Segregation of enlarged vestibular aqueducts in families with non-diagnostic *SLC26A4* genotypes

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Abstract

Background—Hearing loss with enlarged vestibular aqueduct (EVA) can be inherited as an autosomal recessive trait caused by bi-allelic mutations of *SLC26A4*. However, many EVA patients have non-diagnostic *SLC26A4* genotypes with only one or no detectable mutant alleles.

Methods and results—In this study, the authors were unable to detect occult *SLC26A4* mutations in EVA patients with non-diagnostic genotypes by custom comparative genomic hybridisation (CGH) microarray analysis or by sequence analysis of conserved non-coding regions. The authors sought to compare the segregation of EVA among 71 families with two (M2), one (M1) or no (M0) detectable mutant alleles of *SLC26A4*. The segregation ratios of EVA in the M1 and M2 groups were similar, but the segregation ratio for M1 was significantly higher than in the M0 group. Haplotype analyses of *SLC26A4*-linked STR markers in M0 and M1 families revealed discordant segregation of EVA with these markers in eight of 24 M0 families.

Conclusion—The results support the hypothesis of a second, undetected *SLC26A4* mutation that accounts for EVA in the M1 patients, in contrast to non-genetic factors, complex inheritance, or aetiologic heterogeneity in the M0 group of patients. These results will be helpful for counselling EVA families with non-diagnostic *SLC26A4* genotypes.

Enlargement of the vestibular aqueduct (EVA; MIM 600709) is a common radiological inner ear malformation associated with sensorineural hearing loss (SNHL).[1] It is the most penetrant finding in autosomal recessive SNHL with goitre (Pendred syndrome (PDS); MIM 274600) but can also be observed without goitre in non-syndromic EVA (NSEVA) (DFNB4; MIM 600791).[2] Mutations of *SLC26A4*, which encodes the pendrin protein, can cause either PDS or NSEVA.[3–5] Pendrin is a member of the *SLC26A* family of transmembrane anion transporters.[6]

Two mutant alleles of *SLC26A4* can be identified in approximately 1/4 of Caucasian patients with EVA. The other 3/4 of those patients carry only one or zero mutant alleles of *SLC26A4*. [7–10] In these latter patients with non-diagnostic genotypes, the aetiology of EVA remains unclear. The pathogenic contribution of mono-allelic *SLC26A4* mutations is supported by their detection in up to 44% of NSEVA patients.[7 9–13] Azaiez *et al*[11] used the maximum likelihood method[14] to estimate that 98% of these apparent heterozygotes have an undetected *SLC26A4* mutation in *trans* configuration.

SLC26A4 mutations are detected more frequently in multiplex EVA families than in simplex families.[9] This may reflect a contribution of non-genetic factors to the aetiology of EVA in patients with no *SLC26A4* mutations. However, specific non-genetic causes have not been identified to date. Congenital cytomegalovirus infection, for example, is a very common non-genetic cause of childhood hearing loss but accounts for few, if any, cases of EVA.[15] It has also been proposed that EVA may be inherited as a digenic or a complex trait caused by a single mutant allele of *SLC26A4* in combination with environmental factors or mutations in other genes[15] such as *FOXI1*, a putative upstream regulator of *SLC26A4* expression.[16] However, other studies indicate that *FOXI1* mutations contribute to few, if any, cases of EVA. [13 15 17]

The aetiology and recurrence risk for EVA in patients with non-diagnostic *SLC26A4* genotypes thus remain unknown. In this study, we sought to identify pathogenic copy number variants or mutations of conserved non-coding sequences in these patients. We also analysed the segregation of EVA and *SLC26A4* in families with non-diagnostic *SLC26A4* genotypes. Since we have never observed abnormal perchlorate discharge results from EVA patients with non-diagnostic *SLC26A4* genotypes,[7 18 19] we did not include thyroid phenotype in our analyses. Our results provide insight into the aetiology of EVA and estimates of recurrence risk that will be useful when counselling families.

