



## CLINICAL RESEARCH ARTICLE

## Nonsyndromic craniosynostosis: novel coding variants

Anshuman Sewda<sup>1</sup>, Sierra R. White<sup>1</sup>, Monica Erazo<sup>1</sup>, Ke Hao<sup>1</sup>, Gemma García-Fructuoso<sup>2</sup>, Ivette Fernández-Rodríguez<sup>2</sup>, Yann Heuzé<sup>3</sup>, Joan T. Richtsmeier<sup>4</sup>, Paul A. Romitti<sup>5</sup>, Boris Reva<sup>1</sup>, Ethylin Wang Jabs<sup>1</sup> and Inga Peter<sup>1</sup>

**BACKGROUND:** Craniosynostosis (CS), the premature fusion of one or more neurocranial sutures, is associated with approximately 200 syndromes; however, about 65–85% of patients present with no additional major birth defects.

**METHODS:** We conducted targeted next-generation sequencing of 60 known syndromic and other candidate genes in patients with sagittal nonsyndromic CS (sNCS,  $n = 40$ ) and coronal nonsyndromic CS (cNCS,  $n = 19$ ).

**RESULTS:** We identified 18 previously published and 5 novel pathogenic variants, including three de novo variants. Novel variants included a paternally inherited c.2209C>G:p.(Leu737Val) variant in *BBS9* of a patient with cNCS. Common variants in *BBS9*, a gene required for ciliogenesis during cranial suture development, have been associated with sNCS risk in a previous genome-wide association study. We also identified c.313G>T:p.(Glu105\*) variant in *EFNB1* and c.435G>C:p.(Lys145Asn) variant in *TWIST1*, both in patients with cNCS. Mutations in *EFNB1* and *TWIST1* have been linked to craniofrontonasal and Saethre–Chotzen syndrome, respectively; both present with coronal CS.

**CONCLUSIONS:** We provide additional evidence that variants in genes implicated in syndromic CS play a role in isolated CS, supporting their inclusion in genetic panels for screening patients with NCS. We also identified a novel *BBS9* variant that further shows the potential involvement of *BBS9* in the pathogenesis of CS.

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## INTRODUCTION

Craniosynostosis (CS, MIM #123100) is the second most common craniofacial birth defect after orofacial cleft defects<sup>1</sup> and affects as many as 1 in 1400 live births.<sup>2</sup> Children with CS may experience significant medical problems such as increased intracranial pressure, vision and hearing impairments, breathing and dentition problems, and developmental disabilities.<sup>3</sup> There is no pharmacological treatment available and surgical treatments such as open calvarial reconstruction, strip craniectomy, and cranial distraction or surgical suture opening are frequently used.<sup>4</sup>

CS is known to have a complex etiology with both environmental and genetic risk factors.<sup>4</sup> Although CS may occur as part of approximately 200 syndromes (Table S1), about 65–85% of the patients present with nonsyndromic craniosynostosis (NCS), i.e., no other related major birth defect or recognized syndrome.<sup>1,5</sup> Sagittal NCS (sNCS, 40% of all NCS patients)<sup>2,5</sup> and coronal NCS (cNCS, 18–30% of all NCS patients)<sup>5–7</sup> are two of the most common NCS subtypes. sNCS is three times more prevalent in males relative to females, whereas unilateral cNCS is two times more common in females relative to males.<sup>8</sup>

A positive family history of CS has been reported for ~6% of sNCS,<sup>9</sup> and 8–15% of cNCS patients.<sup>6,7</sup> Also, twin studies of sNCS report a higher concordance rate in monozygotic (30%) than dizygotic (0%) twins.<sup>3,10</sup> Additionally, a recent study reported that 3% of patients with no clinical evidence of a syndrome had mutations in genes that are commonly associated with syndromic CS.<sup>5</sup>

In a genome-wide association study (GWAS), our group identified regions downstream of *BMP2* and in introns of *BBS9* gene that were associated with an increased risk of sNCS.

