



De novo ALX4 variant detected in child with non-syndromic craniosynostosis

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Abstract

Current understanding of the genetic factors contributing to the etiology of non-syndromic craniosynostosis (NSC) remains scarce. The present work investigated the presence of variants in *ALX4*, *EFNA4*, and *TWIST1* genes in children with NSC to verify if variants within these genes may contribute to the occurrence of these abnormal phenotypes. A total of 101 children (aged 45.07 ± 40.94 months) with NSC participated in this cross-sectional study. Parents and siblings of the probands were invited to participate. Medical and family history of craniosynostosis were documented. Biological samples were collected to obtain genomic DNA. Coding exons of human *TWIST1*, *ALX4*, and *EFNA4* genes were amplified by polymerase chain reaction and Sanger sequenced. Five missense variants were identified in *ALX4* in children with bilateral coronal, sagittal, and metopic synostosis. A *de novo* *ALX4* variant, c.799G>A: p.Ala267Thr, was identified in a proband with sagittal synostosis. Three missense variants were identified in the *EFNA4* gene in children with metopic and sagittal synostosis. A *TWIST1* variant occurred in a child with unilateral coronal synostosis. Variants were predicted to be among the 0.1% (*TWIST1*, c.380C>A: p. Ala127Glu) and 1% (*ALX4*, c.769C>T: p.Arg257Cys, c.799G>A: p.Ala267Thr, c.929G>A: p.Gly310Asp; *EFNA4*, c.178C>T: p.His60Tyr, C.283A>G: p.Lys95Glu, c.349C>A: Pro117Thr) most deleterious variants in the human genome. With the exception of *ALX4*, c.799G>A: p.Ala267Thr, all other variants were present in at least one non-affected family member, suggesting incomplete penetrance. Thus, these variants may contribute to the development of craniosynostosis, and should not be discarded as potential candidate genes in the diagnosis of this condition.

Key words: Congenital abnormalities; Mutation; Missense mutation; DNA; Saliva; Cranial sutures

Introduction

During early childhood, the fusion of cranial sutures must follow a pre-programmed physiological time table in order to allow adequate growth of the developing brain (1). Different signaling pathways that enable continuance of suture patency may be disrupted, resulting in craniosynostosis. With a prevalence of approximately 3.1–6.4 per 10,000 live births, craniosynostosis can affect one or

more cranial sutures, occurring as an isolated clinical entity or in association with a group of craniofacial syndromes. A recent survey in the Netherlands showed an overall rise in the prevalence of craniosynostosis and synostosis of the sagittal and metopic sutures (2).

In 2015, Twigg and Wilkie (3) published a review reporting a total of 57 genetic variants identified in at least

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[†]This paper is dedicated to the memory of the late Dr. Timothy M. George, whose devotion to the care of children with craniofacial disorders was unsurpassed.

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2 patients with craniosynostosis. Recently, Goos and Mathijssen (4) found 39 novel genes that cause craniosynostosis. Twenty-two of these variants were reported in at least 2 individuals, rendering a total of at least 79 craniosynostosis variants. These variants may interfere with essential processes in the preservation of suture patency during growth and development, such as osteogenic differentiation, maintenance of mesoderm-neural crest lineage boundary, and bone remodeling (5). Specific missense variants in fibroblast growth factor receptor genes (FGFR) 1, 2, and 3 are frequently linked to early suture fusion in various autosomal dominant syndromes that express craniosynostosis, including: Apert, Crouzon, Muenke, Pfeiffer, and Jackson-Weiss syndromes (6). However, variants in these genes have not been associated with non-syndromic craniosynostosis (NSC).