METHODS

Subjects

This study was approved by the Combined Neuroscience Institutional Review Board, National Institutes of Health (NIH), Bethesda, Maryland. Written informed consent was obtained from all subjects or their legal guardians. Our cohort comprised 71 probands, 85 siblings (19 affected and 66 unaffected), and 137 parents from 71 families. Two hundred and thirty-one of 293 subjects were evaluated at the NIH Clinical Center, including 132 family members that were previously reported.[7 19] The evaluations included medical–otolaryngological history interviews and physical examinations, pure tone and speech audiometry and, in most subjects, computed tomography (CT) and/or magnetic resonance imaging (MRI) of the temporal bones. The phenotypes of 62 subjects were inferred from existing medical records or medical and/or family history interviews. We performed *SLC26A4* sequence analysis as described.[7]

We grouped families according to the number of mutant alleles of *SLC26A4* detected in the proband: zero (M0), one (M1), or two (M2). Four hypofunctional variants (p.F335L, p.C565Y, p.L597S, and p.M775T) and two variants (c.−60A>G and c.−3−2A>G) of indeterminate pathogenicity were considered non-pathogenic unless they were in *trans* configuration with *SLC26A4* mutations previously reported to be pathogenic.[19 20] Our cohort comprised 43 M0 families, eight M1 families, and 20 M2 families. Three of 20 M2 and 13 of 43 M0 families segregated variants of indeterminate pathogenicity, including one M2 and seven M0 families with p.L597S.[7 19]

Comparative genomic hybridisation (CGH) microarray analysis

We analysed the DNA of probands from seven M1 families (142, 156, 217, 242, 264, 280 and 293) and two M0 families (182 and 255). We used a custom microarray with 15 bp probe spacing (2006-07-31_HG18_SLC26A4_FT; Nimblegen Sytems Inc, Madison, Wisconsin, USA) representing a 3.8 Mb region encompassing *SLC26A4* on chromosome 7 (nt 105,238,316–109,038,316; UCSC version hg18, March 2006). DNA samples from parents were used for hybridisation controls. For M1 families, we used the heterozygous carrier parent as the control.

Sequence analysis of non-coding regions

We designed primers (supplemental table S1) to polymerase chain reaction (PCR) amplify and sequence non-coding regions of *SLC26A4* from probands from seven M1 families (142, 156, 217, 242, 264, 280 and 293) and one M0 family (118).[7] We sequenced intronic and upstream conserved regions predicted by the Vertebrate Multiz Alignment and PhastCons Conservation (28 species) tool of the University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/). We also sequenced candidate inner ear specific promoter regions[17] and Genscan predicted exons (NT_007933.614) (fig 1) (supplemental table S1). Variants were considered non-pathogenic when they were detected among >1% of normal Caucasian control chromosomes from Coriell Cell Repositories (HD200CAU; Camden, New Jersey, USA).

Zygosity analysis

The zygosity of twin pairs in families 219, 213, and 245 was previously established.[7] [19] We analyzed 14 unlinked STR markers in twins 1750 and 1751 (family 261) to determine zygosity.

Calculation of segregation ratio

We assumed single incomplete ascertainment to calculate segregation ratios among M0, M1, and M2 groups. We used Weinberg's proband method to correct for ascertainment bias against carrier parents with normal progeny.[21] In this method, each proband is considered to be providing information that his or her parents are capable of producing affected progeny. An unbiased estimate of the segregation ratio (p) is calculated from remaining members of the sibship,

$$p = \frac{\Sigma\,(r-1)}{\Sigma\,(s-1)}$$

where r and s indicate the number of affected offspring and the total number of offspring in each family, respectively. Monozygotic twin pairs were each treated as a single observation in the analysis, reducing the total number of siblings for our analysis from 85 to 83. We first calculated the ratio among 48 siblings from 36 families (22 M0, 5 M1, and 9 M2) that were evaluated at the NIH Clinical Center. We repeated the calculations for the same cohort with 35 additional siblings (from 4 M0, 1 M1 and 8 M2 families) that were not evaluated at the NIH Clinical Center. These siblings were considered to be affected if they had radiologically confirmed EVA or a history of pre- or perilingual onset deafness in the absence of radiologic imaging data. We also calculated segregation ratios among heterozygous mutation carriers in M1 and M2 families.

