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A common *SLC26A4*-linked haplotype underlying non-syndromic hearing loss with enlargement of the vestibular aqueduct

Parna Chattaraj[1], **Tina Munjal**[1], **Keiji Honda**[1], **Nanna D Rendtorff**[2,3,4], **Jessica S Ratay**[1], **Julie A Muskett**[1], **Davide S Risso**[5], **Isabelle Roux**[1], **E Michael Gertz**[6], **Alejandro A Schäffer**[6], **Thomas B Friedman**[7], **Robert J Morell**[8], **Lisbeth Tranebjærg**[2,3,4], and **Andrew J Griffith**[1]

[1]Otolaryngology Branch, National Institute on Deafness and Other Communication Disorders (NIDCD), Bethesda, Maryland, USA

[2]Institute of Clinical Medicine, University of Copenhagen, The Panum Institute, Copenhagen, Denmark

[3]Department of Otorhinolaryngology, Head & Neck Surgery and Audiology, Rigshospitalet, Copenhagen, Denmark

[4]Department of Clinical Genetics, Rigshospitalet/The Kennedy Center, Glostrup, Denmark

[5]Laboratory of Communication Disorders, NIDCD, Bethesda, Maryland, USA

[6]Computational Biology Branch, National Center for Biotechnology Information, Bethesda, Maryland, USA

[7]Laboratory of Molecular Genetics, NIDCD, Bethesda, Maryland, USA

[8]Genomics and Computational Biology Core, NIDCD, Bethesda, Maryland, USA

Abstract

Background—Enlargement of the vestibular aqueduct (EVA) is the most common radiological abnormality in children with sensorineural hearing loss. Mutations in coding regions and splice sites of the *SLC26A4* gene are often detected in Caucasians with EVA. Approximately one-fourth of patients with EVA have two mutant alleles (M2), one-fourth have one mutant allele (M1) and one-half have no mutant alleles (M0). The M2 genotype is correlated with a more severe phenotype.

Correspondence to: Dr Andrew J Griffith, Otolaryngology Branch, National Institute on Deafness and Other Communication Disorders, 35A Convent Drive, Rm. GF-103, Bethesda, MD 20892, USA; griffita@nidcd.nih.gov.
PC, TM and KH contributed equally.

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Methods—We performed genotype–haplotype analysis and massively parallel sequencing of the *SLC26A4* region in patients with M1 EVA and their families.

Results—We identified a shared novel haplotype, termed CEVA (Caucasian EVA), composed of 12 uncommon variants upstream of *SLC26A4*. The presence of the CEVA haplotype on seven of ten 'mutation-negative' chromosomes in a National Institutes of Health M1 EVA discovery cohort and six of six mutation-negative chromosomes in a Danish M1 EVA replication cohort is higher than the observed prevalence of 28 of 1006 Caucasian control chromosomes ($p<0.0001$ for each EVA cohort). The corresponding heterozygous carrier rate is 28/503 (5.6%). The prevalence of CEVA (11 of 126) is also increased among M0 EVA chromosomes ($p=0.0042$).

Conclusions—T he CEVA haplotype causally contributes to most cases of Caucasian M1 EVA and, possibly, some cases of M0 EVA. The CEVA haplotype of *SLC26A4* defines the most common allele associated with hereditary hearing loss in Caucasians. The diagnostic yield and prognostic utility of sequence analysis of *SLC26A4* exons and splice sites will be markedly increased by addition of testing for the CEVA haplotype.

Introduction

Enlargement of the vestibular aqueduct (EVA (MIM#6 00 791)) is the most common radiological malformation associated with childhood sensorineural hearing loss.[1] Hearing loss and EVA can be non-syndromic (NSEVA) or associated with an iodine organification defect that can lead to goitre as part of Pendred syndrome (PS (MIM#2 74 600)).[23] Both phenotypes can be associated with recessive mutant alleles of the *SLC26A4* gene (MIM#605646) on chromosome 7. Approximately one-fourth of North American or European Caucasians with NSEVA or PS have homozygous or compound heterozygous mutations in the coding regions or splice sites of *SLC26A4*, as expected for recessive inheritance.[23] We refer to this genotype as 'M2', in contrast to the one-half of patients with EVA with no detectable mutations (M0) or the one-fourth of patients with only one allele with a detectable mutation of *SLC26A4* (M1).[4]

The number of mutant alleles of *SLC26A4* is strongly correlated with the auditory and thyroid phenotypes. The severity of hearing loss in EVA ears is greater in M2 patients than in M0 or M1 patients.[56] With rare exceptions, M2 patients exhibit bilateral EVA, while patients with unilateral EVA are almost always M1 or M0.[2378] Moreover, the iodine organification defect underlying the development of thyroid goitre is directly correlated with the M2 genotype.[379]

The probability (approximately one in four) of EVA among siblings of M1 probands is not significantly different from the probability of EVA in siblings of M2 probands, suggesting the inheritance of an undetected recessive mutation of *SLC26A4* in M1 EVA subjects.[4] Two previous studies proposed that EVA could be caused by digenic inheritance of a pathogenic variant of *FOXI1* (MIM#601093) or *KCNJ10* (MIM#602208) in combination with a mutation of *SLC26A4*.[10–12] However, the methodologies did not permit definitive conclusions, and subsequent studies have been unable to replicate the findings.[13–15] Alternatively, we have consistently observed co-segregation of *SLC26A4*-linked short tandem repeat markers with EVA in M1 sibships, suggesting the existence of an undetected

pathogenic variant(s) on the allele that has no mutation in the exons or splice sites of *SLC26A4*.[4] The purpose of our current study was to explore this hypothesis in a National Institutes of Health (NIH) discovery cohort and a Danish replication cohort of patients with M1 EVA.

