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AXIN1 mutations in nonsyndromic craniosynostosis

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OBJECTIVE Occurring once in every 2000 live births, craniosynostosis (CS) is the most frequent cranial birth defect. Although the genetic etiologies of syndromic CS cases are well defined, the genetic cause of most nonsyndromic cases remains unknown.

METHODS The authors analyzed exome or RNA sequencing data from 876 children with nonsyndromic CS, including 291 case-parent trios and 585 additional probands. The authors also utilized the GeneMatcher platform and the Gabriella Miller Kids First genome sequencing project to identify additional CS patients with *AXIN1* mutations.

RESULTS The authors describe 11 patients with nonsyndromic CS harboring rare, damaging mutations in *AXIN1*, an inhibitor of Wnt signaling. AXIN1 regulates signaling upstream of key mediators of osteoblast differentiation. Three of the 6 mutations identified in trios occurred de novo in the proband, while 3 were transmitted from unaffected parents. Patients with nonsyndromic CS were highly enriched for mutations in *AXIN1* compared to both expectation ($p = 0.0008$) and exome sequencing data from > 76,000 healthy controls ($p = 2.3 \times 10^{-6}$), surpassing the thresholds for genome-wide significance.

CONCLUSIONS These findings describe the first phenotype associated with mutations in *AXIN1*, with mutations identified in approximately 1% of nonsyndromic CS cases. The results strengthen the existing link between Wnt signaling and maintenance of cranial suture patency and have implications for genetic testing in families with CS.

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KEYWORDS *AXIN1*; de novo mutations; Wnt signaling; nonsyndromic craniosynostosis; craniofacial

Craniosynostosis (CS) refers to the premature fusion of one or more cranial sutures, causing abnormal growth of the infant cranium. Patients are treated surgically in infancy to mitigate the risk of elevated intracranial pressure and associated adverse neurodevelopmental outcomes. Recent efforts to identify the genetic causes of nonsyndromic CS have identified considerable locus heterogeneity for this complex trait.[1] Nonsyndromic cases are sporadic in > 95% of families studied to date, demonstrating a role for both de novo (sporadic) mutations and rare transmitted mutations with incomplete penetrance. In studying a cohort of 291 case-parent trios (composed of an affected child and two unaffected parents), we previously identified a significant excess of de novo mutations in genes encoding negative regulators of the Wnt, BMP, and FGF/MAPK signaling pathways, consistent with observations that many monogenic forms of syndromic CS are caused by mutations within these same pathways.[2] The results identified loss-of-function (LOF) mutations in *SMAD6*, an inhibitor of BMP signaling, as the most frequent genetic cause of nonsyndromic CS, with rare LOF mutations identified in approximately 6% of sag-

ABBREVIATIONS CS = craniosynostosis; GOF = gain-of-function; LOF = loss-of-function.

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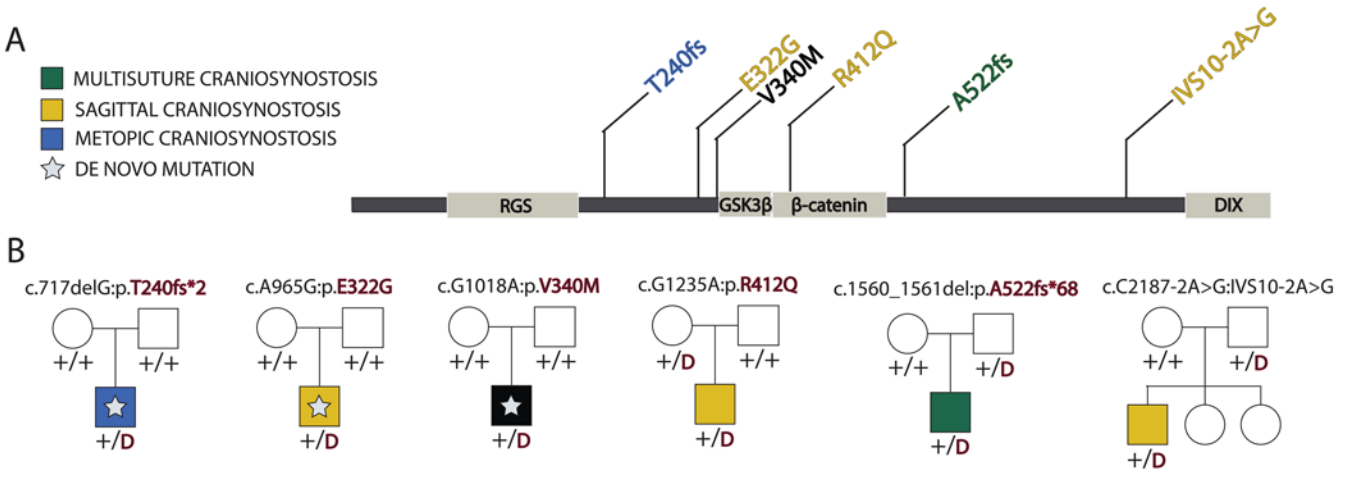

