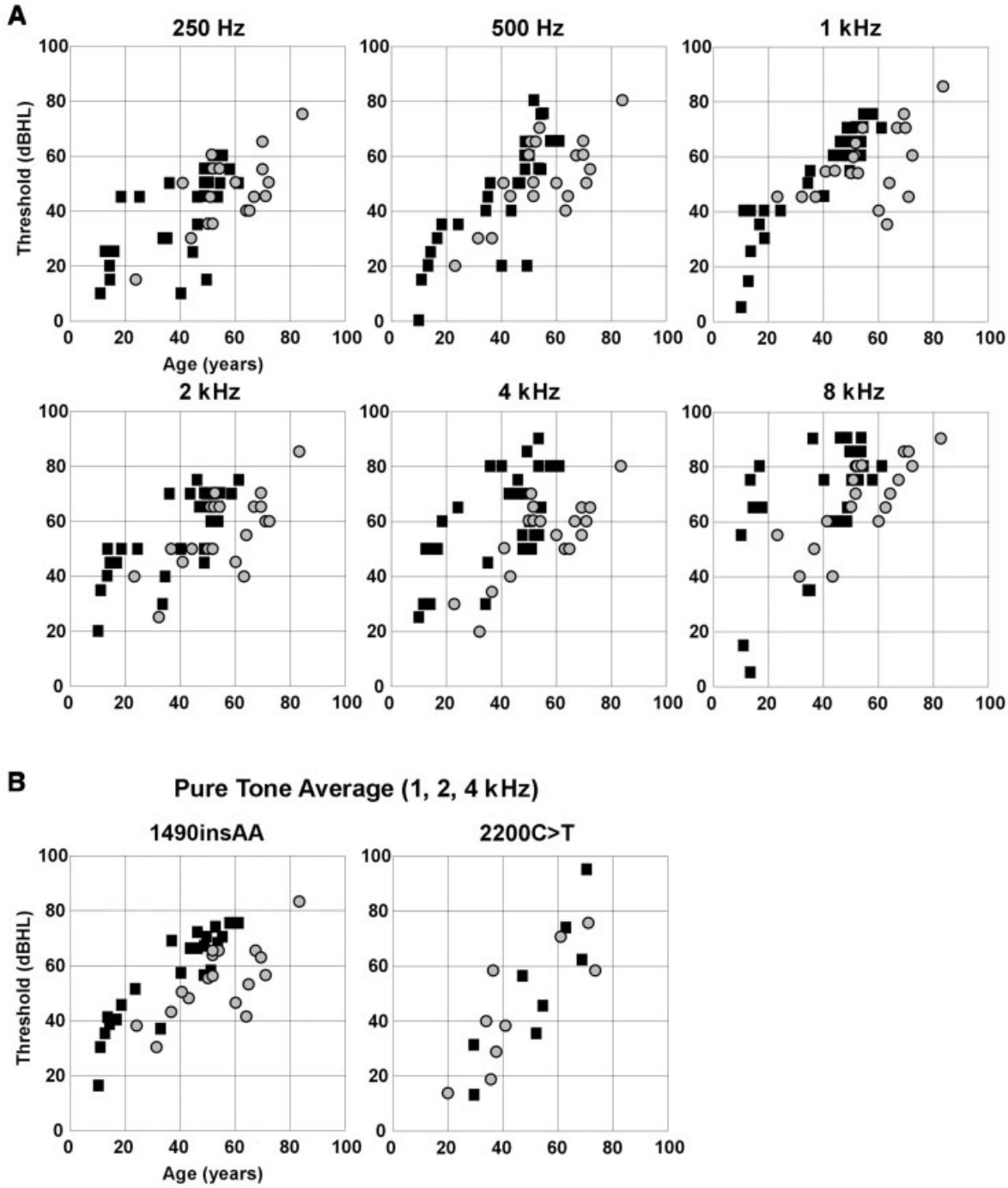

FIG. 4. Comparison of *EYA4* mutant auditory phenotypes. **A**: Pure-tone air conduction thresholds for the better hearing ears of affected male (black squares) and female (gray circles) family members of LMG265 are plotted against age for each stimulus frequency (indicated above each graph). Females had better hearing than males at 0.5, 1, 2, and 4 kHz ($P < 0.05$). **B**: Pure-tone (1, 2, and 4 kHz) threshold averages plotted against age for individuals carrying the DFNA10 mutations 1490insAA (this study) and 2200C > T [Verstreken et al., 2000]. The 1490insAA carriers had slower progression in comparison with the carriers of 2200C > T ($P < 0.05$). The male carriers of 1490insAA had more severe SNHL than female carriers ($P < 0.05$), whereas there was no gender-specific difference in hearing associated with 2200C > T ($P > 0.05$).