Methods

Study and subjects

The current study was approved by the Combined Neuroscience Institutional Review Board, NIH, Bethesda, Maryland, USA. Danish subjects were studied in accordance with approval from the Danish Research Ethical Committee (KF 01-108/03, 11-112/04, KF120/03). Written informed consent was obtained for all subjects. NIH subjects were evaluated at the NIH Clinical Center as described.[35–916] Some subjects have been previously reported.[36–81617] We originally defined a vestibular aqueduct as enlarged if the midpoint diameter was >1.5 mm or if it had a grossly malformed overall morphology,[3] but we later modified the midpoint diameter criterion to >1.0 mm for the NIH cohort.[16] The midpoint diameter criterion was >1.5 mm for the Danish cohort. To distinguish between PS and NSEVA, the thyroid gland was evaluated by serological tests of thyroid function, ultrasonographic examination of thyroid volume and texture, and perchlorate discharge testing.[9] Results were interpreted according to published objective criteria.[9] We classified the pathogenicity of *SLC26A4* variants based on the American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) variant interpretation guidelines.[1819] Based on these criteria, we classified EVA subjects as M2, M1 or M0 if they carried two, one or zero mutations in the coding regions or splice sites of *SLC26A4*.[4] We classified a variant as indeterminate if it is benign in *trans* with an allele with no mutations but pathogenic in *trans* with a mutant allele of *SLC26A4*.[7] There were 34 M2 subjects, 15 M1 subjects and 83 M0 subjects in the NIH cohort.

Massively parallel sequencing

We used Agilent SureDesign to design biotinylated RNA probes targeting the smallest region of overlap (SRO) and subsequently the shared chromosomal segments defined by homozygosity haplotype mapping. The designs achieved 95% coverage of targeted intervals. Library preparation and capture were prepared using SureSelect^{XT} Target Enrichment System (see additional details in online supplementary material). Sequences were aligned to the hg19 version of the human genome using Burrows-Wheeler Aligner (version 0.7.13).[20] Variants were called using the best-practices pipeline for GATK variant analysis package (version 3.5).[2122] After deduplication, the average read coverage over the targeted region was 234×. ANNOVAR[23] software was used to annotate predicted pathogenicity, allele frequency in reference populations and dbSNP number (see online supplementary methods).

Statistical analyses

We used GraphPad Prism 7 (http://www.graphpad.com/) to perform a two-tailed Fisher's exact test to compare the prevalence of the CEVA (Caucasian EVA) haplotype, as defined by genotypes of all 12 variants, among chromosomes from different cohorts (see online supplementary methods) and from the 1000 Genomes database (http://phase3browser.

1000genomes.org/index.html). For EVA cohorts, identical-by-descent chromosomes within a single family were counted as only one chromosome for the comparisons (see online supplementary methods).

Homozygosity haplotype mapping

For each sibling pair in the NIH cohort, we identified homozygous SNPs throughout the region until we reached a SNP at each end of the region that was discordant within that pair of affected siblings.[24] We then performed pairwise comparisons of homozygous SNP genotypes between all possible combinations of sibling pairs.

In silico prediction of pathogenic potential

We used Combined Annotation Dependent Depletion (CADD; http://cadd.gs.washington.edu/) to predict the pathogenic potential of sequence variants. CADD integrates a range of diverse annotations into a single measure (C-score) of the likelihood of deleteriousness for each variant.[25] The scaled C-scores are based on the rank of the C-score of any variant relative to those of 8.6 billion possible single nucleotide variants from the human genome.[25]

Results

SLC26A4-linked haplotype shared among M1 EVA chromosomes

Our strategy was based on the hypothesis that many M1 EVA families co-segregate the same undetected pathogenic variant affecting the expression of an allele of *SLC26A4* that has no detectable mutations in the coding regions or splice sites of this gene. We henceforth refer to these chromosomes as ‘mutation-negative.’

We performed a haplotype analysis of short tandem repeat markers (see online supplementary methods) spanning the *SLC26A4* region of chromosome 7 in six NIH sibling pairs affected with EVA. Five were M1 families (142, 156, 242, 280 and 380), and one family (147) was initially included with the M1 cohort but was later moved to the M0 cohort based on reclassification of p.Met775Thr as a variant of indeterminate pathogenic potential.[7] We identified a centromeric meiotic breakpoint on the mutation-negative chromosome in family 380 and a telomeric breakpoint on the mutation-negative chromosome in family 242 (figure 1A). These breakpoints define an 8.218-Mb SRO of the mutation-negative chromosomes co-segregating with EVA in the six sibling pairs.

