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ARTICLE

Novel variants in *GNAI3* associated with auriculocondylar syndrome strengthen a common dominant negative effect

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Auriculocondylar syndrome is a rare craniofacial disorder comprising core features of micrognathia, condyle dysplasia and question mark ear. Causative variants have been identified in *PLCB4*, *GNAI3* and *EDN1*, which are predicted to function within the EDN1–EDNRA pathway during early pharyngeal arch patterning. To date, two *GNAI3* variants in three families have been reported. Here we report three novel *GNAI3* variants, one segregating with affected members in a family previously linked to 1p21.1-q23.3 and two *de novo* variants in simplex cases. Two variants occur in known functional motifs, the G1 and G4 boxes, and the third variant is one amino acid outside of the G1 box. Structural modeling shows that all five altered GNAI3 residues identified to date cluster in a region involved in GDP/GTP binding. We hypothesize that all *GNAI3* variants lead to dominant negative effects.

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INTRODUCTION

Auriculocondylar syndrome (ACS, OMIM 602483 and 614669) is a rare disorder of the first and second pharyngeal arches, mainly characterized by micrognathia, agenesis or hypoplasia of the mandibular condyle and a typical auricular malformation known as a question mark ear (QME). Other frequently associated malformations include abnormal palate, microstomia, full cheeks, glossoptosis, respiratory distress and hearing loss. A wide range of inter- and intrafamilial clinical variability is observed in ACS.[1]

We previously mapped the first ACS locus to 1p21.1-q23.3 (ACS1)[2] in a large Brazilian family that was initially described by Guion-Almeida *et al*.[3] Genetic heterogeneity was also suggested as affected members of two other families were not associated with this locus.[2,4] Rieder *et al*[5] subsequently showed that variants in phospholipase C beta 4 (*PLCB4*), at 20p12.2, and in guanine nucleotide binding protein (G protein) alpha-inhibiting activity polypeptide 3 (*GNAI3*), located within the 1p21.1-q23.3 candidate interval, are responsible for most ACS cases. GNAI3 and PLCB4 are predicted to be signaling molecules of the endothelin 1 (EDN1)–endothelin receptor type A (EDNRA) pathway, which is important for patterning of the pharyngeal arches in animal models.[1,5] The involvement of this pathway in ACS was recently confirmed by the finding of *EDN1* variants in ACS and in isolated QMEs (OMIM 612798).[6]

Thus far only two GNAI3 variants, c.118G>C and c.141C>A (predicted consequence p.(Gly40Arg) and p.(Ser47Arg), respectively) in three unrelated familial cases have been reported.[5,7] No *de novo* variants have been described. Both variants are located within the G1 box, one of the five conserved motifs (G1–G5) involved in binding guanosine diphosphate (GDP)/guanosine triphosphate (GTP) in the catalytic domain of G-alpha proteins and RAS family members.[8] It is unclear whether these variants have a gain-of-function[5] or dominant negative effect.[7] A larger number of cases is necessary to elucidate these questions.

Here we report the molecular analysis of *GNAI3* in the original ACS1 Brazilian family linked to 1p21.1-q23.3[2] and in two sporadic ACS cases without previous genetic investigations.[9] We describe a novel heterozygous variant in *GNAI3* in each case. These variants are predicted to interfere with GDP/GTP binding, supporting a dominant negative mode of action for *GNAI3* variants in ACS.

MATERIALS AND METHODS

Patients and DNA samples

Approval for this study was obtained from the Biosciences Institute Research Ethics Committee of the University of São Paulo (USP) and from the Comité de Protection des Personnes Ile-de-France II. Clinical descriptions have been previously reported for family ACS1, referred to as F2 in Masotti *et al*,[2,3] and the sporadic case in Propst *et al*,[9] hereafter referred to as Sp1. Sporadic case 2

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(Sp2; Supplementary Figure S1), initially referred for hemifacial microsomia, presented with micrognathia, microstomia, bifid uvula, right lateral tongue polyp, full cheeks, a right QME and normal left ear, conductive hearing loss, severe obstructive sleep apnea and systolic murmur. His development is within normal limits. He had normal full spine X-ray, renal ultrasound scan and SNP microarray (HumanCoreExome-12 v1.0) (Illumina, San Diego, CA, USA). A clinical summary of all cases is in Supplementary Table S1. Methods for extraction of genomic DNA, Sanger sequencing and microsatellite analysis, and programs used for analysis of variants are provided in Supplementary Information. Variants were submitted to the *GNAI3* gene variant database (http://www.LOVD.nl/GNAI3).