Previous studies identified variants in *ALX* homeobox 4 (*ALX4*: NM_021926.3; MIM #605420) and *Homo sapiens* ephrin A4 (*EFNA4*: NM_005227; MIM #601380) in association with non-syndromic forms of craniosynostosis (7,8). In addition, new variants associated with NSC were identified in the *Homo sapiens* Twist family bHLH transcription factor 1 (*TWIST1*: MIM #601622; NM_000474.3), and other genes known to cause syndromic forms of these malformations were also identified in affected individuals (9,10). In spite of these previous findings, *ALX4* and *EFNA4* genes are not usually considered potential candidate genes when investigating genetic causes of NSC, and earlier work failed to demonstrate the presence of mutations associated with syndromic craniosynostosis within the exons of *TWIST1* in suture cells of individuals with single suture craniosynostosis (11). In light of this information, we aimed to evaluate if children with different subtypes of NSC expressed variants in these genes. We have investigated patient medical histories, and have Sanger DNA-sequenced all coding regions pertaining to *ALX4*, *EFNA4*, and *TWIST1* genes in a population of 101 children diagnosed with NSC. Samples from the children's mother and/or father and siblings were also investigated when available.

Material and Methods

Patient recruitment and study design

This cross-sectional study followed the Strengthening the Reporting of Observational Studies (STROBE) guidelines. Children of all ages (both sexes) presenting to the Dell Children's Craniofacial and Reconstructive Plastic Surgery Center at the Dell Children's Medical Center of Central Texas (USA) from May 2015 to August 2016, with a diagnosis of craniosynostosis were screened for participation. Diagnosis of craniosynostosis was based on physical examination by a craniofacial team, medical history review, cranial computed tomography, and magnetic resonance imaging scans. Postoperative surgical reports were also taken into consideration when making

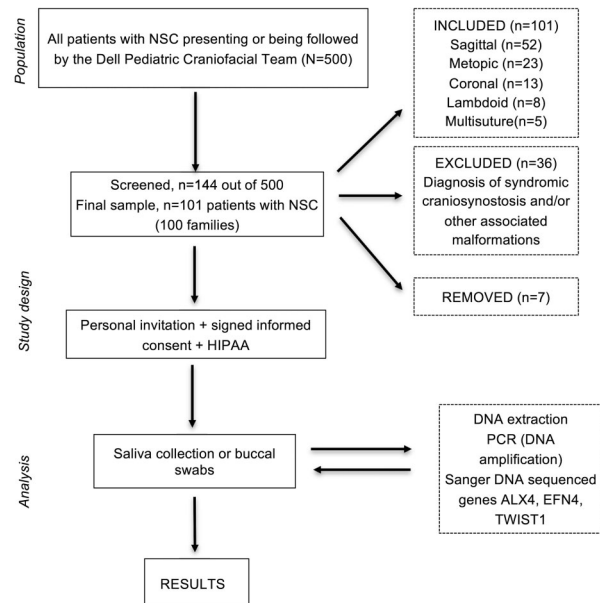


Figure 1. Flowchart showing study design, patient recruitment, and final study sample. NSC: non-syndromic craniosynostosis; HIPAA: Health Insurance Portability and Accountability Act.

the definitive diagnosis. Craniosynostosis syndromes were identified and excluded from the study (Figure 1). Patient screening and recruitment and sample collection occurred during the child's preoperative evaluation for surgical correction of craniosynostosis or during post-surgical follow-up visits. Sample size was determined by the number of families and patients who agreed to participate within the time of patient recruitment (convenience sample). Biological parents and siblings of affected children were also invited to participate in the study. Information regarding medical/family history, ethnicity, sex, and age was provided by the parents and by accessing patients' electronic files. Patient and family enrollment in the study occurred only after the written informed consent forms were appropriately signed. Signature of HIPAA (Health Insurance Portability and Accountability Act) forms allowed access to the patient's electronic files. This study was approved by the Seton Hospital Institutional Review Board (Austin, TX, USA).

Collection of biological samples

Biological samples were collected from children and their families to obtain genomic DNA. For saliva collection, a minimum of 2 mL of saliva was collected from children using the Oragene Discover kit (DNA Genotek Inc., Canada). Buccal cell sampling utilizing Isohelix DNA buccal swabs (SK-2 swabs; Cell Projects Ltd., UK) was the alternative sampling method when there was no cooperation for saliva collection. These kits provided a safe and reliable method of obtaining genomic DNA for

future sequencing analysis, allowing sample storage and transportation to the research lab at room temperature. Genomic DNA was subsequently extracted through an established protocol using the Puregene DNA Purification kit (Qiagen, USA) for saliva and the Isohelix DNA Isolation kit for buccal cell samples.