We identified 23 159 variants by massively parallel sequencing (MPS) of the entire SRO in the six sibling pairs. There are 16 820 variants, located in non-repetitive genomic regions, which co-segregate among any one or more affected sibling pairs. We searched for variants with a minor allele frequency (MAF) ≤0.05 that are shared among the six mutation-negative chromosomes. We used MAFs calculated for the Caucasian-European superpopulation that includes CEU (Utah residents with Northern and Western European ancestry), TSI (Toscani in Italy), FIN (Finnish in Finland), GBR (British in England and Scotland) and IBS (Iberian populations in Spain) populations. The Caucasian-European population has 1785 variants with MAF ≤0.05 in the SRO. None of the 1785 variants are shared among all six families,

but 12 variants (table 1) with MAFs between 0.019 and 0.049 are shared by five of six families (142, 147, 156, 280 and 380, but not 242). This indicates that the pathogenic variant(s) in family 242 may be different than that (those) in the other five families. Since the telomeric breakpoint of the original SRO was identified in family 242, the actual SRO for the other five families extends further toward the telomere than shown in figure 1A. The 12 variants include ten single-nucleotide substitutions and two single-nucleotide deletions spanning a 613-kb region upstream of *SLC26A4*. Some are located in intergenic regions, and the others are found within introns of other genes (figure 1B). We confirmed the genotypes of all 12 variants by Sanger sequencing. Sequencing of parental DNA samples demonstrated that the 12 variants are in *cis* with each other and in *trans* with the known mutation(s) of *SLC26A4*. The 12 variants thus comprise a previously unrecognised haplotype that we refer to as the CEVA haplotype.

To understand the haplotype structure of the region containing the CEVA haplotype and *SLC26A4*, we used Haploview to generate a linkage disequilibrium (LD) map (see online supplementary methods) of all SNPs with MAF >0.01 among 503 unrelated Caucasian-European individuals (figure 1C). The 12 CEVA variants are located within a single region of LD that spans from upstream of the *PRKAR2B* gene to intron 3 of *SLC26A4*. Pairwise comparison of the 12 variants showed that they are all in modest LD (r^2 >0.6) with each other (figure 1C, see online supplementary table 1).

Association of *SLC26A4*-linked CEVA haplotype with M1 EVA

We queried the 1000 Genomes database for the prevalence of the 12-variant CEVA haplotype in the Caucasian-European and other superpopulations. Of a total of 1006 Caucasian-European alleles, 947 had the reference haplotype, and 28 had the CEVA haplotype (table 2). Twenty-two other alleles were composed of different combinations of CEVA and reference genotypes (table 2). The prevalence of the CEVA haplotype is lower in other superpopulations: 1 (0.0756%) of 1322 African chromosomes, 11 (1.59%) of 694 Admixed American Chromosomes, zero of 1008 East Asian chromosomes and 1 (0.102%) of 978 South Asian chromosomes (table 2).

In order to determine the prevalence of the CEVA haplotype among all M1 mutation-negative chromosomes in the NIH cohort, we analysed five additional M1 EVA subjects from simplex families (table 3). Three of the five subjects have the CEVA haplotype in *trans* with their *SLC26A4* mutation. One subject (1580) had the reference haplotype, and one subject (1805) had the reference-CEVA combination haplotype GTTCATG-GC-C (CEVA genotypes are underlined; table 2). Taken together with the familial M1 EVA data, seven of ten M1 mutation-negative chromosomes have the CEVA haplotype (table 3). This is significantly different from the observed prevalence (28/1006) in Caucasian-European controls ($p<0.0001$).

Interestingly, the mixed reference-CEVA haplotype in subject 1805 was present on one chromosome, of Finnish ancestry, among the 1006 Caucasian-European alleles in the 1000 Genomes database (table 2). The different haplotype in subject 1805 may indicate the existence of a different pathogenic variant than is present in *cis* with the 12-variant CEVA haplotype in other M1 subjects. Alternatively, it could refine the pathogenic CEVA

haplotype to the nine telomeric variants (figure 1C, table 2). The nine telomeric variants are located in their own region of higher LD adjacent to and including the 5′ end of *SLC26A4* (figure 1C; see online supplementary table 1).

We then determined the prevalence of the 12-variant CEVA haplotype in a Danish replication cohort of six unrelated M1 EVA subjects. Six of the six Danish M1 mutation-negative chromosomes carry the CEVA haplotype in *trans* with a pathogenic variant of *SLC26A4* (table 3). This is significantly different from the observed prevalence of the CEVA haplotype in Caucasian-European controls ($p<0.0001$). We conclude that there is a specific association of the CEVA haplotype with EVA in M1 subjects with a mutation of the *trans* allele of *SLC26A4*.

To explore the penetrance of the CEVA haplotype in *trans* with an *SLC26A4* mutation, we analysed all unaffected members of M1 families in the NIH and Danish cohorts. We identified two unaffected individuals, one from the NIH cohort (2086, family 380) and one from the Danish cohort (15 283–14), who have the CEVA haplotype in *trans* with a mutation of *SLC26A4*. NIH subject 2086 had normal audiometric test results at the age of 10 years and no evidence of EVA by MRI. Danish subject 15 283–14 had normal audiometric test results at the age of 22 years and did not undergo any temporal bone imaging studies. Seventeen (85%) of 19 subjects in the NIH and Danish cohorts who have the CEVA haplotype in *trans* with a mutation of *SLC26A4* thus have hearing loss and EVA (table 3). Therefore, the penetrance of the CEVA haplotype in *trans* with an *SLC26A4* mutation is incomplete. This result contrasts with the complete penetrance of EVA in homozygous or compound heterozygous carriers of mutations affecting splice sites or exons of *SLC26A4*.[3–68]