FIG. 1. Pedigrees with mutations in *AXIN1* and nonsyndromic CS. **A:** Domain structure of AXIN1 showing the locations of the RGS, GSK3β, β-catenin binding, and DIX domains. De novo or rare damaging mutations identified in the CS probands are indicated. The *color of text* denotes suture(s) showing premature closure. **B:** Pedigrees harboring de novo (denoted by *stars* within pedigree symbols) or rare transmitted mutations in *AXIN1*. *Filled and unfilled symbols* denote individuals with and without CS, respectively. The *AXIN1* mutation identified in each kindred is noted above each pedigree.

ittal and metopic cases.[2,3] No other gene had more than one damaging de novo mutation in this cohort; however, additional genes within the Wnt, BMP, and FGF signaling pathways had transmitted damaging mutations in addition to a single damaging de novo mutation. After *SMAD6*, the second most commonly mutated gene within the implicated pathways was *AXIN1*, encoding an inhibitor or Wnt signaling with no known phenotype associated with germline mutations. Here, we sought to determine the potential contribution of *AXIN1* mutations to nonsyndromic CS risk in an expanded cohort.

Methods

Patients were enrolled in the Yale Craniofacial Surgery Clinic or via social media recruitment on the Cranio Kids-Craniosynostosis Support Facebook page. Each patient provided DNA via buccal swab or saliva samples, along with medical records including diagnostic imaging. DNA was extracted from samples via standard protocols. Exome sequencing was performed using the IDT xGen or Roche V2 capture reagent, followed by either 75 or 99 base paired-end sequencing on the Illumina platform, with variants called and analyzed as previously described.[4] The impact of missense mutations on protein function was inferred using MetaSVM. We assessed the probability of observing a damaging de novo missense mutation in *AXIN1* in a cohort of this size by using denovolyzeR,[5] as previously described, and the probability of observing transmitted damaging mutations in *AXIN1* by using the binomial test as previously described.[2] p values were combined using Fisher's method. We compared the frequency of rare, damaging variants (LOF or damaging missense as called by MetaSVM, with gnomAD minor allele frequency $< 5 \times 10^{-5}$) identified in the cohort of nonsyndromic CS probands to the number in gnomAD v2.1.1 by using the median allele number across all *AXIN1* variant positions (n = 249,763) and Fisher's exact test. Sequencing data from the Gabriella Miller Kids First Pediatric Research Program was obtained from the program's data resource center (https://kidsfirstdrc.org/), and RNA-sequencing data from 391 CS probands (affected patients without both parents available for sequencing) enrolled at the University of Washington were analyzed as previously described.[6]