However, no functionally significant coding variants within the GWAS association peaks were identified.<sup>11</sup> Despite the evidence supporting the role of genetics in the pathogenesis of NCS and recent studies reporting pathogenic variants in known syndromic genes among patients with NCS,<sup>12–14</sup> our understanding of the role of functionally relevant mutations in the genes associated with syndromic CS, as well as within additional NCS susceptibility loci, in NCS development remains limited.

The goal of this study was to identify variants associated with sNCS and cNCS by conducting targeted next-generation sequencing (NGS) of previously known syndromic CS genes, as well as the susceptibility loci linked to sNCS in our previous GWAS.<sup>11</sup>

## MATERIALS AND METHODS

## Study samples

Patients were live-born children with sagittal or coronal (unilateral or bilateral) CS enumerated from the Iowa Registry for Congenital and Inherited Disorders, the New York State Congenital Malformations Registry,<sup>15</sup> and the Hospital Sant Joan de Déu in Barcelona, Spain. The patients with CS that had any other major birth defect or had a monogenic or chromosomal abnormality were excluded. Saliva specimens were collected from the patient and available parents from all sites, except patients from Barcelona for which blood samples were collected during preoperative tests. The patient diagnosis was confirmed by clinicians and clinical geneticists at each institution through review of clinical and imaging records. Overall, 59 NCS specimens from 28 patients (19 sNCS and 9 cNCS) from Iowa/New York State and 31 patients

<sup>1</sup>Department of Genetics & Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA; <sup>2</sup>Department of Pediatric Neurosurgery, Hospital Sant Joan de Déu, Barcelona, Spain; <sup>3</sup>University Bordeaux, CNRS, MCC, PACEA, UMR5199, Bordeaux Archaeological Sciences Cluster of Excellence, Pessac, France; <sup>4</sup>Department of Anthropology, Pennsylvania State University, University Park, PA, USA and <sup>5</sup>Department of Epidemiology, College of Public Health, University of Iowa, Iowa City, IA, USA  
Correspondence: Anshuman Sewda (anshuman.sewda@mssm.edu)

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**Table 1.** Description of selected characteristics of 59 sagittal (sNCS) and coronal (cNCS) nonsyndromic craniosynostosis patients

	sNCS (n = 40)	cNCS <sup>a</sup> (n = 19)
Gender		
Female	13 (32.5%)	14 (73.7%)
Male	27 (67.5%)	5 (26.3%)
Race		
Caucasian	37	18
Other or Unknown	3	1
Recruitment site		
Iowa, USA <sup>b</sup>	14	1
New York State, USA <sup>c</sup>	5	8
Barcelona, Spain <sup>d</sup>	21	10

<sup>a</sup>The counts include one white male patient with bilateral cNCS and one white male patient with multi-suture NCS (bilateral cNCS + sNCS); both patients were recruited from Barcelona

<sup>b</sup>The Iowa Registry for Congenital and Inherited Disorders, Iowa, USA

<sup>c</sup>The New York State Congenital Malformations Registry, New York, USA

<sup>d</sup>Hospital Sant Joan de Déu, Barcelona, Spain

(21 sNCS and 10 cNCS) from Spain were selected for further study (Table 1). The study was performed with the approval of the Institutional Review Boards at Icahn School of Medicine at Mount Sinai and the University of Iowa, and the Helsinki Committee at Hospital Sant Joan de Déu, Barcelona, Spain. All participants provided signed informed consent.

#### Targeted next-generation sequencing

A custom-designed NGS panel included genes previously reported in association with syndromic CS, NCS, and top susceptibility loci identified in our previous sNCS GWAS.<sup>11</sup> The regions we targeted were selected based on extensive literature review (Table S1). In total, we analyzed 60 genes, 54 sequenced at the genome level, including 1000 bases upstream and downstream of each gene (Table S1), and 6 sequenced for exons, including 100 bases upstream and downstream regions of each exon (Table S2). Our custom capture array, covering approximately 7 million bases, was designed using NimbleGen Seqcap EZ Choice kit (Roche, Basel, Switzerland). NGS was done at the DNA Core, Icahn School of Medicine at Mount Sinai, New York, using the HiSeq 2500 high-throughput sequencing platform (Illumina, San Diego, California) (Supplementary Methods).