We also determined the prevalence of the 12-variant CEVA haplotype among M2 EVA chromosomes from the NIH cohort. Since all of the M2 chromosomes already have an *SLC26A4* mutation, we hypothesised that the prevalence of the CEVA haplotype would not be increased in comparison to normal control chromosomes. Four of 54 NIH M2 chromosomes have the CEVA haplotype, which is not different ($p=0.0749$) from the prevalence among Caucasian-European controls. We obtained a similar result ($p=0.0871$) using a non-conservative estimate (see ‘Statistical analyses’ in the Methods section) of the total number (57) of M2 chromosomes. When we excluded chromosomes from subjects whose ethnicity was either unknown, multiracial or not Caucasian, we obtained the same results ($p=0.06$) for both conservative and non-conservative estimates of numbers of chromosomes, respectively. When we compared the prevalence of the CEVA haplotype among M1 chromosomes versus M2 chromosomes in the NIH cohort, the difference was statistically significant irrespective of whether we employed conservative or non-conservative estimates of the number of M2 chromosomes ($p<0.0001$). These results further support the specific association of the CEVA haplotype with EVA in M1 subjects.

Association of *SLC26A4*-linked CEVA haplotype with M0 EVA

Since Mendelian inheritance is a rare pathogenic mechanism for M0 EVA,[4] we hypothesised that the prevalence of the CEVA haplotype would be the same or only slightly increased among M0 EVA chromosomes in comparison to Caucasian-European controls. However, we

observed that 11 of 126 chromosomes from the NIH M0 cohort carry the 12-variant CEVA haplotype, which is significantly higher (p=0.0042) than the observed prevalence among Caucasian-European controls. When we excluded chromosomes from subjects whose ethnicity was either unknown or not Caucasian, we obtained similar results (p=0.0129 and p=0.0117) for conservative and non-conservative estimates of numbers of chromosomes, respectively. One possible reason for this observation is that homozygosity for the CEVA haplotype can cause EVA. Indeed, four of 84 M0 EVA subjects in the NIH cohort are homozygous for the CEVA haplotype. One CEVA haplotype homozygote (subject 1693, family 258) is a singleton with an indeterminate thyroid phenotype.[7] Two CEVA haplotype homozygotes are dizygotic twins (subjects 1702 and 1703, family 147) who each carry one of their CEVA haplotypes in *cis* with an *SLC26A4* variant (p.Met775Thr) with residual pendrin activity (50%–60% of wild type).[7] p.Met775Thr is a hypomorphic 'indeterminate' variant previously proposed to be pathogenic in *trans* with a mutation of *SLC26A4*.[7] It seems likely that the combination of p.Met775Thr with homozygosity for the CEVA haplotype is aetiologic in these subjects. The fourth CEVA haplotype homozygote (subject 2246, family 443) is the oldest of three affected siblings.[16] His father (subject 2244, family 443) and siblings (subjects 2247 and 2248, family 443) are heterozygous for the CEVA haplotype. The discordant segregation of EVA with homozygosity for the CEVA haplotype suggests the existence of other factors, genetic or non-genetic, which contribute to the aetiology of EVA in family 443 (online supplementary figure 1).

There are seven heterozygotes for the CEVA haplotype among five NIH M0 families, but the CEVA haplotype does not co-segregate with EVA in four of the five families (online supplementary figure 1). The EVA subject in the fifth family has no siblings, so co-segregation cannot be assessed. Therefore, a Mendelian contribution of heterozygosity for the CEVA haplotype to EVA seems unlikely in M0 subjects.

In addition to subjects 1702 and 1703 of family 147, there are 21 additional M0 EVA subjects from 17 families in the NIH cohort that carry an *SLC26A4* coding or splice site variant whose pathogenic potential is indeterminate or is thought to be pathogenic only in *trans* with a coding or splice site mutation of *SLC26A4* (table 4). Two of the 23 subjects are black, and two are multiracial. None of the 21 subjects carry the CEVA haplotype, extending our previous conclusion that their *SLC26A4* variants are either benign or pathogenic only in *trans* with a mutation of exons or splice sites of *SLC26A4*.[7] Moreover, this result further demonstrates that the association of the CEVA haplotype with EVA in M1 subjects is specific.

Other *SLC26A4*-linked haplotypes in M0 EVA subjects

Four M0 subjects from three families have haplotypes with a combination of reference and CEVA genotypes. Two affected monozygotic twins in family 379 have the reference haplotype GTTTGCATATTA and a CEVA-reference combination haplotype ACACATG-GTTA (CEVA genotypes are underlined) which is present in nine European chromosomes in 1000 Genomes (table 2). A third subject (1422) has the reference haplotype and the CEVA-reference combination haplotype GTTTGTG-GT-A. This haplotype was not observed in 1000 Genomes (table 2). The reference-CEVA combination haplotype in these three subjects

may be coincidental and unrelated to EVA, since they were not present in *trans* with a mutation or another CEVA haplotype. In contrast, another subject (1470) has two reference-CEVA combination haplotypes, GTTCATG-GC-C and GCACATG-GC-C. The former haplotype is composed of the nine telomeric variants of the CEVA haplotype and is identical to that observed in M1 subject 1805 and a single Finnish chromosome in 1000 Genomes (table 2). The latter haplotype carries the 11 telomeric variants of the CEVA haplotype and is identical to that observed in a single Iberian-Spanish chromosome in 1000 Genomes. Taken together, these results suggest that the CEVA haplotype can be refined to its nine telomeric variants (figure 1C).

