Disruption of Teashirt Zinc Finger Homeobox 1 Is Associated with Congenital Aural Atresia in Humans

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Congenital aural atresia (CAA) can occur as an isolated congenital malformation or in the context of a number of monogenic and chromosomal syndromes. CAA is frequently seen in individuals with an 18q deletion, which is characterized by intellectual disability, reduced white-matter myelination, foot deformities, and distinctive facial features. Previous work has indicated that a critical region for CAA is located in 18q22.3. We studied four individuals (from two families) with CAA and other features suggestive of an 18q deletion, and we detected overlapping microdeletions in 18q22.3 in both families. The minimal region of deletion overlap (72.9–73.4 Mb) contained only one known gene, *TSHZ1*, which was recently shown to be important for murine middle-ear development. Sequence analysis of the coding exons in *TSHZ1* in a cohort of 11 individuals with isolated, nonsyndromic bilateral CAA revealed two mutations, c.723G>A (p.Trp241X) and c.946_947delinsA (p.Pro316Thr*fs*X16), and both mutations predicted a loss of function. Together, these results demonstrate that hemizygosity of *TSHZ1* leads to congenital aural atresia as a result of haploinsufficiency.

Congenital aural atresia (CAA) is a rare malformation of the ear that occurs in approximately 1 in 10,000 live births.[1] It presents unilaterally more often than bilaterally. Its characteristics can vary from a narrow external auditory canal and hypoplasia of the tympanic membrane and middle ear cleft to a complete absence of middle-ear structures and anotia (bony atresia of the external auditory canal and hypoplasia of inner ear structures). In the past, different classifications of CAA have been introduced on the basis of clinical findings. In 1955, Altmann was the first to describe a CAA classification,[1] which has been modified over the years by others.[2–4] CAA type I is classified by a bony or fibrous atresia of the lateral part of the external auditory canal and an almost normal medial part and middle ear. CAA type II is the most frequent type and is characterized by partial or total aplasia of the external auditory canal. In type IIA, the external auditory canal is either affected by a complete bony atresia of its medial part or partially aplastic, ending blindly in a fistula that leads to a rudimentary tympanic membrane. CAA type IIB is characterized by a bony stenosis of the total length of the external auditory canal. Finally, CAA type III is characterized by bony atresia of the external auditory canal and a very small or absent middle-ear cavity.[2]

CAA might be present as an isolated malformation but is also seen as a feature of complex syndromes such as Crouzon syndrome [MIM 123500], Treacher Collins syndrome [MIM 154500], Townes Brocks syndrome [MIM 107480], and branchiootorenal syndrome [MIM 113650], as well as aneuploidy syndromes including Turner syndrome (45,X) and trisomies 13, 18, and 21.[5–10] Although not fully penetrant, CAA type IIA in the absence of microtia or anotia is most frequently seen in individuals with a deletion of the long arm of chromosome 18 (MIM 601808).[11] In 1964, De Grouchy described individuals with an 18q deletion, stating that they displayed CAA and a wide range of associated features including short stature, characteristic facial features, intellectual disability, and foot deformities.[11,12]

The majority of individuals with an 18q deletion carry a microscopically visible terminal deletion of the long arm of chromosome 18.[13] Yet, in a small subset of individuals, routine cytogenetic studies reveal a normal G banded karyotype.

Several genotype-phenotype studies have been performed in persons with 18q deletions of various sizes so that critical regions corresponding to the different clinical symptoms of the 18q deletion syndrome could be defined.[14–16] These efforts have resulted in overlapping critical regions for white-matter disorders and delayed myelination, growth hormone insufficiency, foot deformities, and CAA, all nested within the region from 18q22.3 to 18q23.[14,16] Given that multiple genes reside in the region of deletion overlap, it was concluded that further studies would be needed to determine whether the typical 18q deletion syndrome represents a single gene disorder or whether it should be considered a contiguous deletion syndrome.[14] Fine mapping of microscopically visible deletions via molecular techniques, such as a chromosome 18q BAC array, mapped CAA to a 2.3 Mb