RESULTS

Family ACS1

A novel heterozygous, predicted missense variant was identified in exon 7 of *GNAI3*: c.805A>T; p.(Asn269Tyr) (RefSeq: NM_006496.3) (Figure 1a). Except for one non-penetrant individual (II-7), the variant segregates with the ACS phenotype. Reconstruction of previously published haplotypes of the chromosome 1 linkage region[2] along with the *GNAI3* genotypes showed that individual II-7 shares only a proximal region of the at-risk haplotype (Supplementary Figure S2).

Sp1

Sequencing of *GNAI3* revealed a heterozygous variant in exon 2: c.134G>T predicted to give the missense change p.(Gly45Val) (Figure 1b). The variant was *de novo* in the patient as it was not present in parental DNA.

Sp2

A *de novo*, heterozygous, predicted missense variant was identified in exon 2 of *GNAI3*: c.143C>A; p.(Thr48Asn) (Figure 1c).

Variants affecting Asn269, Gly45 or Thr48 of GNAI3 are absent from dbSNP137 and the Exome Variant Server. Variants affecting Asn269 are also not present in 275 ethnically matched control Brazilian samples. To confirm that the Sp1 and Sp2 variants were *de novo*, polymorphic microsatellites were tested in each family; all microsatellites (11/11 in Sp1 and 8/8 in Sp2) were consistent with paternity.

All three *GNAI3* variants are predicted to disrupt a nucleotide and amino acid highly conserved in vertebrates (Figure 2), suggesting important roles for these residues in protein function. Each amino-acid change was predicted as probably damaging by PolyPhen-2 and damaging by SIFT.

GDP/GTP binding in the catalytic domain of GNAI3 involves five small motifs, the G1–G5 boxes. The p.(Gly45Val) and p.(Thr48Asn) variants fall within and one amino acid outside of the G1 box (residues 40-47), respectively. The p.(Asn269Tyr) variant falls within the G4 box (Figure 2a–c). Mapping of the amino acids that show variants in ACS to a published GNAI3 crystal structure[10] indicates that the side chain of Asn269 forms hydrogen bonds that contact GDP and the G1 box, whereas the backbone of Gly45 and the backbone and side chain of Thr48 also form hydrogen bonds with GDP (Figure 3). Variant of these residues may therefore directly compromise binding of GNAI3 to GDP/GTP.

DISCUSSION

In this study, we demonstrate that the GNAI3 variant, p.(Asn269Tyr), is the likely cause of ACS in the original ACS1 family, thus confirming our previous linkage analysis.[2] The non-penetrant individual (II-7) in this family is consistent with the incomplete penetrance observed in