#### Data analysis

Alignment and variant calling using raw paired-end sequence reads was completed using the in-house “GATK Best Practices” based pipeline (Supplementary Methods). Variants were filtered to keep only high-quality single nucleotide variants (SNVs) that are either novel, that is, not previously reported in public databases (gnomAD, Bravo, 1000 Human Genomes Project, Exome Sequencing Project or dbSNP; Table S3) or known but rare variants (minor allele frequency, MAF < 1% in the gnomAD non-Finnish Europeans database). Functional annotation of these variants was performed using SnpEff version 3.5,<sup>16</sup> SIFT,<sup>17</sup> Polyphen2,<sup>18</sup> and Combined Annotation Dependent Depletion (CADD).<sup>19</sup> SNVs were considered pathogenic, if they met all of the following criteria: CADD phred-scaled score > 20, SnpEff “moderate” or “high” functional impact prediction, “deleterious” according to SIFT and “damaging” or “probably damaging” according to Polyphen2. We sorted all novel SNVs by predicted impact on protein function, using evolutionary conservation patterns through integration of the functional predictions and variant distribution statistics as implemented in Mutation Assessor.<sup>20</sup>

#### Sanger sequencing analysis

Novel SNVs were further validated in probands and available parents using Sanger sequencing. The primers were designed using PrimerQuest Tool (Integrated DNA Technologies, Skokie, Illinois) (Table S4). Standard methods were used to prepare the samples before sending them for Sanger sequencing (Genewiz, South Plainfield, New Jersey) (Supplementary Methods). Analysis was done through Sequencher<sup>®</sup> v5.4.6 DNA sequence analysis software (Gene Codes Corporation, Ann Arbor, Michigan) where assembly parameters were kept as default and SNPs were called with a 20% secondary peak height and confirmed through the UCSC Genome Browser (<https://genome.ucsc.edu/>). All novel SNVs identified and validated in our study have been submitted to the Leiden Open Variation Database (<http://www.lovd.nl>)

#### RESULTS

Of the 59 patients in our study, 32.5% sNCS and 73.7% cNCS patients were females, and the cohort was predominantly Caucasian (Table 1). Targeted NGS was performed on 60 genes including their regulatory regions (total 6,858,924 bases) spanning across autosomes and the X-chromosome (Figure S1, Table S1, and Table S2). There were 96.1% paired-end reads that aligned successfully and the mean depth of coverage was 90x across all targeted bases and specimens. After performing standard quality control procedures, 30,571 high-quality SNVs were functionally annotated.

A total of 23 rare SNVs (novel or known) predicted to be pathogenic were identified in 17 genes. Seven of 19 cNCS patients and 17 of 40 sNCS patients had at least one of these pathogenic novel or known SNVs.

#### Novel variants

We identified five novel, heterozygous coding SNVs predicted to be pathogenic (Table 2). All SNVs were singletons whereby each variant was observed in only one proband in our cohort and had a CADD-phred scaled score > 25. After Sanger sequencing of probands and available parents, we determined that three novel SNVs were de novo and two were paternally transmitted to probands. No craniofacial defects were reported in these parents. A novel paternally transmitted c.2209C>G:p.(Leu737Val) variant was identified in *BBS9* (Figure S2) in a patient with right cNCS. This variant lies 121 kb away from the *BBS9* peak identified in our previous sNCS GWAS.<sup>11</sup> Another novel paternally inherited c.126G>C:p.(Lys42Asn) variant was identified in *ALX4* in a patient with sNCS. In *EFNB1* we detected a novel, de novo c.313G>T:p.(Glu105\*) nonsense variant in a patient with left cNCS, whereas two novel de novo SNVs were identified in *TWIST1* (c.435G>C:p.(Lys145Asn) and c.421G>C:p.(Asp141His)) (Table 2). We predicted that all novel missense variants had functional impact and the amino acid substitutions were evolutionarily conserved across several species according to Mutation Assessor (Fig. 1).