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Table 1. Overview of Clinical Features in Eight Individuals with an 18q22.3q23 Microdeletion or *TSHZ1* Mutation

Individual	Deletion/Mutation	Age (yr)	Intellectual Disability	Hearing Loss[a] (L/R)	Type of CAA	Other
1 (family 1)	4.3 Mb deletion	8	mild	17 dB/22 dB	narrow external auditory canals	epilepsy, autism
2 (family 2)	2.5 Mb deletion	30	borderline	60 dB/60 dB	IIA, bilateral	bilateral vertical talus, strabismus
3 (family 2)	2.5 Mb deletion	5	normal	65 dB/65 dB	IIA, bilateral	bilateral vertical talus
4 (family 2)	2.5 Mb deletion	1	mild motor delay	70 dB/45 dB	IIA, bilateral	bilateral vertical talus
5 (family 3)	c.723G>A	10	normal	42 dB/42 dB	IIA, bilateral	no
6 (family 4)	c.946_947delinsA	41	normal	40 dB/40 dB	IIA, bilateral	no
7 (family 4)	c.946_947delinsA	12	normal	50 dB/55 dB	IIA, bilateral	no
8 (family 4)	c.946_947delinsA	12	normal	48 dB/45 dB	IIA, bilateral	no

Mb, megabase; L, left; R, right; CAA, congenital aural atresia; Type IIA, complete bony atresia of the medial part of the external auditory canal, or the canal is partially aplastic and ends blindly in a fistula that leads to a rudimentary tympanic membrane.
[a] Measurement of air conduction before surgical intervention or use of bone-anchored hearing aid.

region in 18q22.3q23 (between markers D18S489 and D18S554). However, it remained unclear whether CAA could be separated from the other common features of the 18q deletion syndrome.[17,18]

Here, we report that isolated CAA and the CAA phenotype in the 18q deletion syndrome are both caused by haploinsufficiency that results from a heterozygous deletion or loss-of-function mutation of *TSHZ1*, whose ortholog is essential for murine middle-ear development.[19]

All procedures were performed in accordance with the ethical standards of the Radboud University Nijmegen Medical Centre Ethical Committee. After obtaining informed consent, we evaluated the presence of microdeletions in four individuals (from two different families) who had a phenotype consistent with the 18q deletion syndrome. We used 250K (*Nsp1)* single-nucleotide polymorphism (SNP) microarrays (Affymetrix, Inc., Santa Clara, CA, USA).[20] SNP microarray experiments were performed according to the manufacturer's instructions. Copy-number estimates were determined with the CNAG software package v2.0, and the genomic locations of the SNP positions were mapped according to the Genome Reference Consortium Human Genome Build 37 (GRCh37).

Individual 1 is the second son from nonconsanguineous parents. He was born at term after an uncomplicated pregnancy and had a normal birth weight. Psychomotor development was delayed: He started walking at 21 months, and at the age of 4 years, a 10–15 month delay in speech and language development was detected. A pure-tone audiogram was performed when the child was cooperative at 4 years of age and showed mild left- and right-sided conductive low-frequency hearing loss of 17 and 22 dB, respectively (Table 1). At eight years of age, he developed epileptic seizures, and EEG abnormalities occurred after sleep deprivation. He was also noted to have mild intellectual disability (an IQ of 60) and behavioral problems involving autistic features. He was a cooperative, healthy boy with a normal height and head circumference. His dysmorphic features included hypertelorism, an upturned nasal tip, and a thin upper lip (Figure 1A). CT scans showed bilateral, narrow external auditory canals. This finding was confirmed by otoscopy, from which only a small part of the tympanic membrane could be visualized (Figure 3A).