In addition, we calculated the ratios under either of two assumptions: p.F335L, p.C565Y, p.L597S, p.M775T, c.−60A>G and c.−3−2A>G are always or never pathogenic, irrespective of the *trans* allele genotype.

Haplotype analysis

We performed haplotype analyses of *SLC26A4* linked short tandem repeat (STR) markers (D7S496, D7S2459 and D7S2456) on the M0 (n = 17) and M1 (n = 1) families with participating siblings. The results for 11 other families (seven M0, four M1) were previously described.[7]

Statistical analysis

Fisher's exact test was used to compare segregation ratios among genotype groups. A value of p<0.05 was considered to be significant.

RESULTS

Occult *SLC26A4* mutation analyses

CGH microarray analysis revealed only a 338 bp deletion (rs6150268) located 1.56 Mb upstream of *SLC26A4* (chr 7: 105,521,227–105,521,564; UCSC version hg18, March 2006) in four of seven M1 probands. We designed primers flanking this copy number variant and detected it by PCR amplification in 46 of 63 Caucasian control subjects. These results provided no evidence for the presence of pathogenic copy number variations in the 3.8 Mb region encompassing *SLC26A4*.

We did not identify any pathogenic variations in non-coding conserved regions from eight (one M0, seven M1) probands (supplemental table S1 available online).

Segregation analyses

The overall segregation ratio of EVA in 48 siblings evaluated at the NIH Clinical Center was 0.27 (95% confidence interval (CI) 0.17 to 0.41). However, the segregation ratio differed among the genotype groups M0, M1, and M2. The M1 ratio of 0.67 (95% CI 0.29 to 0.90) was significantly higher (p = 0.011) than the M0 ratio of 0.13 (95% CI 0.052 to 0.29), but did not differ significantly (p = 0.62) from 0.45 (95% CI 0.21 to 0.72), the ratio for the M2 group in which EVA is inherited as an autosomal recessive trait. The segregation ratio in the M0 group was significantly lower (p = 0.037) than that in the M2 group (table 1).

The differences between genotype groups persisted when we calculated ratios for the extended cohort including 35 siblings who were not evaluated at the NIH Clinical Center (table 2). The differences persisted among M0 versus M1 (p = 0.02), M0 versus M2 (p = 0.067), and M1 versus M2 groups (p = 0.230) (table 2), although the difference between the M0 and M2 groups was not statistically significant.

We observed the same pattern of relative differences in the segregation ratios among genotype groups under the assumption that the four hypofunctional variants and two variants of indeterminate pathogenicity were pathogenic, irrespective of *trans* allele genotype: M0 versus M1 (0.04 *vs* 0.38; p = 0.003), M0 versus M2 (0.04 *vs* 0.27; p = 0.013), and M1 versus M2 (0.38 *vs* 0.27; p = 0.53). The observed pattern of differences persisted when the same six variants are assumed to be non-pathogenic: M0 versus M1 (0.11 *vs* 0.45, p = 0.018), M0 versus M2 (0.11 *vs* 0.26, p = 0.079), and M1 versus M2 (0.45 *vs* 0.26, p = 0.28).

The segregation ratio among the four heterozygous carrier siblings from M1 families was 1.00. As expected, the ratio among the five heterozygous carrier siblings from M2 families was 0.00, since they do not carry the second mutant allele segregating in their respective families.