Results

Among 291 trios studied with nonsyndromic sagittal or metopic (midline) CS, we identified 3 damaging mutations in *AXIN1*, including 1 LOF and 2 damaging missense mutations as called by MetaSVM (Fig. 1, Table 1). The first was a mutation affecting a canonical splice site (IVS10–2A > G), identified in a child with sagittal CS. This mutation was inherited from an unaffected parent. The first damaging missense mutation (p.E322G) arose de novo in a proband who also had isolated sagittal CS. The final missense mutation (p.R412Q), identified in a child with isolated sagittal CS, was inherited from an unaffected parent. Interestingly, this mutation has been identified as a somatic mutation in colorectal adenocarcinoma samples,[7] in which LOF mutations have been identified as frequent driver mutations,[8] supporting a LOF mechanism for this mutation as well. Taken together, both of the transmitted mutations are plausible LOF mutations (IVS10–2A > G and p.R412Q), and the third mutation occurred de novo producing the same phenotype, supporting an LOF mechanism for each of these 3 mutations. The probability of seeing a de novo missense along with 2 other transmitted damaging mutations in *AXIN1* in unrelated patients in a cohort of this size was highly unlikely to occur by chance ($p = 8.0 \times 10^{-4}$, Fisher's combined P; see *Methods*).

We used the GeneMatcher platform to search for additional patients with *AXIN1* mutations.[9] We matched with 2 additional clinicians caring for patients with *AXIN1* mutations, and both patients had CS (Table 1, Fig. 1). One patient

TABLE 1. Mutations identified in AXIN1

AXIN1 Nucleotide Variant (NM_181050.2)	AXIN1 Protein Alteration	Parental Test Results	Ethnicity	CS	Additional Features
c.717delG	p.T240fs*2	De novo	Middle Eastern	Metopic CS	Mild developmental delay, thick hair, 5th digit clinodactyly, cardiac murmur, unilat cryptorchidism
c.1560_1561del	p.A522Efs*68	Transmitted from father	European	Lt lambdoid & posterior sagittal CS	Mild developmental delay, ADD, thick hair, patent foramen ovale, ketotic hypoglycemia, hypertransaminasemia
c.A965G	p.E322G	De novo	European	Sagittal CS	None
c.G1018A	p.V340M	De novo	Unknown	Unknown type	Unknown
c.G1235A	p.R412Q	Transmitted from mother	European	Sagittal CS	None
c.A2187-2G	IVS10-2A>G	Transmitted from father	European	Sagittal CS	None

ADD = attention-deficit disorder.

with a transmitted frameshift mutation (p.A522Efs*68) had both sagittal and left lambdoid CS (Table 1, Fig. 2A), and a second patient had a de novo frameshift mutation (p.T240fs*2) and was diagnosed with metopic CS in early infancy (Fig. 2B). Additional clinical features, including fifth digit clinodactyly, thick hair, patent foramen ovale, and cryptorchidism were identified in these patients, as described in Table 1.

Next, we queried genome sequencing data from 320 trios (affected patients and both unaffected parents) with sporadic nonsyndromic CS sequenced as part of the Gabriella Miller Kids First project for mutations in *AXIN1*. We identified an additional damaging missense mutation in *AXIN1* in a proband with nonsyndromic CS (p.V340M) (Fig. 1). The specific suture(s) involved were not indicated in the database. Although we relied on the supplied pedigree and could not prove definitive kinship, this mutation appears to have arisen de novo as well.

In order to replicate these findings, we queried exome sequencing data from 194 isolated probands with nonsyndromic forms of midline CS, as well as RNA sequencing data from 391 isolated probands with nonsyndromic mid-