Candidate pathogenic sequence variants

One or more of the variants comprising the CEVA haplotype could be pathogenic alone or in combination. It is also possible that they are in LD with a true pathogenic variant(s) that we did not detect because it was not annotated in 1000 Genomes or had a MAF >0.05. Therefore, we performed MPS to search for additional variants linked to the CEVA haplotype region in three additional simplex trios. We also performed MPS of DNA from parents from four initial M1 multiplex families (142, 156, 280 and 380) to define the meiotic phase configuration of variants with the CEVA haplotype. To define the regions to be sequenced by MPS, we used homozygosity haplotype mapping (figure 2) to delineate chromosomal segments that were not shared among the M1 families and could thus be excluded from MPS analysis.[24] The longest contiguous segment (756 kb) for MPS included the 12 CEVA variants and the entire *SLC26A4* gene. We designed a second set of Agilent XT SureSelect probes for MPS to cover this segment as well as the other top 32 longest (>35 kb) shared segments. We also included exons and splice sites for *FOXI1* and *KCNJ10*, since Yang *et al*[1011] reported potential pathogenic roles for these genes in EVA. We did not find any exonic or splice site variants in *FOXI1* or *KCNJ10*. We could not identify any variants, with MAF <0.1 or not annotated in the 1000 Genomes database, in *cis* configuration with the 12 CEVA variants.

We used the CADD analysis to estimate the pathogenic potential of each of the 12 CEVA sequence variants.[25] Any variant with C≥20 is considered to be within the top 1% of variants likely to have a deleterious effect.[25] The scores for our variants range from 0.071 to 21.6 (table 1). The variant (rs199915614) with the highest score (C=21.6) is located within a sequence with partial similarity to the consensus binding sequence for the FOX family of transcription factors (RegulomeDB accessed on 11/4/16). However, we have thus far not detected binding of FOXI1 protein to the reference sequence in an electrophoretic mobility shift assay.

Discussion

We have identified a haplotype, which we have designated the CEVA haplotype, composed of 12 variants upstream of wildtype exonic and splice site sequence of *SLC26A4*, associated with EVA in patients with a mutation of the coding regions or splice sites of the *trans* allele. We did not detect any other potential pathogenic variants in *cis* with the CEVA haplotype by MPS. It is possible that we missed the true pathogenic variant due to the technical caveats of

assembling the short reads produced by this methodology. However, we were previously unable to detect copy number variants by comparative genomic hybridisation microarray analyses of *SLC26A4* and its flanking regions in M0 and M1 EVA subjects.[4] We conclude that one or more of the 12 variants of the CEVA haplotype, either alone or in combination, exert a pathogenic effect on the expression or function of *SLC26A4*. The mixed reference-CEVA haplotype in subjects 1470 and 1805 suggests that it is one or more of the nine telomeric variants of the CEVA haplotype that are pathogenic (figure 1C). It is possible that these variants act in combination with other variants that have a minor allele frequency greater than 0.1 and thus escaped our analysis.

The phenotype in M1 patients is generally less severe than in M2 patients. Since our current study shows that nearly all of these M1 patients carry the CEVA haplotype, this indicates that a modest effect of the CEVA haplotype on *SLC26A4* expression underlies this genotype–phenotype correlation. This difference in expression would be difficult to detect with conventional measures of RNA levels. Furthermore, alternative transcription initiation sites and alternative splicing resulting in a family of shorter transcripts may contribute to complex regulation and function of the 'full-length' transcript.[26] Finally, inner ear tissue from human patients is not accessible for research purposes, and peripheral leucocytes express *SLC26A4* at low levels that cannot be reliably measured or compared (unpublished observations). Other potential surrogate tissues for measurement of *SLC26A4* expression levels include the thyroid gland, but the mature thyroid may not be an appropriate surrogate for embryonic endolymphatic sac.[2728] In our view, an invasive procedure to obtain thyroid or other tissue is not justified.

We presently lack data supporting the hypothesis that the CEVA haplotype affects expression of *SLC26A4*. However, rs199915614 had the highest CADD score of any of the variants associated with the CEVA haplotype, rendering it a top candidate for exploration of a pathogenic role in EVA. The reference sequence, TGTTCGA, matches five (underlined) of seven nucleotide positions of the consensus binding sequence, TRTTKRY (R=A/G; K=G/T; Y=C/T), reported for FOXI1 proteins.[11] rs199915614 is a deletion of the first T in the reference sequence, resulting in CGTTCGA in chromosomes with the CEVA haplotype and a reduced match to the consensus binding sequence. Although we have not detected binding of FOXI1 protein to the reference sequence, this may be a false-negative result due to experimental procedures and conditions or the affected sequence may have some other functional role. Alternatively, rs199915614 may not be pathogenic.

This study's elucidation of the contribution of the CEVA haplotype to NSEVA will have an important impact on routine genetic testing for EVA. The detection of the CEVA haplotype can provide a conclusive diagnostic result for most Caucasian patients with EVA previously classified as having only one (M1) detectable mutation of *SLC26A4*. Moreover, our study advances the interpretation of *SLC26A4* test results in which a single hypomorphic variant is detected.[7] We observed that the only indeterminate *SLC26A4* coding variant (p.Met775Thr) in *trans* with the CEVA haplotype is also in *cis* with the CEVA haplotype (table 4). Similarly, there are two unrelated Danish EVA subjects who are heterozygous for the indeterminate variant p.Glu29Gln and homozygous for the CEVA haplotype (not shown). These observations suggest that hypomorphic variants such as p.Met775Thr and p.Glu29Gln

can be pathogenic when they are in *cis* with the CEVA haplotype. Conversely, none of the other indeterminate or benign variants were observed in *trans* with the CEVA haplotype, indicating that they are coincidental non-pathogenic variants in those subjects. Finally, detection of homozygosity for the CEVA haplotype may even provide a diagnostic result for patients with M0 EVA. We expect the diagnostic yield and prognostic utility of sequence analysis of *SLC26A4* exons and splice sites to be markedly increased by the addition of testing for the CEVA haplotype.