We found no bias in the gender of the heterozygous carrier parents (two males, two females) transmitting the mutant allele of *SLC26A4* to their affected M1 offspring. We detected no

audiologic or radiologic evidence of vertical transmission of EVA in any of our M0 or M1 families.[7]

Haplotype analyses of *SLC26A4* linked STR markers in 24 M0 and five M1 families with participating siblings revealed concordant segregation with EVA in 21 families and discordant segregation in eight families (fig 2). In families 144, 213, 255, 259, 261, and 283, we observed unaffected siblings and the proband carrying the same *SLC26A4* linked haplotypes. This result could reflect either discordant inheritance or non-penetrance. Non-penetrance (bilateral) of EVA is not unexpected from the frequent observation of unilateral non-penetrance (that is, unilateral EVA) in M0 patients.[7] In contrast, families 133 and 219 each have affected sibling pairs with different *SLC26A4* linked haplotypes. Families 133 and 219 were considered to be M1 in our previously published haplotype analysis,[7] but we now recognise their variants (p.R776C and c.-3-2A>G) as likely to be benign polymorphisms.[19] [20] [22] We did not observe discordant segregation of *SLC26A4* linked STR markers with EVA in any of five M1 families (fig 3).

Twinning

There were three dizygotic (DZ) and two monozygotic (MZ) twin pairs among 77 live births in M0 families (supplemental table S1 available online). Three DZ twin births but no MZ twin births were reported among 73 live births in the combined group of M1 and M2 families; all three M1/M2 twin births were in one M2 family (family 188).[7]

DISCUSSION

We were unable to identify any previous published reports of segregation ratios for EVA.

Our segregation ratios for M1 families confirm the pathogenic contribution of detected mono-allelic mutations to EVA and support the hypothesis that EVA is inherited as a mono- or digenic recessive trait in those families. Although our M1 segregation ratio was greater than the expected Mendelian result of 0.25, it was similar to the ratio observed among the M2 families in whom EVA was inherited as a monogenic autosomal recessive trait. The lack of a significant difference between M1 and M2 ratios might be due to the small number of families, especially M1 families. However, the high M1 ratio seems unlikely to reflect dominant inheritance because we have not observed vertical transmission of EVA in M0 or M1 families. The ratios for all of the genotype groups likely reflect an ascertainment bias for multiplex sibships in the families referred to our study.

If the hypofunctional alleles p.R776C and c.-3-2A>G are considered to be non-pathogenic,[19] [22] there is no longer evidence for digenic or complex inheritance among our M1 families. The lack of discordant segregation of EVA with *SLC26A4* linked STR markers in M1 families is thus consistent with the hypothesis of autosomal recessive inheritance of bi-allelic mutations of *SLC26A4*, although we cannot exclude the possibility of digenic inheritance in M1 families.

Although we were unable to find evidence of pathogenic copy number variations or non-coding sequence mutations, they may reside elsewhere in intronic or regulatory regions that we did not evaluate. Occult mutant alleles of *SLC26A4* would be predicted to encode some residual pendrin since the thyroid and auditory phenotypes in M1 patients are generally less severe than in M2 patients. Abnormal perchlorate discharge results are observed almost exclusively among M2 patients,[7] [18] unilateral EVA is more prevalent among M1 patients whereas EVA is almost always bilateral in M2 patients, and M1 patients tend to have better hearing thresholds than M2 patients.[7] [11] [23]

Given the probable influence of ascertainment bias on our segregation ratios, the actual M0 ratio may be closer to zero than our observed ratio of approximately 0.1. This low ratio and discordant segregation of EVA with *SLC26A4* in eight of 24 M0 families are consistent with a non-genetic or complex aetiology, or aetiologic heterogeneity that may include Mendelian inheritance in some families. However, we did not detect phenotypic evidence for etiologic heterogeneity among the M0 group since there was no significant difference in hearing thresholds between familial and sporadic patients (not shown).

The twin pairs in our M0 cohort raise the possibility of a causal relationship or a shared aetiology with twinning such as that described for other phenotypes including Beckwith–Wiedemann[24][25] and Goldenhar syndromes[26] and symmelia.[27] However, we identified no patterns among the M0 twin pregnancies to indicate a causal relationship between twinning and EVA (supplemental table S2 available online).