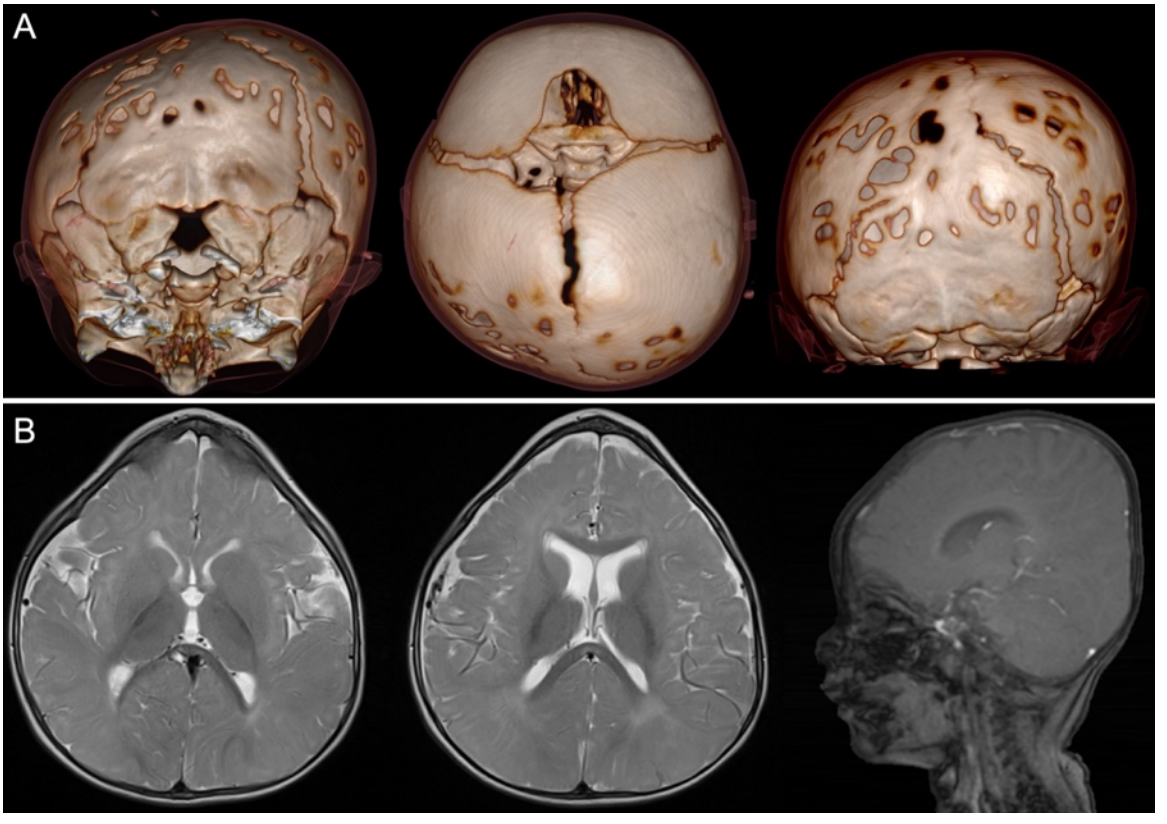

FIG. 2. Cranial imaging of CS patients with *AXIN1* mutations. **A:** 3D CT reconstructions of a child with posterior sagittal and left lambdoid CS, with evidence of thinning of the posterior cranial bones. **B:** MR images of a child with severe metopic CS.