SNP-array analysis of DNA isolated from blood revealed a 4.3 Mb interstitial deletion in 18q22.3q23, extending between the genomic coordinates 69.2 and 73.4 Mb (arr snp 18q22.3q23 [SNP_A-2065000 > SNP_A-184607]×1; Figure 2A). SNP-array analysis of the phenotypically normal mother revealed no abnormalities. The father was not available for testing.

Individual 2 is a 30-year-old woman, born at 39 weeks gestation after an uneventful pregnancy to unrelated, healthy parents. Directly after birth, bilateral forefoot deformities were noted and classified as congenital vertical talus (Figure 1B), for which she received surgical treatment. She could walk from the age of 2 years. At the age of 15 months, she presented with a bilateral conductive 60 dB hearing loss, which was caused by bilateral atresia of the external auditory canals, consistent with CAA type IIA (Table 1). She received hearing aids and consequently developed speech, although a delay remained. At ten years of age, the aural atresia of her right ear was successfully operated on, and at the age of 26, she received a bone-anchored hearing aid (BAHA) on the left side. Physical examination at the age of 30 years showed a healthy woman with normal height, weight, and head circumference. Dysmorphic features included hypertelorism, midfacial hypoplasia, and a broad mouth with prominent lips. There was a normal implantation of the ears, which showed a prominent superior crus of the antihelix and underdevelopment of the descending part of the helix (Figure 1B).

SNP-array analysis revealed a 2.5 Mb interstitial deletion between the genomic coordinates 72.9 and 75.4 Mb (arr