This is the first study to estimate the recurrence risk of EVA, which will be useful for counselling families segregating EVA and one or no detectable mutations of *SLC26A4*. Families with EVA and one detectable mutation of *SLC26A4* seem likely to be segregating EVA as a trait caused by that mutation in combination with a second occult mutant allele of *SLC26A4* or another autosomal gene. In contrast, EVA appears to be a non-genetic or complex trait with a significantly lower recurrence rate in families with no detectable *SLC26A4* mutations. The discovery of a source of occult mutations in families with non-diagnostic *SLC26A4* genotypes could clarify the aetiology and recurrence risk of EVA in those families.

Key points

- ▶ We calculated segregation ratios and analysed the segregation of *SLC26A4* linked markers in enlargement of the vestibular aqueduct (EVA) families with non-diagnostic *SLC26A4* genotypes.

- ▶ Our results support the hypothesis of a second, undetected *SLC26A4* mutation in M1 patients, whereas EVA appears to be caused by mutations in other genes, non-genetic factors, or both as a complex trait in M0 patients.

- ▶ These results will be helpful for recurrence risk counselling of EVA families with non-diagnostic *SLC26A4* genotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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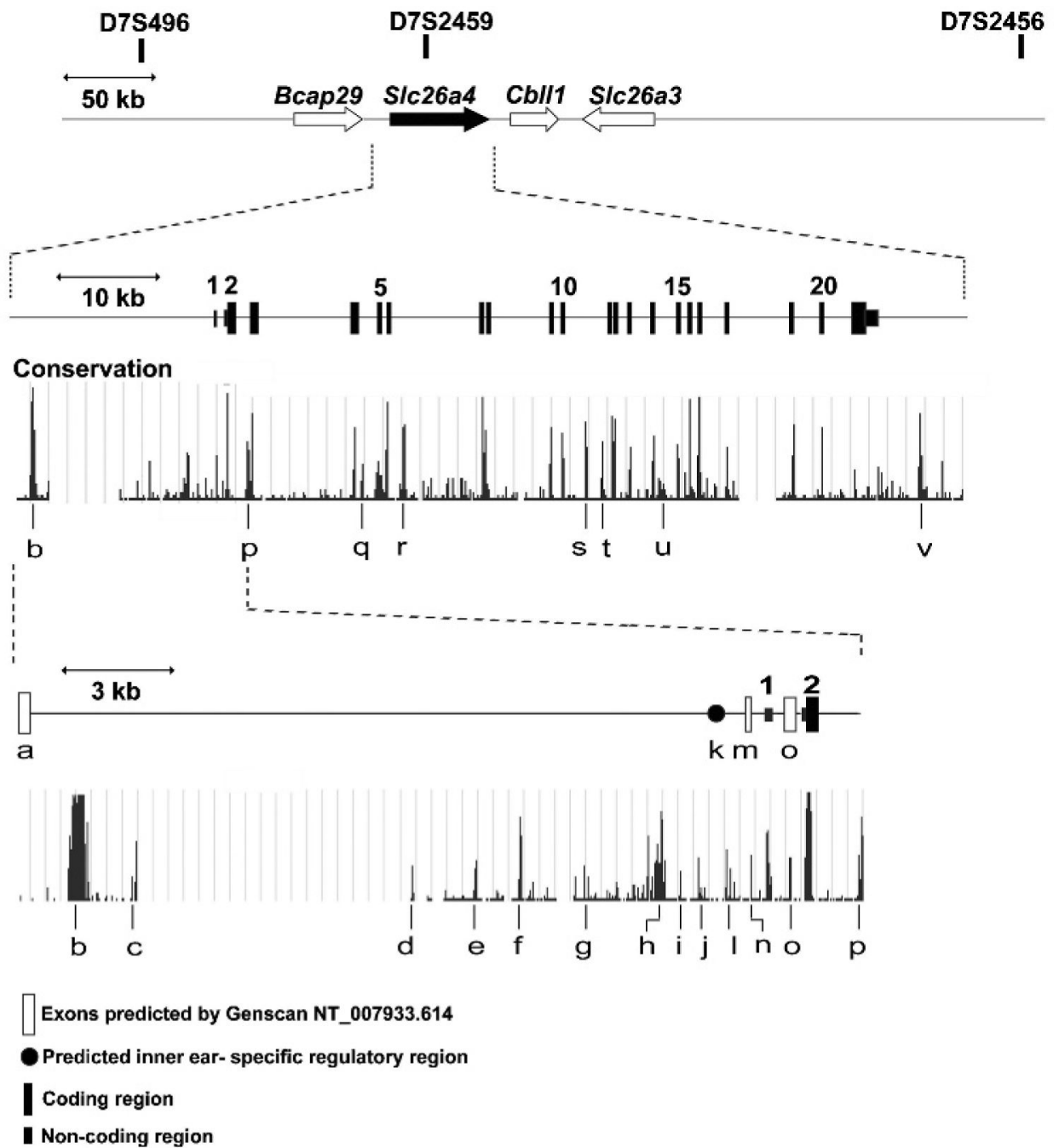
Figure 1.
Schematic illustration of chromosome 7q31 encompassing the *SLC26A4* gene with STR markers shown above. Non-coding regions (labelled "a" to "v") that are most highly conserved among 28 species were sequenced in seven M1 families and one M0 family. These regions include an inner ear specific positive regulatory element (*k*) predicted by Adler *et al*[17] and potential additional exons predicted by Genescan (NT_007933.614) ("a", "m" and "o").
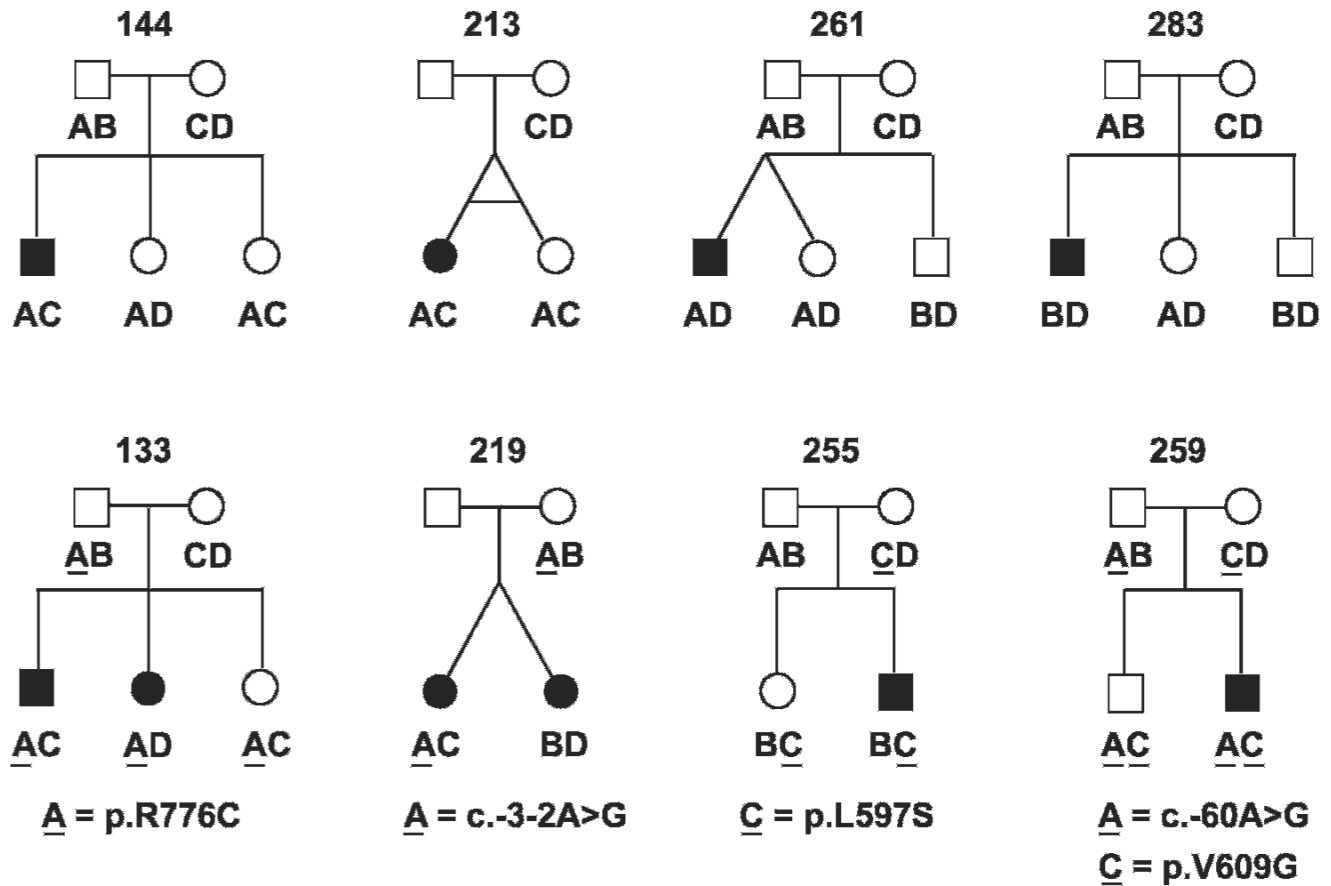
Figure 2.