We previously reported two unilateral EVA subjects in the NIH cohort with two mutant alleles of *SLC26A4*, leading us to propose an expansion of the indications for *SLC26A4* testing to include unilateral EVA.[8] In our current study, two (18%) of 11 NIH M1 subjects carrying the CEVA haplotype had unilateral hearing loss and EVA (table 3). These results further support the diagnostic utility of *SLC26A4* testing for unilateral EVA.

The heterozygous carrier frequency (5.567%) of the CEVA haplotype is high among Caucasian controls. The CEVA haplotype defines, to our knowledge, the most common pathogenic allele of any gene implicated in hereditary hearing loss in these populations. In contrast, we did not observe the CEVA haplotype in East Asian populations, such as Koreans and Japanese, in 1000 Genomes (table 2). This may explain why M1 genotypes are rare in Japanese and Korean patients with EVA.[35–929–32] If there are EVA-associated haplotypes in East Asians, they likely have a lower prevalence or lower penetrance.

In summary, this study elucidates the genetic architecture of non-syndromic hearing loss associated with EVA, the most common radiological abnormality observed in ears of children with sensorineural hearing loss. Elucidating the mechanistic link between the CEVA haplotype and its phenotypic associations may reveal therapeutic opportunities to restore *SLC26A4* expression and preserve hearing in ears with EVA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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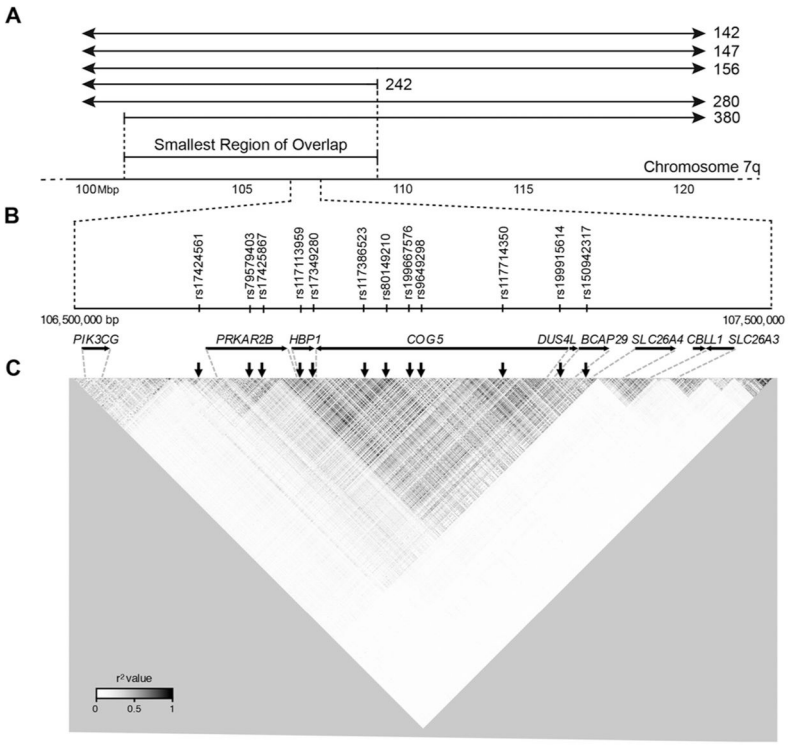

Figure 1.

Region of the 'mutation-negative' allele of *SLC26A4* on chromosome 7q that co-segregates with enlargement of the vestibular aqueduct (EVA). (A) Region on chromosome 7q that co-segregates with EVA among families 142, 147, 156, 242, 280 and 380. The dotted vertical lines delineate the centromeric recombination breakpoint in family 380 and the telomeric breakpoint in family 242 that define a smallest region of overlap among the families. (B) Twelve uncommon variants that define the Caucasian EVA haplotype that co-segregates with EVA in families 142, 147, 156, 280 and 380. Two variants (rs199667576 and rs199915614) are single-nucleotide deletions and ten are SNPs. (C) Linkage disequilibrium (LD) map shows the 12 variants in a single region of LD spanning from upstream of *PRKAR2B* to intron 3 of *SLC26A4*. The colour scale represents pairwise r^2 values with darker shades reflecting higher co-inheritance. The 12 variants are all in modest LD with each other ($r^2>0.6$). The nine telomeric variants are in a large region of higher LD, and the three centromeric variants are in an adjacent region of weaker LD. Genes and their orientations are shown as horizontal arrows. Vertical arrows indicate the location of the variants.