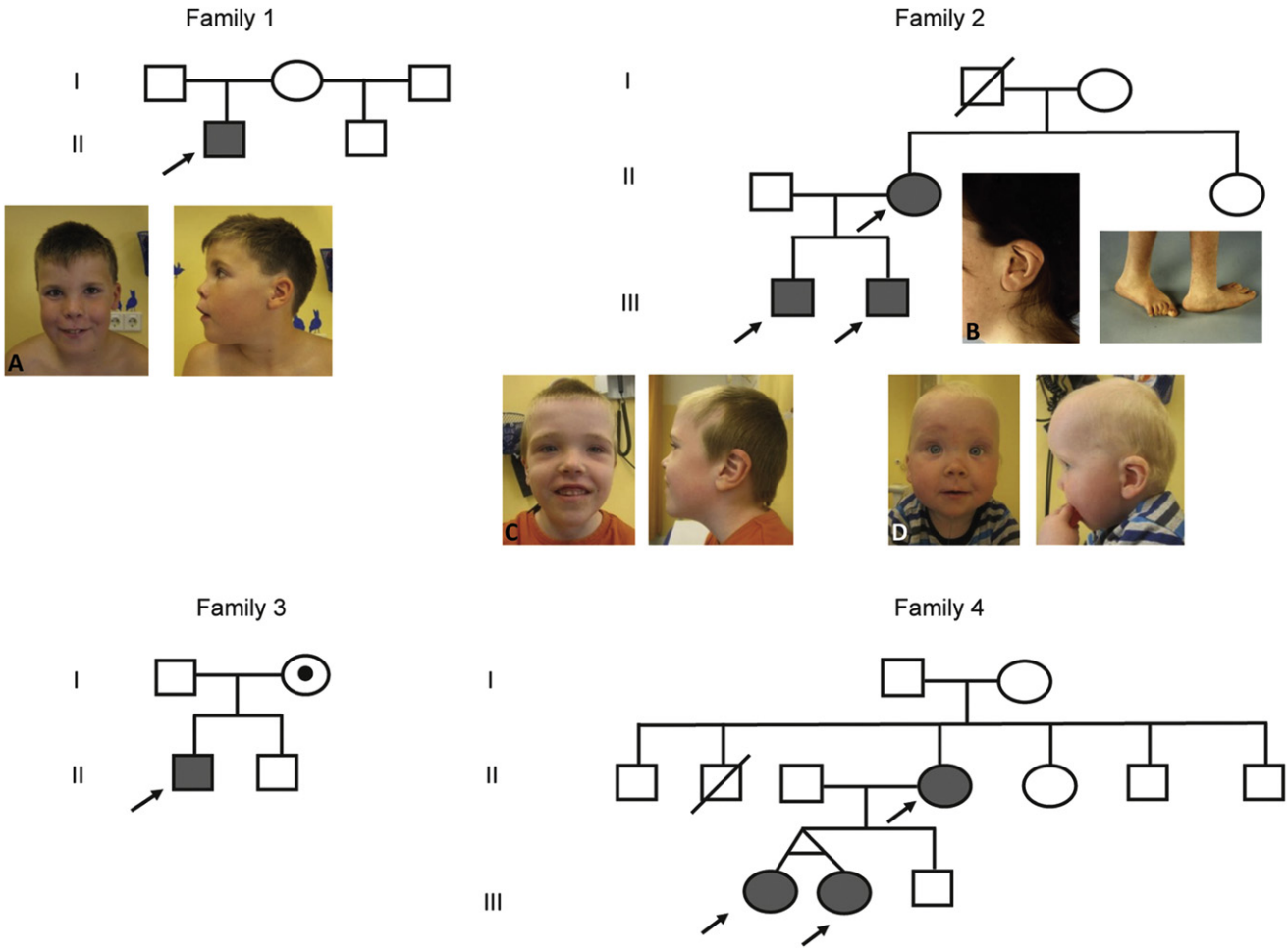

Figure 1. Pedigrees and Clinical Pictures of Affected Individuals
The individuals with an 18q22.3q23 microdeletion are depicted above (families 1 and 2), and the pedigrees of individuals with a *TSHZ1* mutation are depicted below (families 3 and 4).
Note the mild dysmorphic features in individual 1 (A), including hypertelorism, an upturned nasal tip, and a thin upper lip. The mother (B) and sons (C and D) of family 2 display several features in common, consisting of hypertelorism, down-slanting palpebral fissures, a broad mouth, characteristic low-set ears, and bilateral foot deformities.

snp 18q22.3q23 [SNP_A-1893660 > SNP_A-1815424]×1; Figure 2A).

Individual 3 is the first son of individual 2 and her healthy husband. He was born after an uneventful pregnancy at term and had normal birth parameters. Like his mother, he had congenital bilateral vertical talus and bilateral CAA type IIA (Table 1). Computed tomography (CT) scans showed significant narrowing of the external auditory canals, opacification of the mastoid and middle ear probably related to otitis media, and normal anatomical aspects of the inner ear.

During the first years of life, his hearing was assisted by a bone-conductive hearing aid on a softband. The latter was replaced by a percutaneous titanium screw at the age of 4 3/4 years. Examination at 5 1/2 years of age showed a healthy, cooperative boy with normal height, weight, and head circumference. Dysmorphisms included hypertelorism, mild down-slanting palpebral fissures, a broad mouth, and characteristic low-set ears with a prominent superior crus of the antihelix and hypoplasia of the descending helix (Figure 1C). SNP-array analysis revealed the same 2.5 Mb interstitial deletion (arr snp 18q22.3q23 [SNP_A-1893660 > SNP_A-1815424]×1) as observed in his affected mother (data not shown).

Individual 4 is the second child of individual 2. A prenatal ultrasound at 20 weeks gestation showed congenital bilateral vertical talus. He was born at term after an otherwise uncomplicated pregnancy and had a normal birth weight. Similar to his mother and older brother, he had congenital bilateral vertical talus and distinctive dysmorphic features including hypertelorism and low-set ears with hypoplasia of the descending helix (Figure 1D). Otoscopic examination showed bilateral narrowing of the auditory canals, consistent with CAA type IIA.