Discordant segregation of *SLC26A4* linked short tandem repeat (STR) marker haplotypes with enlargement of the vestibular aqueduct (EVA). The haplotypes within each family are arbitrarily represented as A, B, C, or D—for example, an A haplotype in one family does not necessarily imply the same combination of genotypes in the A haplotype for another family. Families 133, 144, 213, and 219 were previously described by Pryor *et al.*[7] Haplotypes are underlined when linked to a *SLC26A4* sequence variant. p.V609G is a common polymorphism (dbSNP rs17154335). If p.R776C, c. -3-2A>G, p.L597S and c.-60A>G are considered non-pathogenic in *trans* with a wild type allele,[19][20][22] all of these eight families are M0 families (tables 1 and 2).
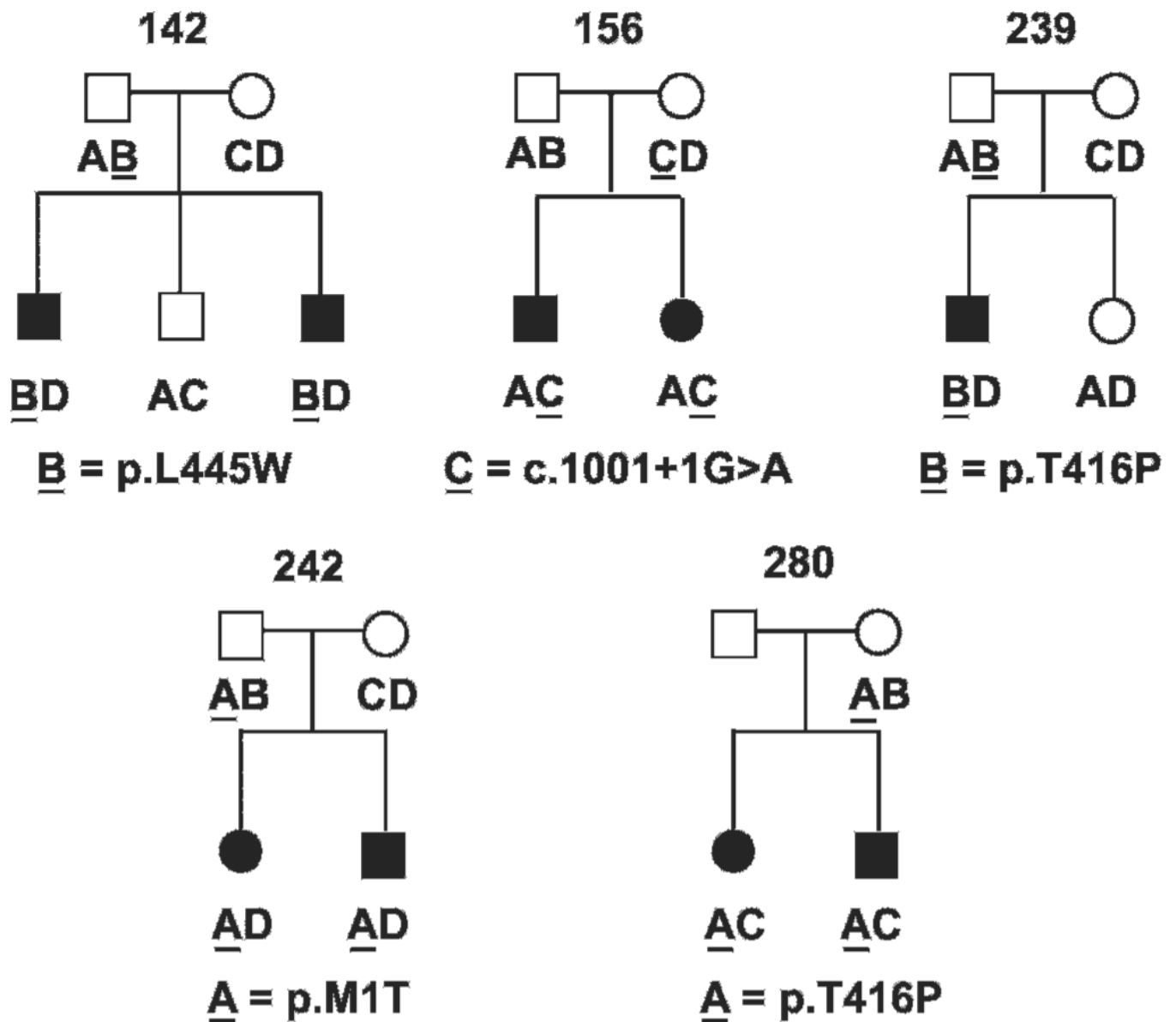
Figure 3.
Segregation of *SLC26A4* linked short tandem repeat (STR) marker haplotypes and enlargement of the vestibular aqueduct (EVA) in M1 families. The haplotypes within each family are arbitrarily represented as A, B, C, or D. Haplotypes are underlined when linked to an *SLC26A4* mutation. We did not include families 133 and 219 which we formerly considered to be M1[7] but are now considered M0.