#### Known variants

We identified 18 previously observed, heterozygous, rare SNVs (including 12 singletons) predicted to be pathogenic (Table 2). Thirteen SNVs were present in patients with sNCS, three SNVs in patients with cNCS, and two SNVs were present in both sNCS and cNCS. The top gene with the largest number of SNVs identified was *RECL4* with five different variants identified, one in each of five different patients with sNCS. A known SNV in *NOTCH1* was detected in three patients with sNCS, whereas another SNV in *NOTCH2* was detected in two patients with sNCS (Table 2).

In patients with sNCS, we identified pathogenic mutations in genes that were selected in the custom panel based on our previous sNCS GWAS,<sup>11</sup> namely c.1663C>T:p.(Arg555Trp) variant in *BMPER* of two patients with sNCS, c.467C>T:p.(Thr156Met) variant in *ADCK1* of a patient with sNCS, c.1810A>G:p.(Ile604Val) variant in

**Table 2.** Variants predicted to be pathogenic in 59 sagittal (sNCS) and coronal (cNCS) nonsyndromic craniosynostosis patients

Gene	Variant	gnomAD MAF <sup>a</sup>	CADD score <sup>b</sup>	Inheritance/ Rs id <sup>c</sup>	Gender <sup>d</sup>	Affected suture <sup>e</sup>
<b>Novel variants</b>						
<i>ALX4</i>	NM_021926:c.126G>C; p.(Lys42Asn)	.	29.9	Paternal	M	S
<b><i>BBS9</i></b>	NM_198428:c.2209C>G; p.(Leu737Val)	.	25.9	Paternal	F	Right C
<i>EFNB1</i>	NM_004429:c.313G>T; p.(Glu105*)	.	37.0	De Novo	F	Left C
<i>TWIST1</i>	NM_000474:c.435G>C; p.(Lys145Asn)	.	29.3	De Novo	M	Bilateral C+S
<i>TWIST1</i>	NM_000474:c.421G>C; p.(Asp141His)	.	27.7	De Novo	M	Bilateral C
<b>Known variants</b>						
<b><i>ADCK1</i></b>	NM_020421:c.467C>T; p.(Thr156Met)	1.7E-03	33.0	rs144436820	F	S
<i>ALPL</i>	NM_000478:c.212G>C; p.(Arg71Pro)	3.64E-05	33.0	rs121918003	M	S
<b><i>BMPER</i></b>	NM_133468:c.1663C>T; p.(Arg555Trp)	8.0E-03	34.0	rs10249320	M (2)	S (2)
<i>FREM1</i>	NM_144966:c.3819T>A; p.(Asp1273Glu)	3.3E-03	26.5	rs7025814	M, F	S (1), C (1)
<i>FREM1</i>	NM_144966:c.1394G>C; p.(Gly465Ala)	8.3E-03	24.9	rs41298151	M, F	S (1), C (1)
<i>JAG1</i>	NM_000214:c.2740G>A; p.(Gly914Arg)	3.00E-05	33.0	rs376630327	F	C
<i>NELL1</i>	NM_006157:c.368A>G; p.(Asp123Gly)	1.51E-05	22.9	rs763010935	F	Left C
<i>NOTCH1</i>	NM_017617:c.2734C>T; p.(Arg912Trp)	3.3E-03	31.0	rs201620358	F, M (2)	S (3)
<i>NOTCH2</i>	NM_024408:c.7223T>A; p.(Leu2408His)	3.6E-03	25.5	rs35586704	F, M	S (2)
<b><i>PDILT</i></b>	NM_174924:c.1243C>T; p.(Pro415Ser)	6.5E-03	27.3	rs139748181	F, M	S (2)
<i>RECQL4</i>	NM_004260:c.2435G>A; p.(Cys812Tyr)	6.71E-05	33.0	rs372372052	M	S
<i>RECQL4</i>	NM_004260:c.2340G>T; p.(Pro780Pro)	6.76E-05	33.0	rs369488194	M	S
<i>RECQL4</i>	NM_004260:c.2237C>T; p.(Ala746Val)	1.0E-04	26.8	rs201883228	M	S
<i>RECQL4</i>	NM_004260:c.1565G>A; p.(Arg522His)	2.0E-04	24.6	rs35842750	M	S
<i>RECQL4</i>	NM_004260:c.212A>G; p.(Glu71Gly)	8.0E-03	21.6	rs34642881	M	S
<i>SH3PXD2B</i>	NM_001017995:c.970C>T; p.(Arg324Trp)	6.68E-05	24.2	rs199739437	F	S
<b><i>SHC4</i></b>	NM_203349:c.1810A>G; p.(Ile604Val)	0	25.3	rs145850141	F	C
<i>TGFBR2</i>	NM_001024847:c.1732T>A; p.(Ser578Thr)	3.0E-04	23.6	rs112215250	M	S