During the last examination at the age of 1 year, his parents reported a motor delay. SNP-array analysis revealed the same 2.5 Mb interstitial deletion (arr snp 18q22.3q23 [SNP_A-1893660 > SNP_A-1815424]×1) as observed in

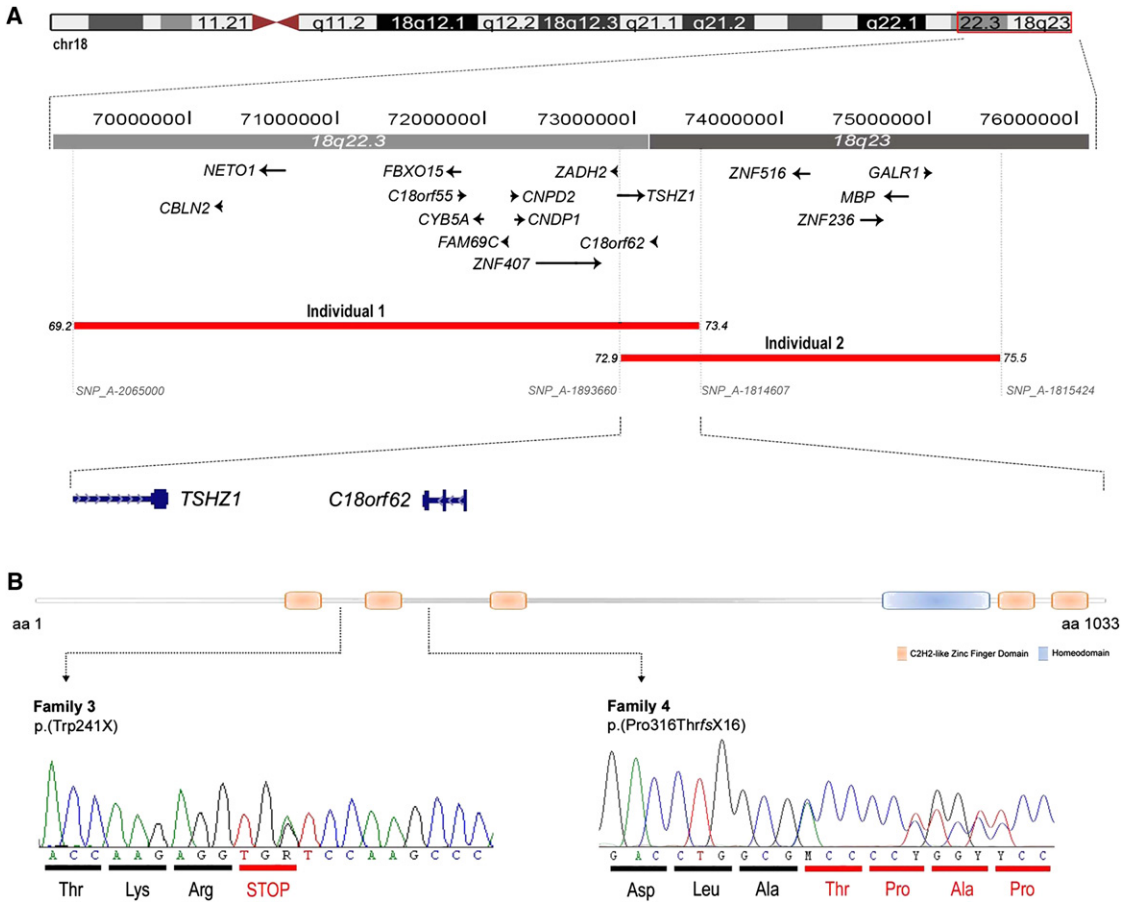

Figure 2. Detailed Genomic View of 18q23.3q23; Organization of *TSHZ1* and Mutations Detected
(A) A schematic representation shows the transcripts mapped to the 18q23.3q23 region. Deletions detected by SNP-array analysis in individual 1 and family 2 are shown by red solid lines, and details on the first and last deleted SNPs are annotated in gray. The shortest region of deletion overlap contains a known gene, *TSHZ1,* and an open reading frame, c18orf62, of unknown function.
(B) A schematic representation of the TSHZ1 protein shows its domain structure. Targeted Sanger sequencing of *TSHZ1* in families 3 and 4 revealed mutations in both families; the mutations are shown as partial electropherograms for both families, respectively. Of note, the protein sequence denoted below the electropherograms only shows the mutated sequence.

his affected mother and brother (data not shown), indicating full cosegregation of the deletion with the phenotype in this family.