DNA sequencing

All coding exons of human *TWIST1* (NM_000474.3), *ALX4* (NM_021926), and *EFNA4* (NM_005227) genes were amplified by polymerase chain reaction (PCR) (T100™ Thermal Cycler, Bio-Rad Laboratories, USA). Primer3 was used to design primers. Primers covered intro/splice sites. We included at least 50 nt flanking the splice sites of target exon. Primer sequences and PCR conditions are available upon request. The PCR products were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA), in addition to either a specific forward or reverse primer. Samples were processed in the 3730 DNA Analyzer (Thermo Fisher Scientific, USA). DNA sequences were aligned and analyzed using the Mutation Surveyor V4.0.7 (Softgenetics, USA). The obtained sequences were mapped using Genome Reference Consortium Human Build 38 (GRCh38/hg38). Previously reported variants in human *TWIST1*, *ALX4*, and *EFNA4* were determined based on their presence in four public databases: dbSNP (<http://www.ncbi.nlm.nih.gov/snp>), the 1000 Genome Project (<http://www.1000genomes.org>), the NHLBI GO Exome Sequencing Project (<https://esp.gs.washington.edu/drupal/>), and the Exome Aggregation Consortium (ExAC) Browser (<http://exac.broadinstitute.org/>). Additional PCR and sequencing analyses were performed to confirm the presence of the identified rare variants (<1%). The outcome of rare missense variants on the coded protein was predicted *in silico* using the PolyPhen-2 (Polymorphism Phenotyping version 2.1.0; <http://genetics.bwh.harvard.edu/pph/>), SIFT (Sorting Intolerant From Tolerant; <http://sift.jcvi.org/>), and CADD (Combined Annotation Dependent Depletion; <https://cadd.gs.washington.edu/score>) programs (12). Pathogenicity of these variables was also assessed by applying the American College of Medical Genetics and Genomics Standards and Guidelines (ACMG) (13).

Data analysis

The outcome measure was presence of at least one *TWIST1*, *ALX4*, or *EFNA4* variant described as being probably damaging (PolyPhen.) and damaging (SIFT) in children with craniosynostosis. The main exposure was defined as having a confirmed diagnosis of NSC. In order to control for potential confounders, biological samples were collected from parents and siblings. Presence of coinciding genotypes between the proband and at least one other family member that did not express craniosynostosis led to the understanding that the proband's genotype was not pathogenic. Association of sex, ethnic-

ity, craniosynostosis subtypes, family history of craniosynostosis, presence/absence of variants, and affected genes was performed using chi-squared test. The Fisher exact test was used to compare the frequency of the rare (MAF < 0.001) *ALX4* and *EFNA4* variants and controls in the Genome Aggregation Database (gnomAD). Significance was set at $P < 0.05$.

Results

Population, ethnicity, and family history

A total of 101 children (aged 45.07 ± 40.94 months) with NSC, including 43 parent-offspring trios, 49 mother-offspring pairs, and 2 father-offspring pairs participated in the present study. Six children were enrolled in the study without the participation of other family members. A male-to-female ratio of 2.74:1 was observed within this population, (males: $n=74$, 73.3%; females: $n=27$, 26.7%). Siblings of affected individuals from 26 families also agreed to participate in the study. Fifteen patients (14.9%) within the study population reported a positive family history of craniosynostosis.

Craniosynostosis of the sagittal suture (51.5%, $n=52$) was the most frequent subtype, followed by synostosis of the metopic (22.8%, $n=23$), unilateral coronal (12.9%, $n=13$), and lambdoid (7.9%, $n=8$) sutures (Figure 1). Five children (5.0%) expressed a multi-suture synostosis, including bilateral coronal synostosis ($n=3$) and involvement of the lambdoid and sagittal sutures ($n=2$). Demographic distribution of patients in our study was as follows: Hispanic (48.5%, $n=49$) and White children (42.6%, $n=43$) were more frequently affected than children of Asian (5.0%, $n=5$) and African (4.0%, $n=4$) descent. There was no significant association between craniosynostosis subtype and sex ($P=0.184$) or ethnicity ($P=0.494$). Children with a family history of craniosynostosis were affected by the isolated forms of sagittal, metopic, or unilateral coronal craniosynostosis. Families with children affected by unilateral lambdoid ($n=8$) or multi-suture craniosynostosis ($n=5$, bilateral coronal craniosynostosis included) did not report having a family history of craniosynostosis.