The gene names in bold text were within the genomic regions selected in our custom panel based on the previous GWAS<sup>11</sup>

<sup>a</sup>Minor allele frequency (MAF) in the Genome Aggregation Database (gnomAD) non-Finnish European population. The Exome Aggregation Consortium (ExAC) non-Finnish European minor allele frequencies (italicized) are reported for the variants that were not reported in the gnomAD database

<sup>b</sup>Combined Annotation-Dependent Depletion (CADD) phred-scaled score: 20 = 1% percentile highest scores, 30 = 0.1% percentile highest scores

<sup>c</sup>Inheritance status of novel variants and Reference SNP cluster ID (rsID) of known variants

<sup>d</sup>Proband's gender (M = Male, F = Female)

<sup>e</sup>Affected cranial sutures (S = Sagittal, C = Coronal) in the proband with nonsyndromic craniosynostosis. The number in parenthesis refers to the number of patients in which a variant was present. All pathogenic variants were identified in white patients, except *SHC4* and *JAG1* pathogenic variants; both identified in one Asian patient

*SHC4* of a patient with cNCS, and c.1243C>T;p.(Pro415Ser) variant in *PDILT* of two patients with sNCS (Table 2).

## DISCUSSION

Through targeted sequencing of patients with sNCS and cNCS, we identified several rare (MAF < 1%), novel, and previously observed variants, predicted to be functionally pathogenic, within the loci detected in our previous sNCS GWAS<sup>11</sup> or in the genes previously associated with syndromic CS (Table S1).