Interestingly, the microdeletions in individuals 1 and 2 (and both her sons) showed a 459 kb deletion overlap, which contains one hypothetical protein (C180rf62) and a single known gene, Teashirt Zinc Finger Homeobox 1 (*TSHZ1*; NM_005786.4) (Figure 2). *TSHZ1* was considered to be a good candidate gene for the observed CAA phenotype on the basis of the deletion overlap and the fact that all four individuals presented with the common feature of narrow or atretic external auditory canals. This hypothesis was further supported by previously reported *Tshz1* loss-of-function mutations in mice; these mutations lead to specific malformations of the middle ear components[19] and emphasized the importance of *TSHZ1* in the developing middle ear. Therefore, conventional bidirectional Sanger sequencing was performed for this specific gene in 11 persons (6 sporadic and 5 familial individuals) with an isolated, bilateral form of CAA type IIA and normally shaped pinnae. In total, four individuals and one unaffected relative showed heterozygous loss-of-function mutations, including a sporadic affected person (individual 5), his unaffected mother, and a family with three affected individuals consisting of a mother and her two daughters (individuals 6–8) (Table 1).

In individual 5 (family 3), we identified a heterozygous c.723G>A mutation, which was predicted to introduce the premature stop codon p.(Trp241X) (Figure 2B). This boy was the first child of healthy, nonconsanguineous parents (Figure 1). He was born at term after an uneventful pregnancy, had normal birth parameters, and no congenital anomalies were detected. He had normal motor development, but impaired speech and delayed language development were noticed between the ages of 3 and 4 years. Pure-tone audiometry at the age of five demonstrated a 42 dB bilateral conductive hearing loss due to CAA type IIA (Figure 3B). A BAHA Softband and subsequent percutaneous titanium BAHAs were applied successfully.

Physical examination at the age of 10 years showed no facial dysmorphisms or other features associated with the previously determined critical 18q deletion regions that

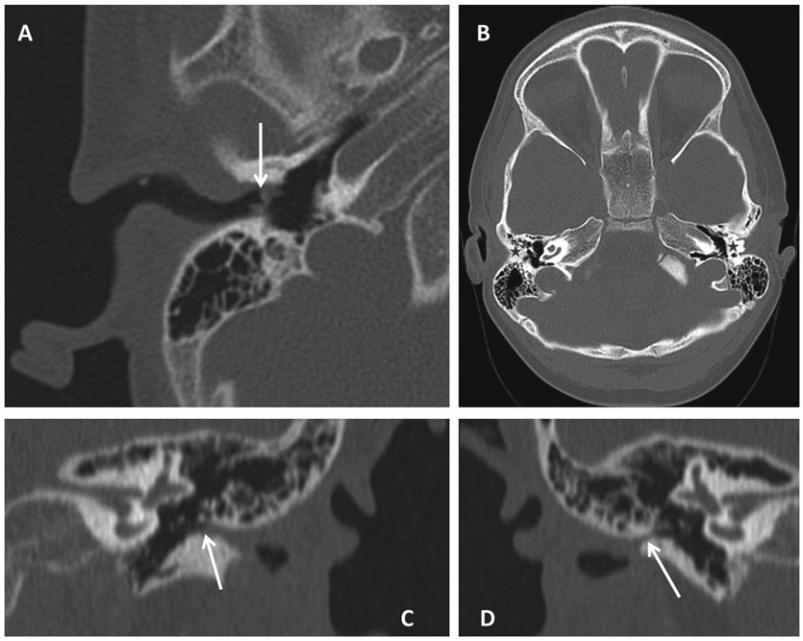

Figure 3. CT Scan Images of Individuals with an 18q Deletion or *TSHZ1* Mutation
An axial CT scan of individual 1 (A) shows a narrow external auditory canal with a normal tympanic membrane and a grommet (white arrow) in place of the right ear. (B) shows an axial-plane bilateral CT scan of individual 5 with bilateral CAA type IIA (stars). (C) shows a coronal CT reconstruction of individual 7, demonstrating a CAA type IIA (arrow) and (D) shows a coronal CT reconstruction of individual 8, demonstrating a CAA type IIA (arrow).