DNA sequencing analysis

Five different missense variants were identified in the *ALX4* gene (c.769C>T: p.Arg257Cys; c.799G>A: p.Ala267Thr; c.929G>A: p.Gly310Asp; c.304C>T: p.Pro102Ser; and c.104G>C: p.Arg35Thr) (Supplementary Table S1). These variants were present in children with the following phenotypes: bilateral coronal (*ALX4*, c.769C>T: p.Arg257Cys), sagittal (*ALX4*, c.799G>A: p.Ala267Thr, c.929G>A: p.Gly310Asp), and metopic synostosis (*ALX4*, c.304C>T: p.Pro102Ser, c.104G>C: p.Arg35Thr). Variant *ALX4*, c.799G>A: p.Ala267Thr was identified as a *de novo* mutation affecting the HOX domain of the *ALX4* protein. It was not identified in the proband's parents or siblings (Figures 2 and 3). Three missense

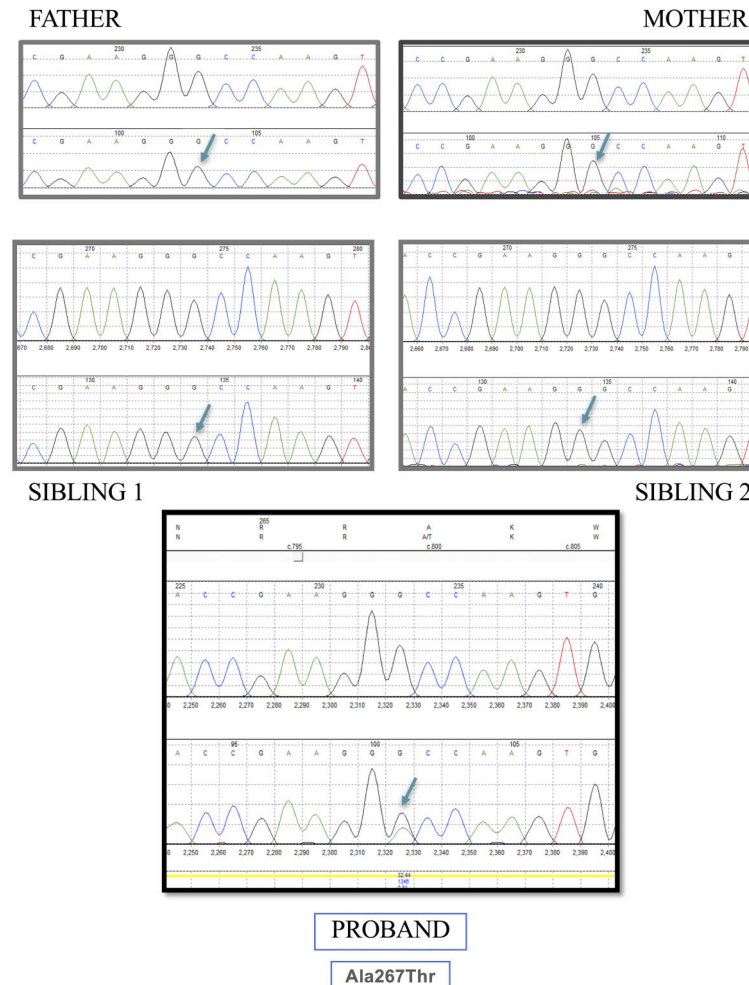


Figure 2. Chromatogram showing presence of *de novo* *ALX4* variant in proband and absence in family members.

variants were identified in *EFNA4* in children with metopic (c.178C>T: p.His60Tyr and C.283A>G: p.Lys95Glu) and sagittal synostosis, whereas a *TWIST1* missense variant (c.380C>A: p.Ala127Glu) affected a child with unilateral coronal synostosis. One child with metopic synostosis expressed concomitant variants in the *ALX4* (c.304C>T: p.Pro102Ser and c.104G>C: p.Arg35Thr) and *EFNA4* (C.283A>G: p.Lys95Glu) (Supplementary Table S1) genes. The variant *ALX4* (c.304C>T: p.Pro102Ser) was predicted as being tolerated and benign by SIFT and PolyPhen databases, respectively. Types of variants (*ALX4*, *EFNA4*, or *TWIST1*) were not significantly associated with specific craniosynostosis subtypes ($P=0.221$). The presence/absence of variants was not significantly associated with craniosynostosis subtypes ($P=0.708$), ethnicity ($P=0.414$), or sex ($P=0.605$).