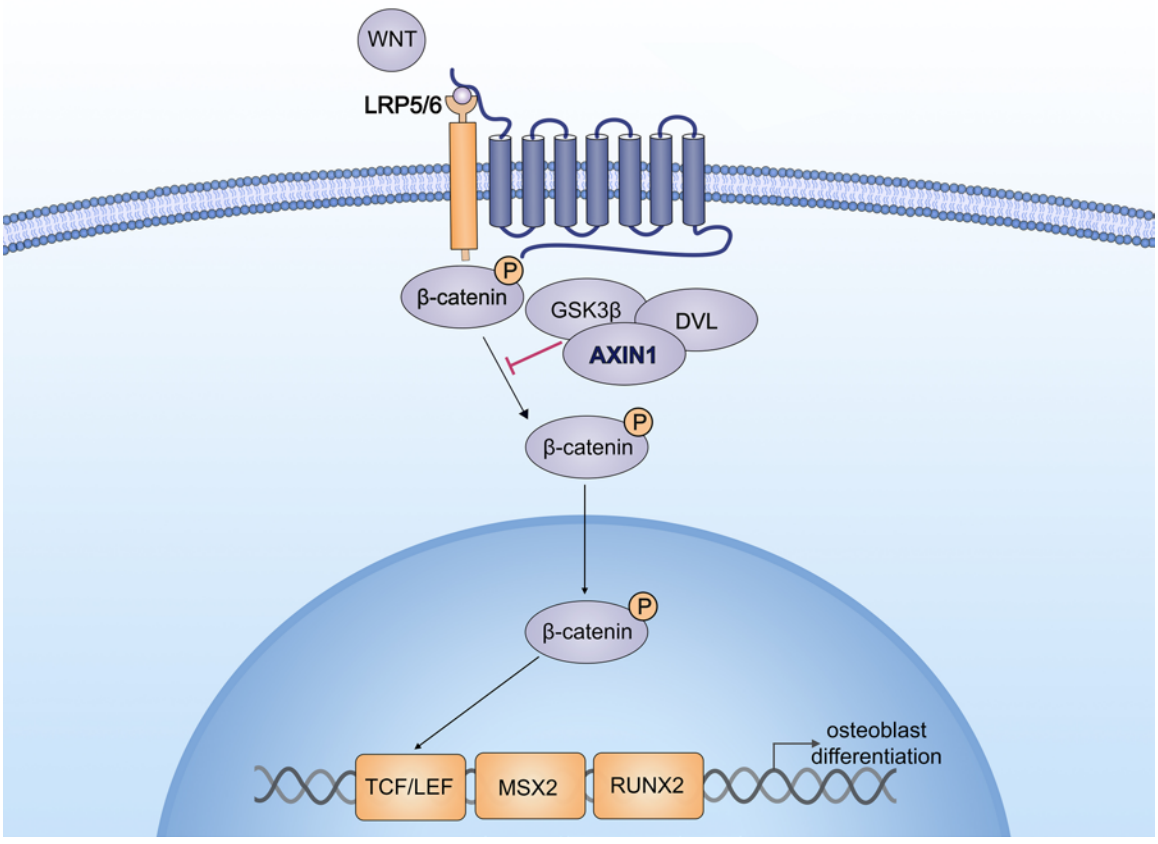

FIG. 3. AXIN1 is an inhibitor of Wnt/β-catenin signaling. AXIN1 is a part of the regulatory complex that targets activated β-catenin for ubiquitin proteasome-mediated degradation. LOF mutations in AXIN1 lead to augmented Wnt signaling, which is upstream of transcription of master regulators of osteoblast differentiation.

line CS, for additional mutations in *AXIN1*. In addition to the mutations described above, we identified 5 additional rare, damaging missense mutations in these probands. Within the 194 exomes analyzed, we identified p.N319K in a proband with sagittal CS (gnomAD frequency = 0), p.T336M in a proband with metopic CS (gnomAD frequency = 4.8×10^{-5}), and p.P697T in a proband with sagittal CS (gnomAD frequency = 0). In the RNA-Seq dataset containing 391 probands, we identified 2 additional damaging missense mutations in probands. These included p.E642Q in a child with metopic synostosis (gnomAD allele frequency = 0) and p.G804S in a child with sagittal CS (gnomAD allele frequency = 0). Each of these 5 mutations was found in an isolated proband, so we were unable to determine whether they arose de novo in the affected child or were transmitted from an unaffected parent; the potential contributions of these additional 5 mutations to CS will require further study.

In the comparison of the total number of rare (gnomAD minor allele frequency < 5×10^{-5}), damaging mutations identified in the CS cases studied (n = 1196) to those found in > 76,000 healthy controls in the gnomAD database,[10] the CS cases were highly enriched for damaging mutations (OR 8.6, $p = 2.3 \times 10^{-6}$, Fisher's exact test, see *Methods*). We estimate that damaging mutations in AXIN1 cause approximately 1% of nonsyndromic CS cases, with mutations most frequently identified in probands with sagittal CS.

In sum, we identified 11 unrelated individuals in distinct kindreds with damaging mutations in *AXIN1* who all had CS. Among the 6 mutations identified in trios, 3 of these arose de novo, 2 of the transmitted mutations were LOF mutations, and the final 1 has been identified as a somatic mutation in adenocarcinoma samples. AXIN1 LOF has been described as a frequent driver mutation in diverse cancer subtypes.[11] These findings suggest that these mutations cause loss of protein function, with haploinsufficiency of *AXIN1* contributing to CS risk.