include *TSHZ1*. Segregation studies revealed that the detected stop mutation was also present in his phenotypically normal mother. Examination of her ears demonstrated no abnormalities to the external auditory canal or to the tympanic membrane or her hearing.

In family 4 (Figure 1), we identified a frameshift mutation due to a single base pair insertion (c.946_947delinsA), which is predicted to cause the premature stop codon p.Pro316Thr*fs*X16 (Figure 2B). As expected, this mutation showed an autosomal-dominant segregation pattern. The affected mother of this family (individual 6) had isolated bilateral conductive hearing loss due to CAA type IIA, for which she had bilateral surgical treatment at the age of 3. Her hearing declined slowly over the following decades, and she recently received a BAHA on the left side at 42 years of age.

Individuals 7 and 8, a monozygotic twin pair, are the daughters of individual 6 and also both displayed CAA type IIA (Figures 3C and 3D). They had undergone a canalplasty on one ear, and on the contralateral ear, they wore a bone-anchored hearing aid. The mother and daughters showed no notable dysmorphic facial features or any other abnormalities associated with 18q deletion syndrome. It is worth noting that a sequence analysis of the phenotypically normal maternal parents did not reveal the presence of this frameshift mutation, indicating that the mutation occurred de novo in the index person of this family.

The remaining seven individuals did not show any base pair mutations in the coding sequence of *TSHZ1*.For these individuals, whole-gene deletions were excluded with the Cytogenetics Whole-Genome 2.7M Array (Affymetrix) according to the manufacturer's instructions.

To test the (clinical) specificity of *TSHZ1* mutations, we subsequently tested a cohort of 24 individuals with a unilateral form of CAA type I, IIB, or III (Table S1, available online). In 10 of the 24 individuals, the CAA phenotype was accompanied by mild to severe developmental malformation of the external ear(s), such as microtia or anotia. Sanger sequencing did not reveal any causal mutations in this cohort.

In previous studies, we proposed, in accordance with reports of other groups, [17] that the critical region for isolated CAA was located on chromosome 18q22.3.[14,18] This region was reported to contain nine candidate genes, yet none of the reports mentioned *TSHZ1* as a potential candidate for CAA.

We detected two small overlapping 18q microdeletions in individuals with CAA as a common feature, narrowing the critical interval for CAA to 72.9–73.4 Mb and establishing *TSHZ1* as a strong candidate for the CAA gene. The subsequent detection of both a nonsense and a frameshift mutation in *TSHZ1* in two families with nonsyndromic CAA clearly shows that hemizygosity of *TSHZ1* indeed leads to isolated CAA through haploinsufficiency. This observation further suggests that other genes in the previously established critical region in 18q22.3 should be more relevant to the intellectual disability, facial dysmorphisms, and foot deformities that are commonly seen in the 18q deletion syndrome.

The members of family 2 (individuals 2, 3, and 4) displayed a collection of features including characteristic facial features, bilateral CAA, and vertical talus. The mother and sons described here very much resembled the phenotype of three males and three females in a family described by Rasmussen in 1979 (MIM 133705).[21] Possibly, hemizygosity of one of the other four genes deleted in individuals 2–4, namely *ZNF516*, *ZNF236* (MIM 604760), *MBP* (MIM 159430), and *GALR1* (MIM 600377), could lead to haploinsufficiency and cause congenital foot deformities like vertical talus. Molecular analysis of the family members described by Rasmussen could provide more insight into the hypothesis that Rasmussen syndrome is caused by a microdeletion, which is identical or at least overlapping with the deletion detected in individuals 2–4 in our study.