Discussion

While the etiology of non-syndromic forms of craniosynostosis remains unclear, efforts in recent years have increased our basic understanding of several gene variants associated with these malformations. In the present study, we have investigated the presence of variants in genes previously associated with NSC phenotypes (7,8,14). Despite earlier and recent evidence (15), the *ALX4* and *EFNA4* genes are frequently not considered candidate genes when investigating the genetic causes of NSC. In addition, *TWIST1* has been shown to cause both syndromic and non-syndromic forms of synostosis, but it is more frequently investigated in the presence of coronal and sagittal forms of these malformations. We have therefore chosen to screen these three genes for possible

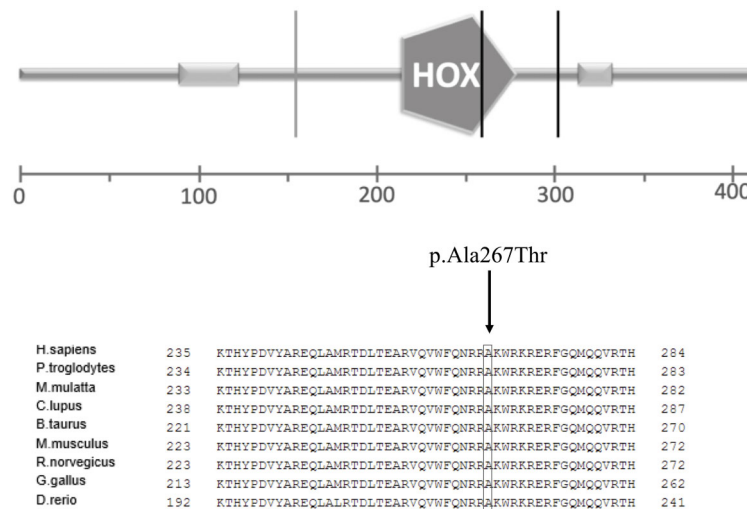


Figure 3. HOX domain within *Homo sapiens* Homeobox protein aristaless-like 4 and sequence alignment between species.

pathogenic variants in a population of children with different forms of non-syndromic single- and multi-suture synostosis. In our cohort, nine different variants affecting these genes were identified in probands with metopic (*ALX4*, *EFNA4*), sagittal (*ALX4*, *EFNA4*), and unilateral or bilateral coronal (*TWIST1*, *ALX4*) craniosynostosis. These variants were not identified in patients with craniosynostosis of the lambdoid suture or with multi-suture synostosis involving other sutures.

An earlier cohort of 203 patients identified five *ALX4* variants in individuals with either sagittal or multi-suture synostosis with the involvement of the sagittal suture (8). Functional analysis of three previously reported variants revealed gain-of-function effect for two of these variants (Val7Phe and Lys211Glu). In the present work, five missense variants were identified in the *ALX4* gene. Two of these variants (c.304C>T: p.Pro102Ser and c.104G>C: p.Arg35Thr) are not rare in the general population and are predicted as benign by SIFT and PolyPhen, while the other three variants (c.769C>T: p.Arg257Cys; c.799G>A: p.Ala267Thr; and c.929G>A: p.Gly310Asp) constitute novel missense variants in the *ALX4* gene. *ALX4* variant Gly310Asp showed conflicting results, being tolerated with a score of 0.1 (Sift), probably damaging in PolyPhen (score of 0.454), and a CAAD of 24.8, thus being classified as likely pathogenic (ACMG). The patient's mother did not present the same variant, and the father was not available for DNA testing. Two of the identified variations in amino acid substitutions (Arg257Cys and Ala267Thr) were predicted by SIFT as being damaging to protein function, with a score of 0 and confirmed to be probably damaging with a PolyPhen score of 1. A white female with bilateral coronal synostosis harbored the Arg257Cys variant, which was also present in the child's unaffected mother. However, the child's unaffected sibling expressed a different genotype, whereas the

father did not enroll in the study. This finding suggested that this variant was not the sole cause of craniosynostosis in this patient, but may show incomplete penetrance, or predispose the individual to craniosynostosis, but not cause it (16,17).