Table 1

Segregation of enlargement of the vestibular aqueduct (EVA) among siblings ascertained at the National Institutes of Health

Number of *SLC26A4* mutations in proband	Number of siblings		Segregation ratio[*] (95% CI)
	Affected	Unaffected	
0	4	27	0.13 (0.052 to 0.29)
1	4	2	0.67 (0.29 to 0.90)
2	5	6	0.45 (0.21 to 0.72)
Total	13	35	0.27 (0.17 to 0.41)

CI, confidence interval.

[*] The M1 ratio of 0.67 was significantly higher (p=0.011) than the M0 ratio of 0.13, but did not differ significantly (p=0.62) from the M2 ratio of 0.45. The segregation ratio in the M0 group was significantly lower (p=0.037) than that in the M2 group.

Table 2

Segregation of enlargement of the vestibular aqueduct (EVA)[*] among the entire cohort of 83 siblings

Number of *SLC26A4* mutations in proband	Number of siblings		Segregation ratio[†] (95% CI)
	Affected	Unaffected	
0	4	34	0.11 (0.043 to 0.24)
1	4	4	0.50 (0.21 to 0.79)
2	10	27	0.27 (0.15 to 0.43)
Total	18	65	0.22 (0.14 to 0.32)

CI, confidence interval.

[*] Includes EVA inferred from outside evaluations and family history interviews.

[†] The M1 ratio of 0.50 was significantly higher (p=0.02) than the M0 ratio of 0.11, but did not differ significantly (p=0.23) from the M2 ratio of 0.27.