Discussion

AXIN1 is a part of the regulatory complex that targets activated β-catenin for ubiquitin proteasome-mediated degradation, thus inhibiting Wnt signaling. β-catenin is a central signaling molecule in the canonical Wnt pathway. Various Wnt ligands bind to Frizzled and low-density lipoprotein receptor–related protein 5 and 6 coreceptors to activate β-catenin via phosphorylation, which accumulates in the cytoplasm and is translocated to the nucleus where it activates transcription of target genes (Fig. 3). Various animal studies have demonstrated mechanisms by which β-catenin signaling promotes osteoblast differ-

entiation and bone formation, and premature differentiation of osteoblast precursors is an established mechanism of CS pathogenesis.[12–14] Axin1 knockout is lethal in mice.[15] Conditional knockout of Axin1 in osteoblast precursor cells results in increased levels of active β-catenin, decreased osteoclast formation, and increased osteoprotegerin expression; however, the patency of the cranial sutures in these mice has not been assessed.[16] Mouse models of Axin2 knockout demonstrate CS and increased bone density.[17] Interestingly, gain-of-function (GOF) mutations in *LRP5*, a Wnt receptor regulated by *AXIN1*, are an established cause of osteopetrosis, with many individuals harboring *LRP5* GOF mutations having CS.[18–20]

We have not identified any multiplex kindreds with *AXIN1* mutations and CS to date, thus it is challenging to estimate the penetrance of these mutations. In 3 of 6 cases identified in trios, the mutation was inherited from an unaffected parent in a kindred with no family history of CS, and no parental phenotypes suggestive of any form of syndromic or nonsyndromic disease, implying some degree of incomplete penetrance. Larger cohorts of patients will be necessary to characterize the penetrance and full phenotypic spectrum of *AXIN1* mutations. Interestingly, one of the patients harboring a de novo AXIN1 mutation (p.T240fs*2) was found to also harbor an inherited LOF mutation in another CS gene, *SMAD6* (NM_005585.4(SMAD6):c.1423delT (p.Cys475fs)). This highlights the importance of genetic testing in complex cases of CS, as composite genotypes such as this one (*SMAD6*/*AXIN1*) have been found in several atypical CS presentations, frequently helping explain abnormal clinical courses.[21,22] At the current time, genetic testing is not standard practice for sporadic cases of nonsyndromic CS. Efforts to date have identified a genetic cause in < 20% of nonsyndromic CS cases, with mutations in different genes having different implications for management and patient counseling. There is no specific phenotype currently that would indicate a need for specific testing for mutations in *AXIN1*; however, as genetic testing becomes increasingly affordable and available, damaging mutations in *AXIN1* should be considered pathogenic when identified in whole exome or genome sequencing analyses.

Conclusions

These findings describe the first phenotype associated with mutations in *AXIN1*, with mutations identified in approximately 1% of nonsyndromic CS cases. The results strengthen the existing link between Wnt signaling and maintenance of cranial suture patency and have implications for genetic testing when indicated in families with CS.

Acknowledgments

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Author Contributions

Conception and design: Timberlake, Hao, Lifton, Persing. Acquisition of data: Timberlake, Gustafson, Hao, Valenzuela, Slavotinek. Analysis and interpretation of data: Timberlake, Hemal, Gustafson, Hao, Kahle, Lifton. Drafting the article: Timberlake, Hao, Kahle. Critically revising the article: Timberlake, Hemal, Hao, Slavotinek, Cunningham, Kahle. Reviewed submitted version of manuscript: Timberlake, Valenzuela, Slavotinek, Cunningham, Kahle, Lifton, Persing. Approved the final version of the manuscript on behalf of all authors: Timberlake. Statistical analysis: Timberlake, Hemal. Administrative/technical/material support: Lifton. Study supervision: Timberlake, Kahle.

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