We identified a *de novo* *ALX4* variant c.799G>A (Ala267Thr) in a proband with sagittal craniosynostosis. The child's parents and siblings did not harbor this variant and had a normal phenotype, suggesting that this variant is pathogenic. The role of *ALX4* in calvarial development appears to be associated with osteogenic differentiation and modulation of osteoblast function, working within a consortium of transcription factors alongside *MSX2*, subsequent to lineage commitment of the mesenchymal progenitor cells (18,19). Further, heterozygous loss-of-function variants in *ALX4* and *MSX2* genes produce similar phenotypic outcomes, including an enlarged parietal foramen (20). It has been demonstrated in *ALX4* knockout mice that inactivation of this gene adversely impacts the transcription of *Spp1*, resulting in a reduction in the expression of *FGFR1* and *FGFR2* within the parietal and frontal bones (18). We do know that *FGFR1* and *FGFR2* signaling pathways fulfill specific regulatory functions on suture osteogenesis by controlling the proliferation of osteogenic stem cells (*FGFR2*) and by regulating cell differentiation (*FGFR1*) (21). On the other hand, *Spp1* acts as a key regulator in the bone remodeling process by directly binding to apatite crystals and inhibiting mineralization, in addition to exerting a role in osteoclast recruitment, stimulating adhesion, migration, and bone resorption by these cells (22). Thus, the Ala267Thr variant may create an imbalanced signal expression downstream of *ALX4*, and the early closure of the sagittal suture in this Hispanic patient may be the negative consequence of such a signaling error. This amino acid substitution affects the HOX domain of the *ALX4* protein that lies between

positions 214 and 276. Reported *ALX4* variants affecting this domain cause parietal foramina 2 (PFM2, MIM # 609597) (23), which is also a clinical feature of Potocki-Shaffer syndrome (MIM # 601224), a rare contiguous gene deletion on chromosome 11p11.2 that involves the *ALX4* gene (24).

In the present work, 3/101 individuals presented with variants in the *EFNA4* gene. Two of these patients had a diagnosis of metopic craniosynostosis, while one patient had sagittal craniosynostosis. Interestingly, the male proband with metopic synostosis that carried protein change Lis95Glu also carried two additional *ALX4* variants (c.304C>T: p.Pro102Ser; c.104G>C: p.Arg35Thr). The *EFNA4* variant (c.283A>G) was predicted as probably damaging (PolyPhen score 0.981) and likely pathogenic (ACMG), with a CADD of 23.8. Concomitant variants should be viewed with caution, since their concurrent presence may alter their disease-causing potential (25). Merrill and colleagues described *EFNA4* variants that were associated with metopic (c.178C>T: p.His60Tyr) and sagittal (c.349C>A: p.Pro117Thr) craniosynostosis in patients with non-syndromic unilateral coronal craniosynostosis (14). The variant c.178C>T: p.His60Tyr was also identified by Clarke et al. in two patients with single suture craniosynostosis (coronal and sagittal) (7). Merrill et al. (14) demonstrated that existent boundary defects between neural crest and mesoderm cell populations in the junction between parietal and frontal bones lead to early closure of the coronal suture in *Twist1*^{+/-} mouse embryos. These *Twist1* mutants showed an increase in *MSX2* expression, with lower tissue distribution of Ephrin-A4, perhaps explaining the importance of *EFNA4* in the etiology of craniosynostosis of the coronal suture. It was also demonstrated that these missense variants (c.349C>A: p.Pro117Thr and c.178C>T: p.His60Tyr) cause loss of function and compromise binding of Ephrin-A4 to the EphA7 receptor. High affinity Eph/ephrin interactions are essential for neural crest cell migration and maintenance of tissue boundary by mediating attractive and repulsive effects between cells (26). Failure in tissue boundary integrity may be one of the mechanistic failures generated by pathogenic *EFNA4* variants. The sagittal suture is also formed at the mesoderm-neural crest interface. Loss of tissue boundary may adversely affect the metopic suture, which is exclusively of neural crest origin (27).