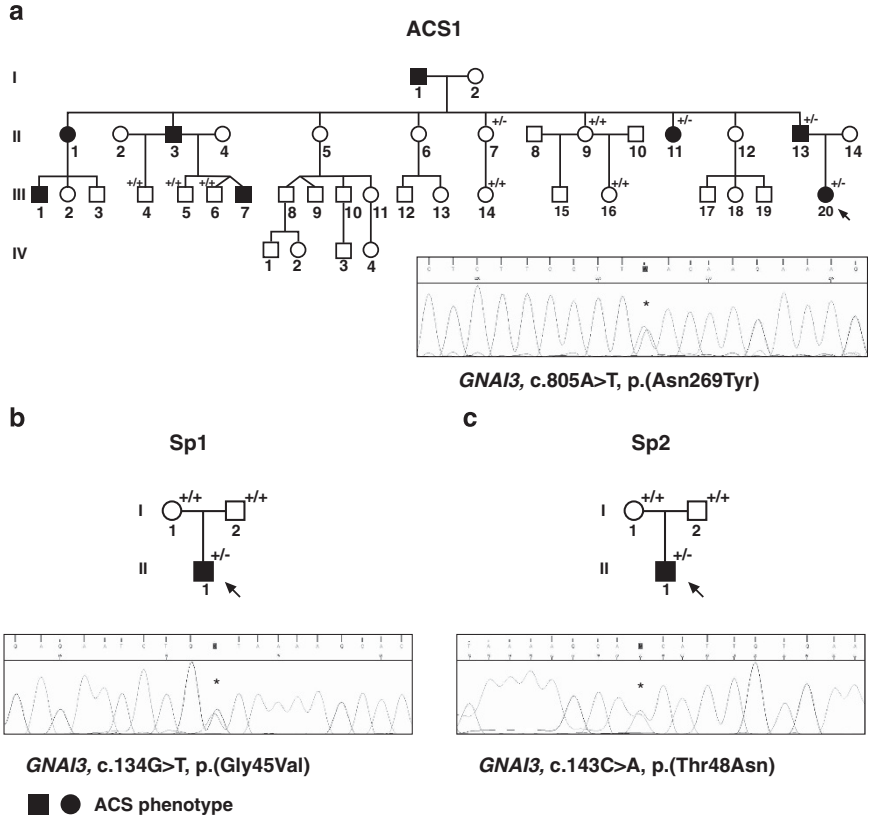

Figure 1 Identification of novel heterozygous, predicted missense *GNAI3* variants. (**a**) *GNAI3* c.805A>T variant in the ACS1 family. (**b**) *GNAI3* c.134G>T variant in Sp1. (**c**) *GNAI3* c.143C>A variant in Sp2. Wild-type allele is indicated by a plus (+) sign; the allelic variant is represented by a minus (−) sign in the pedigree and indicated with an asterisk in the chromatogram; the arrows indicate proband.