Supplementary Table I at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html) demonstrated co-segregation of SNHL with markers at the DFNA10 locus on chromosome 6q23 (Fig. 1) and no other DFNA loci (not shown). LMG265 family member IV-11 was excluded from the initial linkage screen because he had a pure-tone average of 28.3 dB HL at 55 years of age (Fig. 2) and his phenotype status could not confidently be assigned. Since mutations of *EYA4* had previously been shown

TABLE I. LMG265 Cardiac Phenotype

Subject	Age (years)	Gender	Blood pressure	Blood lipids	Electrocardiography	Echocardiography	Cardiac MRI
IV-1	73	Female	High	High	Early precordial transition	Aortic regurgitation, tricuspid regurgitation	Aortic insufficiency, mitral regurgitation, tricuspid regurgitation
IV-2	61	Male	High	High	Normal	Aortic regurgitation, inferior wall hypokinesis	Not tested
IV-3	57	Female	Normal	High	Normal	Normal	Not tested
IV-6	54	Female	High	High	1° atrioventricular block, left atrial abnormality	Normal	Normal
IV-8	51	Female	Normal	High	Normal	Normal	Normal
IV-9	49	Male	High	High	Nonspecific T wave abnormality, right ventricular conduction delay	Mild concentric hypertrophy	Normal
V-1	54	Male	High	High	rSR', early precordial transition, inferior T wave abnormality	Normal	Mild left atrial enlargement
V-4	14	Male	Normal	High	Normal	Normal	Not tested
VI-1	23	Female	Normal	Normal	Normal	Normal	Not tested

to cause DFNA10 hearing loss [Wayne et al., 2001], we PCR-amplified and sequenced all 20 exons and flanking intronic sequences of *EYA4* from genomic DNA. We detected a heterozygous insertion of AA at position 1490 (1490insAA) that co-segregated with SNHL in LMG265. We confirmed the mutation by nucleotide sequence analysis of subcloned amplification products of exon 12 from affected subjects (Fig. 3A). We did not detect 1490insAA in unaffected LMG265 members (including IV-11) or 96 ethnically matched control DNA samples. The 1490insAA allele is predicted to encode a truncated EYA4 protein with an intact variable domain and a deleted Eya domain (Fig. 3B).

LMG265 Cardiac Phenotype

Comprehensive cardiac evaluation of nine affected LMG265 members revealed a variety of abnormalities, including hyperlipidemia and hypertension (not shown), but no evidence of DCM (Table I). The abnormalities we detected are also reported to be present in unaffected family members (data not shown) and, given their high prevalence in the general population, are likely to be unrelated to 1490insAA or SNHL.

DISCUSSION

We identified a novel frameshift mutation, 1490insAA, of *EYA4* co-segregating with dominant hearing loss at the DFNA10 locus in family LMG265. Our study comprises the first detailed cardiac evaluation of DFNA10 hearing loss to support the proposed correlation of *EYA4* mutation position with the presence or absence of DCM [Schonberger et al., 2005]. We cannot rule out the possibility that this is a spurious correlation arising from interfamilial differences in genetic or environmental modifiers of the cardiomyopathy phenotype.

The postlingual-onset, progressive SNHL phenotype segregating in family LMG265 is similar to those which have been reported for DFNA10 in the American (1468insAA) and Belgian (2200C > T) families [Verstreken et al., 2000; De Leenheer et al., 2001]. The affected members of the Belgian family did not show ($P > 0.05$) the gender difference in severity of SNHL that we observed in LMG265. It is possible that our observation was spurious, but we cannot rule out sex-linked genetic or environmental factors modifying the DFNA10 phenotype in LMG265. Similarly, our observation of small but significant differences in hearing loss associated with 1490insAA versus 2200C > T may also be spurious, but could reflect a correlation with *EYA4* genotype.

The vestibular findings in LMG265 family members IV-2, IV-6, V-4, and VI-1 may or may not be direct effects of the *EYA4* mutation since they were unilateral in those individuals and absent in affected relatives. The significance of the caloric response variations in IV-6, V-4, and VI-1 and their causal relationship to DFNA10 are even less clear.

The results of our study can now be used to guide the molecular diagnosis and genetic counseling of patients with these phenotypes and *EYA4* mutations.

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