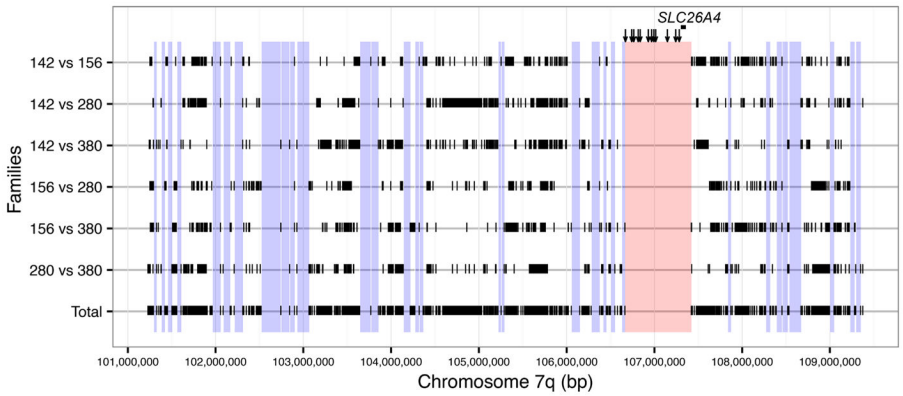

Figure 2.

Homozygosity haplotype analysis of alleles of *SLC26A4* with no detectable mutations in the coding regions or splice sites in M1 families. Homozygous SNPs in the 8.218 Mb smallest region of overlap (figure 1A) were compared between pairs of chromosomes from four M1 families. Black vertical tick marks (|) show unmatched homozygous SNPs between each pair of chromosomes (ie, GG vs CC). Segments bounded by two tick marks indicate regions potentially shared by the two families. All unmatched pairwise homozygous SNPs from the six comparisons are shown at the bottom ('Total'), delineating intervening regions that may be shared among the four chromosomes from the four families. The longest segment (shown in red) includes the Caucasian enlarged vestibular aqueduct haplotype markers (vertical arrows) and *SLC26A4*. Other segments longer than 35 kb (shown in blue) were analysed by massively parallel sequencing.

Table 1

SLC26A4-linked variants comprising the CEVA haplotype

Variant	Chromosome 7q position (bp)*	Reference genotype	CEVA genotype	Minor allele frequency†	CADD score‡
rs17424561	106669858	G	A	0.049	3.9
rs79579403	106741374	T	C	0.048	2.9
rs17425867	106764419	T	A	0.048	11.7
rs117113959	106815154	T	C	0.047	0.1
rs17349280	106837681	G	A	0.047	2.2
rs117386523	106930234	C	T	0.047	13.5
rs80149210	106967931	A	G	0.047	1.6
rs199667576	106993159	T	–	0.046	5.9
rs9649298	107014419	A	G	0.046	13.7
rs117714350	107147622	T	C	0.035	7.8
rs199915614	107242636	T	–	0.035	21.6
rs150942317	107282469	A	C	0.035	10.4

*Based on hg19.

†From European populations in 1000 Genomes phase 3.

‡CADD scaled C-scores for predicted deleteriousness. Larger scores are more strongly predictive of deleteriousness. CADD, Combined Annotation Dependent Depletion; CEVA, Caucasian enlargement of the vestibular aqueduct.

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Table 2

Numbers of *SLC26A4*-linked haplotypes in 1000 Genomes

	EUR									
Haplotype	**CEU**	**GBR**	**TSI**	**IBS**	**FIN**	**ALL**	**AFR**	**AMR**	**EAS**	**SAS**
GTTTGCATATTA (reference)	188	177	197	200	185	947	1314	676	1008	971
ACACATG-GC-C (CEVA)	3	2	8	7	8	28	1	11	0	1
ACACATG-GTTA	2	0	1	3	3	9	5	1	0	0
ATTTGCATATTA	1	1	1	1	0	4	0	3	0	0
ACATGCATATTA	1	1	0	1	0	3	1	0	0	0
GCACATG-GTTA	1	0	1	0	0	2	0	0	0	0
GCACATG-GC-C	0	0	0	1	0	1	0	0	0	0
GTTCATG-GC-C	0	0	0	0	1	1	0	1	0	0
GTTCATG-GTTA	0	0	1	0	0	1	0	0	0	0
ACTCATG-GC-C	0	0	0	0	1	1	0	0	0	0
ACACACGTAC-C	0	1	0	0	0	1	0	0	0	0
GTATGCATATTA	0	0	1	0	0	1	0	0	0	0
GTTTGTATATTA	0	0	0	1	0	1	0	2	0	0
ACACATA-GC-C	1*	0	0	0	0	1	0	0	0	0
GTTTGCGTATTA	1*	0	0	0	0	1	0	0	0	0
ACACATG-GCTC	0	0	1†	0	0	1	0	0	0	0
GTTTGCATAT-A	0	0	1†	0	0	1	0	0	0	0
ACACATG-GCTA	0	0	1‡	0	0	1	0	0	0	0
GTTTGCATAT-C	0	0	1‡	0	0	1	0	0	0	1§
GTTCATG-GCTA	0	0	0	0	0	0	0	0	0	1§
GCATGCATATTA	0	0	0	0	0	0	0	0	0	2
GTTCACATATTA	0	0	0	0	0	0	0	0	0	1
GTTCGCATATTA	0	0	0	0	0	0	0	0	0	1
GTTTGCA-ATTA	0	0	0	0	0	0	1	0	0	0

	EUR									
Haplotype	**CEU**	**GBR**	**TSI**	**IBS**	**FIN**	**ALL**	**AFR**	**AMR**	**EAS**	**SAS**
Total number of haplotypes	198	182	214	214	198	1006	1322	694	1008	978

Superpopulations are shown in italic: European (EUR), African (AFR), Admixed American (AMR), East Asian (EAS) and South Asian (SAS). European subpopulations are Utah residents with Northern and Western European ancestry (CEU), Toscani in Italy (TSI), Finnish in Finland (FIN), British in England and Scotland (GBR), and Iberian populations in Spain (IBS). Genotypes for CEVA variants are underlined. Footnotes indicate individuals with two rare haplotypes due to allele flip error.