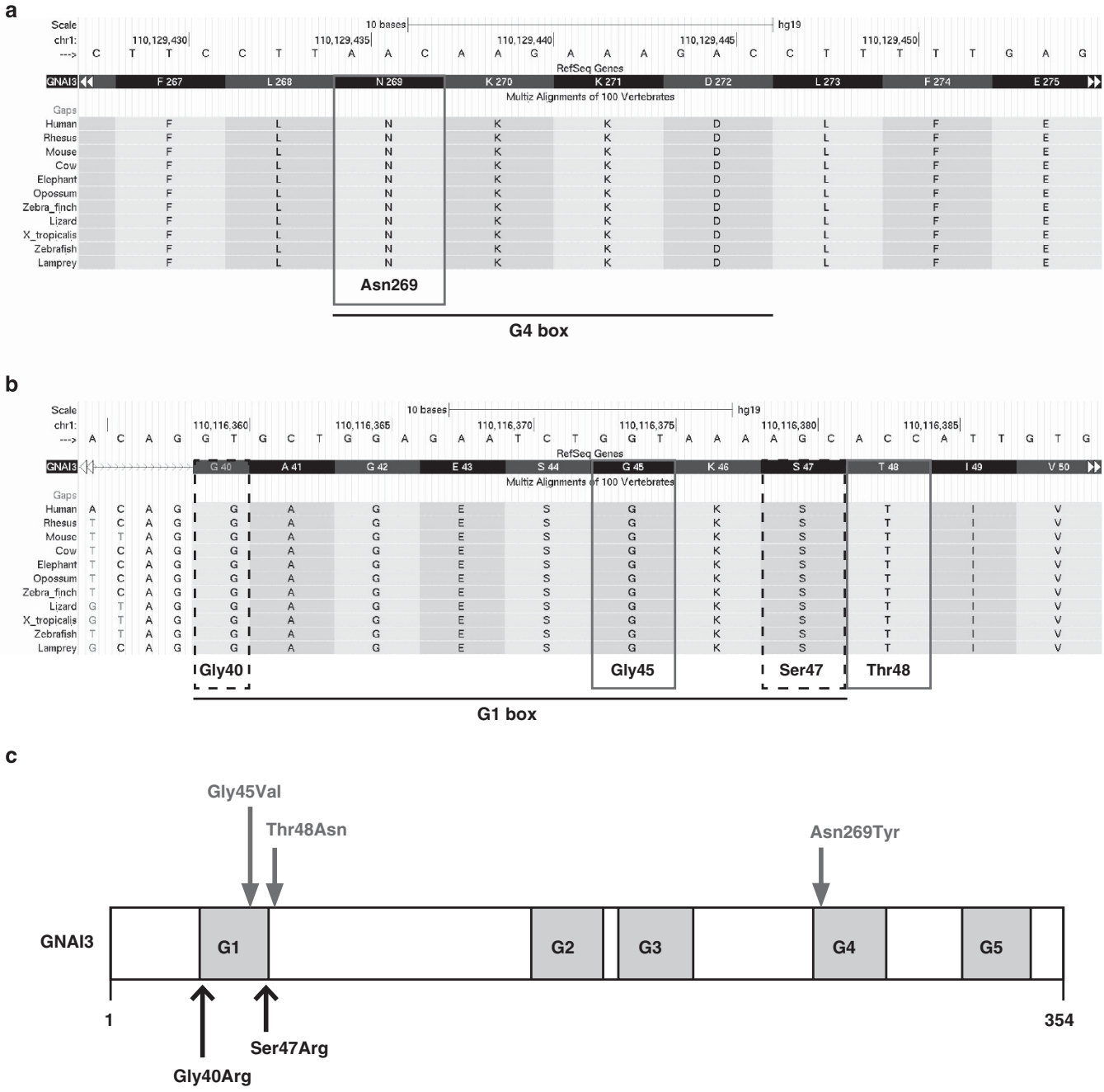

Figure 2 Conservation and location of GNAI3 residues altered in ACS. (**a**, **b**) UCSC screenshot displaying conservation of the GNAI3 G1 and G4 boxes (underlined) throughout vertebrates. The position of the variants identified here and previously reported variants are indicated by full and dashed boxes, respectively. (**c**) Schematic of GNAI3 showing conserved domains as described in the CDD; G1–G5 boxes are depicted in gray; previously described variants are indicated with arrows below the schema; the present variants are indicated with full arrows above the schema.

another family with a *GNAI3* variant.[5] We also report the first *de novo* variants in *GNAI3* in two simplex ACS cases, Sp1 and Sp2. Our study brings the total of ACS cases harboring a *GNAI3* variant to six, comprised of five different variants.

G-alpha proteins and RAS family members share a structural domain involved in GDP/GTP binding that is composed of the G1–G5 boxes.[8] The two previously reported GNAI3 variants fall within the G1 box (at Gly40 and Ser47), as does the p.(Gly45Val) variant identified here in Sp1, whereas the p.(Thr48Asn) variant of Sp2 falls one amino acid outside of the G1 box. p.(Asn269Tyr) is the first variant located within the G4 box. All three of the variant residues identified here (Gly45, Thr48 and Asn269), along with Ser47, are predicted to form hydrogen bonds with GDP in the GNAI3 crystal structure. Supporting the significance of the p.(Asn269Tyr) variant, contact between hydrogen bonds of the G4 box residues of GTPase superfamily members and the guanine ring of GDP/GTP are known to confer specificity to GTP over ATP and provide stabilizing interactions with G1 box residues.[11] Although it was speculated that p.(Gly40Arg) modified the conformation of a portion of the protein that