TSHZ1 consists of two exons, of which only exon 2 is coding and has a genomic size of 79 kb. *TSHZ1* is a member

of the teashirt-type zinc-finger protein family and encodes putative zinc finger transcription factors that are broadly expressed during mouse embryogenesis.[22] In vertebrates, three *TSHZ1*-related genes (*TSHZ1*, *TSHZ2* [MIM 614118], and *TSHZ3* [MIM 614119]) have been isolated on the basis of sequence homology.

Recently, knockout mice have been generated for *Tshz1*.[19] *Tshz1* inactivation in mice leads to neonatal lethality and causes multiple developmental abnormalities, including a severe middle-ear phenotype that mimics defects observed in individuals with isolated CAA. In addition, *Tshz1*-deficient mice show a defect of the soft palate, a feature which was not seen in any of the individuals in the current study. The phenotype of *Tshz1*-deficient mice resembles the phenotype seen in *Hoxa3* and *Sall3* mouse mutants.[23,24] Interestingly, the gene families to which these genes belong—the Hox and Spalt gene families—genetically interact with teashirt (*tsh*) in *Drosophila*.[25] Possibly, mutations in genes of these families give rise to a similar CAA phenotype, either isolated or as part of a more complex syndrome. Possible candidate genes might include other members of the teashirt zinc-finger protein family, such as *TSHZ2* and *TSHZ3*, and members of the human HOX and SPALT families. A member of the HOX family, *HOXA2* (MIM 604685), has indeed been described as playing a crucial role in auditory-system malformations, more specifically in an autosomal-recessive form of bilateral microtia, hearing impairment, and partial cleft palate.[26] Similar to what we now observe for *TSHZ1,* the human phenotype caused by *HOXA2* mutations is in concordance with that of the *Hoxa2* knockout mouse.[27] However, DNA-sequence analysis of *HOXA2* in individuals with isolated microtia did not reveal mutations.[28] In the same fashion, not all individuals with an isolated form of CAA type IIA selected for the present study showed mutations in *TSHZ1*; this finding is in line with the fact that CAA is observed as an endophenotype in multiple syndromes, thereby suggesting that several CAA genes still await discovery.

Most individuals with a terminal 18q deletion have been diagnosed with CAA type I or II.[29] Also, all persons in this study had normal external-ear morphology, a finding that is consistent with the observation that the majority of 18q deletion individuals either have normal external ears or show only minor abnormalities such as low-set ears or prominent helices.

On the basis of our observation that intragenic mutations in *TSHZ1* could only be detected in the four individuals with isolated CAA type IIA who, except for their narrow or atretic auditory canals, have no other congenital malformations, it might be speculated that hemizygosity of *TSHZ1* is limited to and specific to this type of CAA.

The fact that the nonsense mutation in *TSHZ1* in individual 5 was inherited from his phenotypically normal mother can be explained by reduced penetrance. This observation is in accordance with previous reports that describe a CAA incidence of 26% in individuals with an 18q deletion of any kind and an incidence of up to 78% in individuals with a deletion of the critical CAA region that includes *TSHZ1*.[12,16,30] Therefore, nonpenetrance is not unexpected in a carrier of a mutation in the CAA gene.

In conclusion, we have detected both point mutations and copy-number variants leading to haploinsufficiency due to hemizygosity of *TSHZ1* as causes of bilateral CAA type II (in the absence of microtia or anotia) both in isolated nonsyndromic individuals and in persons with the 18q deletion syndrome. Our results provide compelling evidence that the 18q deletion syndrome is a true contiguous gene syndrome, in which the CAA endophenotype is explained by the deletion of *TSHZ1*. Detailed genotype-phenotype studies might further delineate the other phenotypic components of this syndrome.

Supplemental Data

Supplemental Data include one table and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
UCSC Genome Browser, http://genome.ucsc.edu/

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