Previously, *TWIST1* gene variants were identified in patients with Saethre-Chotzen syndrome and syndromic craniosynostosis (28–30). Evidence suggests that variants within this gene are not considered as a causative or contributing factor in non-syndromic, single-suture craniosynostosis (11). Nevertheless, *TWIST1* variants are frequently associated with coronal craniosynostosis, and this type of premature suture closure is more commonly of genetic origin (9). In 2006, Kress et al. (31) reported a rare variant (c.602C>T, p.Ser201Tyr) in a child with sagittal

NSC and his unaffected mother. The authors proposed that this variant was non-pathogenic. Seto et al. (16) identified variants in the *TWIST* box region of *TWIST1* in two different patients presenting with isolated sagittal synostosis (c.563C>T: p.Ser188Leu) and unilateral coronal synostosis (c.556G>A: p.Ala186Thr). In the present work, a *TWIST1* variant was found in a Hispanic child with unilateral coronal NSC and a positive family history of this malformation. This variant was in the top 0.1% of deleterious variants in the human genome, being also a pathogenic variant according to other databases. A sample from the child's unaffected sibling allowed detection of the same variant. We believe that c.380C>A: p. Ala127Glu could be a variant with incomplete penetrance or one that results in variable expressivity, as previously proposed by Seto et al. (16). The possibility of mosaicism must also be considered, since this condition was previously identified for the *TWIST1* gene (32). Furthermore, a *TWIST1* variant within this location (c.380C>T) with a substitution of alanine by valine was previously reported in a patient with Saethre-Chotzen syndrome (33). Hence, variants within this region are likely damaging, emphasizing the need to sequence the *TWIST1* gene in patients with coronal craniosynostosis.

The present study had limitations. First, the study was limited to screening only the *ALX4*, *EFNA4*, and *TWIST1* genes. Previous work has identified variants within other genes in patients with non-syndromic craniosynostosis. Some of these genes include: *BMP2*, *SMAD6*, *BBS9*, *MEGF8*, *SCARF2*, *FBN1*, *IGF1R*, *ATR*, *ERF*, *FAM20C*, *TGFR2*, *FGFR1*, *TCF12*, *IFT122*, *IL11RA*, *MASP1*, *MEGF8*, *POR*, *RAB23*, *RECQL4*, *SKI*, *ALPL*, *FLNA*, *HUWE1*, *IDUA*, *IFT122*, *IRX5*, *KAT6A*, *KMT2D*, and *LRP5* (7,10,25,34,35). The sample size was also considered a limitation. The identified variants showed very low frequency in a given population; therefore, we were unable to demonstrate statistical association between these variants and craniosynostosis subtypes. We had difficulties recruiting parent-offspring trios, as not all parents agreed to participate or only one parent (usually the child's mother) was present for medical appointments at which time a sample could be collected. Moreover, many families resided in distant communities and return consultation visits were scheduled only after our recruitment period had ended.

In the present work, the *ALX4* gene presented the highest frequency of variants in patients with NSC (5/101), followed by the *EFNA4* (3/101) and *TWIST 1* (1/101) genes. It is also important to mention that, with the exception of the *ALX4* variants c.304C>T (gnomAD frequency of 78.070/175.982) and c.104G>C (gnomAD frequency of 121.117/244.066) that are relatively common, all other variants were less frequent alleles, featuring among the top 1% of deleterious variants in the human genome. Based on the current literature and databases, these

variants are likely pathogenic and may play a contributing role in the development of craniosynostosis. Future population-based studies should help clarify this matter. These results suggested that *ALX4* and *EFNA4* genes should not be discarded as potential candidate genes to be used for panel testing in the diagnosis of NSC along with other genes (10). Also, the addition of the *TWIST1* gene in these panels may be of interest when investigating genetic causes of coronal NSC. The *ALX4* c.799G>A (Ala267Thr) is a *de novo* variant affecting the HOX domain of the *ALX4* protein in a proband with premature closure of the sagittal suture. This finding suggested *ALX4* as a gene possibly involved in NSC.

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Supplementary Material

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Acknowledgments

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