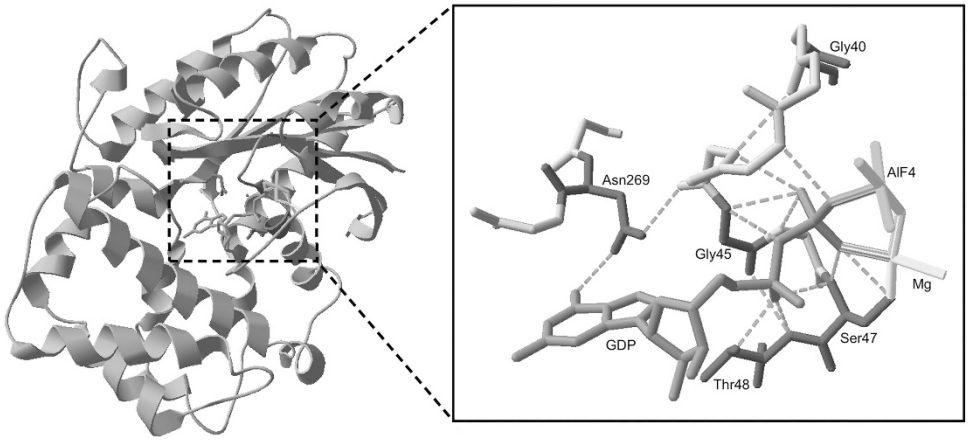

Figure 3 Structure of the GNAI3 protein (PDB ID: 2ODE) and the positions of residues affected in ACS. To the left is a view of the entire protein in ribbon mode. To the right is a magnified view of selected regions surrounding the GDP molecule, in stick mode. The five amino acids that show variants in ACS are in pink (Gly40, Gly45, Ser47, Thr48 and Asn269). For clarity, side chains are only shown for these five. Hydrogen bonds are shown as green dotted lines. AlF_4 (aluminum tetrafluoride) is a substitute for the third phosphate of GTP. Mg, magnesium.

interacts with downstream effectors, resulting in a gain-of-function protein,[5] we suggest rather that all GNAI3 variants may disrupt GDP/GTP binding (directly or indirectly) without disrupting the overall structure of the protein, thereby resulting in dominant negative effects, perhaps via sequestration of GNAI3's cognate beta-gamma G protein subunits or G protein-coupled receptor, as has been shown for other G-alpha proteins.[12] Supporting this idea, the equivalent variant to GNAI3 p.(Asn269Tyr) in the G4 box of HRAS (p.Asn116Tyr) shows a dominant negative effect, inhibiting GTP binding activity and proliferation, and causing induction of apoptosis in human cancer cell lines.[13,14] Similarly, the previously published p.(Ser47Arg) GNAI3 variant is predicted to be a dominant negative, based on the dominant negative action of other G proteins and RAS family members with a variant of the equivalent residue.[7] Although GNAI3 belongs to the inhibitory class of G-alpha proteins, originally described for their ability to inhibit adenylyl cyclase, it has been reported that activation of G protein heterotrimers containing GNAI3 leads to inhibitory or stimulatory responses, depending on the downstream effector: adenylyl cyclase or phospholipase C, respectively.[15] Supporting the idea that *GNAI3* and *PLCB4* variants have a similar negative effect on the EDN1–EDNRA–DLX pathway, expression of *DLX5* and *DLX6* was reduced in mandibular osteoblasts of ACS patients mutated for *GNAI3* or *PLCB4*.[5] Finally, several deletions that remove *GNAI3* have been reported in the DECIPHER database; of the eight cases with phenotypes listed, auricular malformations are not mentioned, supporting the idea that the ACS *GNAI3* variants are not haploinsufficient alleles.

Sp1 and Sp2 have conductive hearing loss, which in Sp1 was associated with fusion of the malleus and incus,[9] and one *GNAI3*-variant individual in the ACS1 family presented with sensorineural hearing loss.[3] Hearing loss was reported in both members (conductive in one case and unspecified in the other) of a family harboring a *GNAI3* variant.[5,16] Interestingly, zebrafish with variants in components of the endothelin pathway display fusion of some jaw cartilage elements.[1] In addition, targeted deletion of *Gnai3* in mice results in rib and vertebral fusions[17] and in defects in cochlear hair cells.[18] Collectively these findings suggest independent roles for GNAI3 in the development of multiple skeletal elements and in the inner ear, suggesting the possibility of conductive and/or sensorineural hearing loss in *GNAI3*-associated ACS.

In conclusion, here we have described three new ACS-associated variants in *GNAI3*. We suggest that these and previously described GNAI3 variants interfere directly or indirectly with GDP/GTP binding, leading to dominant negative effects. Our analysis indicates that interaction with GDP/GTP will be a strong predictor of pathogenicity for future ACS-associated GNAI3 variants.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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