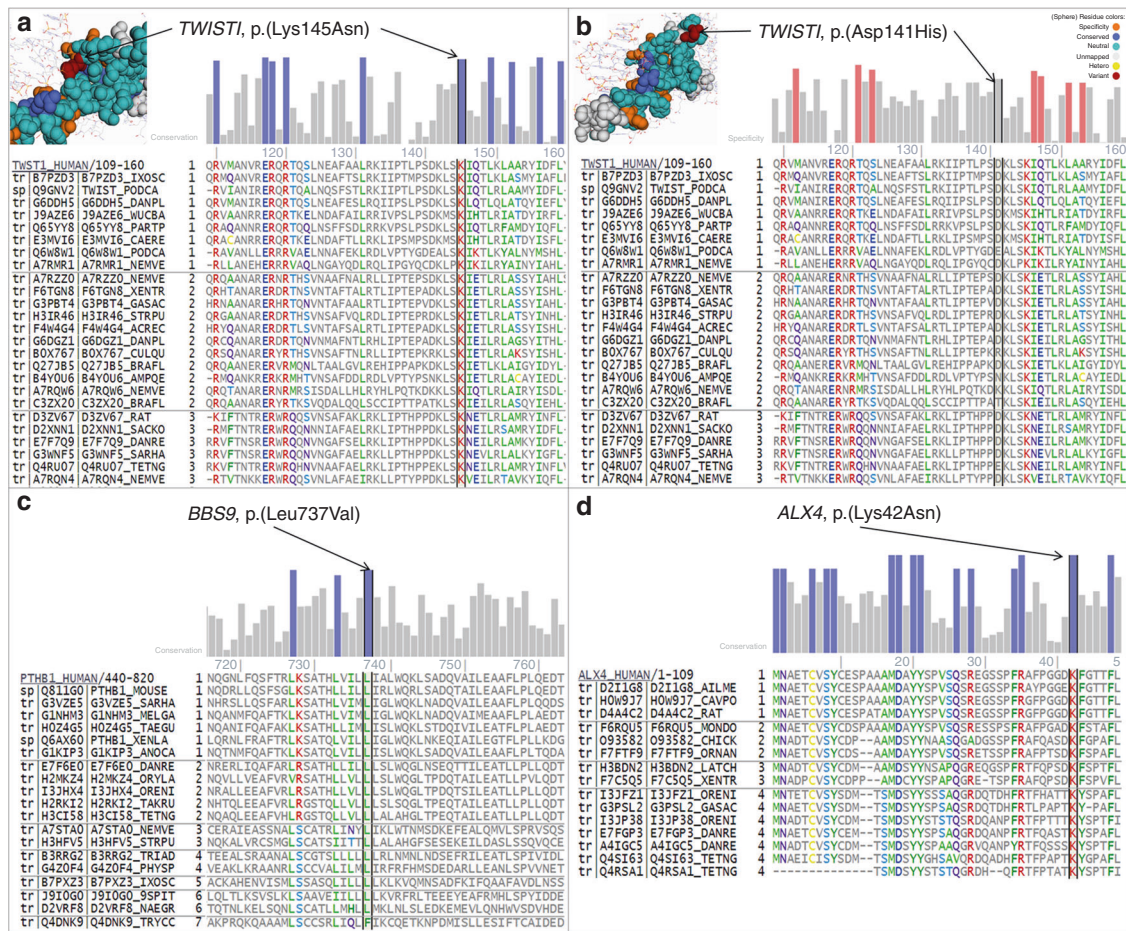
*BBS9* was included in our sequencing panel based on findings from our previous sNCS GWAS<sup>11</sup> showing associations with three intronic SNVs (rs10262453, rs1420154, and rs17724206) spanning a 167 kb region within *BBS9* introns 4 and 15 on chromosome 7p14.3 (Figure S2).<sup>11</sup> Resequencing of *BBS9* in patients with NCS led to identification of a paternally transmitted novel pathogenic c.2209C>G variant in a female patient with right cNCS; the variant is located approximately 121 kb from another GWAS peak at rs17724206 (Figure S2).<sup>11</sup> *BBS9* is essential for proper BBSome complex assembly which is required for ciliogenesis<sup>21</sup> a process involved in cranial suture pathophysiology.<sup>22</sup> Analysis showed that the c.2209C>G variant affects a site that is well-conserved across several species (Fig. 1c) suggesting the role of this locus in key

biological functions. Despite the role of *BBS9* in ciliogenesis, no coding variants in this gene have been linked to CS previously.

Our study also included genes near or containing additional loci that showed suggestive evidence of an association ( $p < 10^{-5}$ ) in the sNCS GWAS.<sup>11</sup> We identified several rare previously observed variants predicted to be pathogenic in *SHC4*, *ADCK1*, and *PDILT* in four patients with both cNCS and sNCS (Table 2; Figure S3). Further replication in an independent cohort and experimental validation are needed to confirm the association between NCS and these variants.

There are numerous genes reported over the last several decades with mutations linked to syndromic forms of CS, as reviewed by Flaherty et al.<sup>1</sup> and Wilkie et al.<sup>5</sup> Although recent studies have established a link between some of these genes and NCS,<sup>12,23,24</sup> systematic screening of these genes in patients with NCS is still lacking. We report a novel, de novo c.313G>T, nonsense variant in *EFNB1* of a female patient with left cNCS. *EFNB1* encodes a type-I membrane protein which is a ligand of Eph-related receptor tyrosine kinases. *EFNB1* has been linked to craniofrontonasal syndrome (MIM #304110), an X-linked inherited syndrome with a coronal CS phenotype. A mouse study identified multiple defects in ephrinB1-deficient mice including shortening of skull and cleft palate.<sup>25</sup> Multiple heterozygous variants have been