[*] NA12400.

[†] NA20761.

[‡] NA20768.

[§] NA20858.

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Table 3

SLC26A4 genotypes, haplotypes and phenotypes of M1 subjects with enlargement of the vestibular aqueduct and one mutated allele

Family	Subject	Ancestry	Allele 1 genotype	Allele 2 haplotype	HL/EVA*	Thyroid
142	1303	Caucasian	p.Leu445Trp	CEVA	B	NS
142	1304	Caucasian	p.Leu445Trp	CEVA	R	NS
156	1425	Caucasian	c.1001+1G>A	CEVA	B	NS
156	1426	Caucasian	c.1001+1G>A	CEVA	B	I
217	1580	Caucasian	p.Glu384Gly	Reference	R	NS
239	1645	Caucasian	p.Thr416Pro	CEVA	B	NS
242	1630	Caucasian	p.Met1Thr	Reference	B	I
242	1631	Caucasian	p.Met1Thr	Reference	B	I
264	1762	Caucasian	p.Leu445Trp	CEVA	B	I
273	1805	Caucasian	p.Met1Thr	Other†	B	NS
280	1802	Caucasian	p.Thr416Pro	CEVA‡	B	I
280	1803	Caucasian	p.Thr416Pro	CEVA‡	B	I
293	1849	Caucasian	p.V138F	CEVA	B	I
380	2083	Caucasian	c.365insT	CEVA	L	NS
380	2085	Caucasian	c.365insT	CEVA	B	NS
DK-1	12 624–11	Caucasian	p.Thr416Pro	CEVA	B	NT
DK-2	11 934–10	Caucasian	p.Thr416Pro	CEVA	B	NT
DK-3	11 890–10	Caucasian	c.1614+1G>A	CEVA	B	NT
DK-4	kc102065	Caucasian	p.Leu236Pro	CEVA	B§	NT
DK-5	2531–02	Caucasian	p.Thr416Pro	CEVA	B	NS
DK-6	13 317–12	Caucasian	p.Val138Phe	CEVA	B	NS

*Hearing loss with enlargement of the vestibular aqueduct affecting right (R), left (L) or both ears (B).

†Neither reference nor CEVA.

‡Phase configuration not definitive but likely to be in *trans* to p.Thr416Pro.

§Bilateral EVA but left ear has normal hearing.

CEVA, Caucasian enlargement of the vestibular aqueduct; EVA, enlargement of the vestibular aqueduct; I, indeterminate; HL, hearing loss; NS, non-syndromic; NT, not tested.

Table 4

SLC26A4 genotypes, haplotypes and phenotypes of NIH subjects with enlargement of the vestibular aqueduct and a benign or indeterminate variant on one allele

Family	Subject	Ancestry	Allele 1 genotype*	Allele 2 haplotype	HL/EVA†	Thyroid
118	1166	Caucasian	p.Leu597Ser	Reference	B	NS
118	1167	Caucasian	p.Leu597Ser	Reference	R	NS
133	1281	Caucasian	p.Arg776Cys	Reference	B	NS
133	1282	Caucasian	p.Arg776Cys	Reference	R	NS
145	1422	Caucasian	p.Leu597Ser	Reference	B	I
147	1702	Caucasian	p.Met775Thr‡	CEVA	R	I
147	1703	Caucasian	p.Met775Thr‡	CEVA	B	I
176	1473	Caucasian	p.Leu597Ser	Reference	B	NS
182	1495	Caucasian	p.Phe335Leu	Reference	B	NS
218	1590	Black	p.Val609Gly	Reference	B	I
219	1598	Caucasian	c.-5A>G	Reference	B	NS
223	1643	Caucasian	c.-66C>G	Reference	B	I
229	1619	Caucasian	p.Leu597Ser	Reference	B	I
231	1639	Caucasian	p.Arg776Cys	Reference	L	I
240	1649	Multiracial	p.Leu597Ser	Reference	B	I
255	1691	Multiracial	p.Leu597Ser	Reference	B	NS
259	1726	Black	c.-60A>G	Reference§	L	I
278	1791	Caucasian	p.Leu597Ser	Reference	B	NS
341	1986	Caucasian	p.Gly6Val;p.Leu597Ser	Reference	B	NS
341	1981	Caucasian	p.Leu597Ser	Reference	B	I
388	2106	Caucasian	p.Asp324Tyr	Reference	B	NS
388	2107	Caucasian	p.Asp324Tyr	Reference	B	NS
388	2108	Caucasian	p.Asp324Tyr	Reference	B	NS

*Alleles are considered benign, indeterminate or pathogenic only in *trans* with a mutation affecting coding regions or splice sites of *SLC26A4*.

†Hearing loss with enlargement of the vestibular aqueduct affecting right (R), left (L) or both ears (B).

‡ p.Met775Thr is in *cis* and *trans* with CEVA; the subject is homozygous for CEVA.

§ p.Val609Gly carried on allele 2.

I, indeterminate; NS, non-syndromic.