**Fig. 1** Functional consequences of novel mutations: **a** *TWIST1* p.(Lys145Asn) acts as a transcriptional regulator for cranial suture patterning and fusion. The mutation affects the evolutionarily conserved residue and one of the key binding site residues. Functional impact predicted by Mutation Assessor is “Medium”. **b** *TWIST1* p.(Asp141His) mutation affects one of the high-scoring specificity residues, i.e., residues conserved within protein subfamilies. Functional impact predicted by Mutation Assessor is “Medium”. **c** *BBS9* p.(Leu737Val) is required for proper BBSome complex assembly which is required for cilogenesis. The mutation affects the evolutionarily conserved residue. Functional impact predicted by Mutation Assessor is “High”. **d** *ALX4* p.(Lys42Asn) encodes a paired-like homeodomain transcription factor expressed in the mesenchyme of developing bones. The mutation affects the evolutionarily conserved residue

identified in association with coronal CS phenotype in another ligand of Eph receptor, ephrin-A4 (*EFNA4*) in humans<sup>26</sup> and mice.<sup>27</sup> However, variants in *EFNB1* have not been reported previously in patients with NCS.

We also identified novel variants in the extensively studied syndromic CS gene, *TWIST1*. *TWIST1* is a transcriptional regulator that helps maintain coronal suture integrity and interacts downstream with ephrin receptors.<sup>26</sup> *TWIST1* haploinsufficiency is associated with Saethre–Chotzen syndrome (MIM #101400) that is characterized by coronal CS as one of the phenotypes.<sup>28</sup> Also, *TWIST1* has been reported in association with sNCS and cNCS previously.<sup>5,15,29</sup> The two novel *TWIST1* SNVs (c.435G>C and c.421G>C) we identified were in two male patients with bilateral cNCS. During surgery, the proband with c.435G>C mutation was also identified as having sagittal suture closure. However, despite a multi-suture phenotype, no known clinical syndrome could be determined in this patient. Analysis with Mutation Assessor showed that p.(Lys145Asn) amino acid change affects the evolutionarily conserved residue and one of the key binding site residues (Fig. 1a), whereas p.(Asp141His) amino acid change affects one of the high-scoring specificity residues (i.e., residues conserved within protein subfamilies) (Fig. 1b).<sup>20</sup>

Our sequencing also identified a novel c.126G>C variant in *ALX4* of a male patient with sNCS. This gene encodes a paired-like homeodomain transcription factor expressed in the mesenchyme

of developing bones<sup>30</sup> and the mutation affects the evolutionarily conserved residue (Fig. 1d). Mutations in *ALX4* cause a form of frontonasal dysplasia (MIM #613451) with alopecia and hypogonadism<sup>30</sup> suggesting a role for this gene in craniofacial development and mesenchymal–epithelial communication. Deletion of a segment of chromosome 11 containing *ALX4*, del(11)(p11p12), causes Potocki–Shaffer syndrome (MIM #601224); a syndrome characterized by craniofacial anomalies.<sup>31</sup> *ALX4* has not been previously reported in patients with NCS.

Given the evidence for familial recurrence of NCS,<sup>6,7,9</sup> chromosomal microarray testing has been recommended in patients with birth defects particularly if syndromic CS is suspected.<sup>5</sup> Recently, targeted NGS of known disease susceptibility genes has become a useful tool to identify novel variants in patients with NCS.<sup>32</sup> Several of our genes, including *ALPL*, *RECQL4*, *SH3PXD2B*, *TGFBR2*, and ephrin family of genes (*EFNA4* and *EFNB1*) have been implicated in NCS in previous DNA resequencing<sup>12</sup> and RNA sequencing studies.<sup>14</sup> Of note, while the same genes have been identified in multiple studies, often associated with the same affected cranial sutures, the individual mutations were unique. This suggests that chromosomal microarray testing genes might not fully capture all susceptibility loci and DNA resequencing should be recommended for genetic testing of patients when NCS is suspected.

Moreover, craniofacial surgery currently remains the only option to repair abnormally closed sutures with no preventive measures

yet available. Pharmacologic strategies are being tested in model systems to alter cranial suture fate at the biomolecular level and prevent CS. These experiments target known genes such as *FGFR2*, mutations in which cause several syndromes with a CS phenotype. Several *in vivo* and *in vitro* studies using MEK1/2 or p38 inhibitors ameliorated the CS phenotype in *Fgfr2*<sup>+/-S252W</sup>, *Fgfr2*<sup>+/-Y394C</sup>, and *Fgfr2*<sup>P253R/+</sup> mice.<sup>33–36</sup> Two similar studies observed that treatment with FGFR signaling inhibitors in organ culture of calvaria derived from *Fgfr2*<sup>C342Y/+</sup> +Crouzon syndrome mouse prevented premature suture fusion.<sup>37,38</sup> These approaches have yet to be clinically translated in humans; nonetheless, they emphasize the importance of detecting genes and biological pathways involved in CS as potential avenues for development of future early diagnostic tools and therapeutics.

Our study has several strengths. We used samples from patients with well-characterized phenotypes for CS. We also conducted a systematic screening of a large number of known genes associated with syndromic CS exclusively in patients with NCS using high-resolution NGS. We identified pathogenic missense variants in several genes that were previously reported only in association with syndromic CS. In addition, we identified novel coding variants predicted to be functionally pathogenic, in the previously reported sNCS GWAS loci, including *BBS9*.<sup>11</sup> We validated novel findings through Sanger sequencing and further sequenced parental samples to identify whether the variants were *de novo* or transmitted through parents.

Our study has some limitations. We performed targeted sequencing of 60 candidate genes and genetic regions with previous evidence of involvement in calvarial development, syndromic CS, or NCS. However, several genes were reported in association with syndromic CS and NCS after we designed our custom panel and, therefore, were not examined in this study including *CDC45*, *FLNA*, *PTH2R*, *SIX2*, *SMO*, *SMURF1*, *SPRY1*, *SPRY4*, and *ZIC1* (Table S1). Screening panels need to be continuously and collaboratively updated to include newly identified CS-associated genes before genome-wide sequencing approaches become more affordable. Also, we have reported only those novel and known variants that were sequenced at high quality and were rare across all reference populations (MAF < 1%). Genome-wide screening of common and low-frequency variants in large cohorts of NCS patients is warranted to confirm our findings and to capture the full spectrum of genes and variants involved in the pathogenesis of NCS. Detailed clinical information on CS status or skull shape was missing on the parents who transmitted high-impact variants to patients with NCS, preventing the assessment of penetrance of variants, especially the novel SNVs. Moreover, the possibility of a syndrome in the recruited patients cannot be ruled out completely as there are certainly recorded instances of reduced penetrance and syndromic clinical manifestations becoming more obvious later in life.<sup>39,40</sup> However, as mentioned, the nonsyndromic diagnosis was confirmed by clinicians and clinical geneticists at each institution through review of clinical and imaging records. Lastly, our study subjects were predominantly of European descent and given the significant differences in the genetic architecture among various ancestries, especially for rare variants, the novel variants identified in our study could be population-specific, limiting the generalizability of our findings. Future studies in diverse racial and ethnic groups are needed to better understand the genetic risks for NCS.

In summary, we identified several previously unreported variants in genes linked to syndromic CS that may play a role in NCS and provide evidence supportive of findings from recent NCS studies of syndromic genes as well as the sNCS GWAS. Given the locus heterogeneity of syndromic genes and increasing evidence of involvement of additional genes in NCS, we recommend targeted resequencing of candidate genes for genetic testing of patients suspected to have NCS.

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## AUTHOR CONTRIBUTIONS

Authors made substantial contributions to conception and design (A.S., I.P., E.W.J., G.G., J.T.R., P.R., M.E.), acquisition of data (A.S., I.P., E.W.J., G.G., J.T.R., P.R., M.E., Y.H., I.F.), or analysis and interpretation of data (A.S., I.P., S.R.W., B.R., K.H.); all authors contributed in drafting the article and revising it critically for important intellectual content, and all contributing authors have approved the final version for publication.

## ADDITIONAL INFORMATION

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