HHS Public Access

Author manuscript

Am J Med Genet A. Author manuscript; available in PMC 2019 February 01.

Published in final edited form as:

Am J Med Genet A. 2018 February ; 176(2): 290–300. doi:10.1002/ajmg.a.38540.

Single Suture Craniosynostosis: Identification of Rare Variants in Genes Associated with Syndromic Forms

Christine M. Clarke[1], **Vincent T. Fok**[1], **Jennifer A. Gustafson**[1], **Matthew D. Smyth**[2,3], **Andrew E. Timms**[1], **Chris D. Frazar**[4], **Joshua D. Smith**[4], **Craig B. Birgfeld**[5], **Amy Lee**[6,7], **Richard Ellenbogen**[6,7], **Joseph S. Gruss**[5,10], **Richard A. Hopper**[5,10], and **Michael L. Cunningham**[1,8,9]

[1]Center for Developmental Biology and Regenerative Medicine, Seattle Children's Research Institute, Seattle, WA 98101

[2]Washington University Pediatric Epilepsy Center at St. Louis Children's Hospital, Washington University School of Medicine, St. Louis, MO 63110

[3]Department of Neurological Surgery, St. Louis Children's Hospital, St. Louis, MO 63110

[4]Department of Genome Sciences, University of Washington, Seattle, WA 98195

[5]Department of Surgery, Division of Plastic Surgery, University of Washington School of Medicine, Seattle, WA 98195

[6]Department of Neurological Surgery, University of Washington School of Medicine, Seattle, WA 98195

[7]Department of Pediatric Neurosurgery, Seattle Children's Hospital, Seattle, WA 98105

[8]Division of Craniofacial Medicine, Department of Pediatrics, University of Washington, Seattle, WA 98195

[9]Seattle Children's Craniofacial Center, Seattle Children's Hospital, Seattle, WA 98105

[10]Division of Craniofacial and Plastic Surgery, Seattle Children's Hospital, Seattle, WA 98105

Abstract

We report RNA-Sequencing results on a cohort of patients with single suture craniosynostosis and demonstrate significant enrichment of heterozygous, rare, and damaging variants among key craniosynostosis-related genes. Genetic burden analysis identified a significant increase in damaging variants in *ATR, EFNA4, ERF, MEGF8, SCARF2* and *TGFBR2*. Of 391 participants, 15% were found to have damaging and potentially causal variants in 29 genes. We observed transmission in 96% of the affected individuals, and thus penetrance, epigenetics, and oligogenic factors need to be considered when recommending genetic testing in patients with nonsyndromic craniosynostosis.

Corresponding Author: Michael L. Cunningham, Seattle Children's Craniofacial Center, 4800 Sand Point Way NE, Seattle, Washington, 98105, michael.cunningham@seattlechildrens.org.

Keywords

Craniosynostosis; Single Suture; Non Syndromic; RNA sequencing; Enrichment; Gene Burden; Protein Domains; Variants

INTRODUCTION

Craniosynostosis is the premature fusion of one or more of the calvarial sutures that occurs in syndromic and non-syndromic forms in approximately 1/2,100–2,500 live births [Boulet et al., 2008; Johnson and Wilkie 2011; Wilkie et al., 2010]. Craniosynostosis, as a major or minor feature, has been associated with over 150 different syndromes [Kabbani and Raghuveer 2004; Ye et al., 2016]; however, single suture fusions account for approximately 85% of all patients diagnosed with craniosynostosis [Heuze et al., 2014]. In some families, single suture craniosynostosis (SSC) follows Mendelian patterns of inheritance, and approximately 6–8% of patients have a positive family history that is consistent with autosomal dominant transmission [Boyadjiev 2007; Lajeunie et al., 1996]. Examples of familial recurrence typically involve the same suture, although large pedigrees with coronal or sagittal synostosis have been described that exhibit significant intrafamilial variability [Boyadjiev 2007; Cohen and MacLean 2000]. This suggests that the pattern of suture fusion may not be a reliable method of clustering affected individuals. In addition, due to clinical overlap in the presentations of SSC and mild or atypical patients with Crouzon, Saethre-Chotzen, or Muenke syndromes [Boyadjiev 2007], phenotypically guided genetic testing may fail to identify rarer causes of disease [Miller et al., 2017].

The use of next generation sequencing (NGS) and RNA-Sequencing (RNA-Seq) technologies offer alternatives to phenotypically guided genetic testing methods. The benefits of RNA-Seq are two-fold: gene variant detection can be conducted in ways analogous to exome sequencing, but with the added benefit of patient specific gene expression. Our use of calvarial osteoblasts cell lines from 391 SSC patients as an RNA source serves to enrich osteoblast candidate gene transcripts, and thus, variant detection, in ways that can be used to identify key pathway drivers in the development of SSC.

To date, most of the genetic testing for SSC has focused on sequencing hotspots of known craniosynostosis syndromes associated with coronal synostosis (*FGFR1–3, TWIST1, EFNB1, TCF12*), with low diagnostic yield [Heuze et al., 2014; Ye et al., 2016]. We sought to address these gaps by using RNA-Seq methodology and stringent filtering criteria to identify coding region variants in individuals diagnosed with SSC while providing patient specific expression data for future functional studies. In this study, we report rare, damaging alleles in 29 genes known to be associated with syndromic forms of craniosynostosis that are enriched in patients with SSC.

MATERIALS AND METHODS

Ethics statement

Written informed consent from all parents and guardians of children with SSC was obtained and a consent waiver was obtained for the use of anonymous control samples.

Participant enrollment and cohort description

Three hundred and ninety-seven children were enrolled in the study at the time of treatment. Computed tomography scans confirmed the diagnosis of isolated SSC. Criteria for exclusion included the presence of major medical conditions or presence of three or more minor extra-cranial malformations. Calvaria and blood samples were obtained from the 397 individuals undergoing surgery and blood or saliva samples were collected from consented parents. Eighty-seven control samples were obtained from patients undergoing a craniotomy for reasons other than craniosynostosis (e.g., brain tumor, isolated hydrocephalus) or at the time of autopsy. All samples were screened for known pathogenic variants in *FGFR1, FGFR2, FGFR3, TWIST1, EFNB1, and MSX2*. Patients with these variants or chromosomal rearrangements were excluded from this analysis.

Cell culture of primary osteoblasts

For participants with craniosynostosis, calvarial bone fragments were obtained from otherwise discarded tissues during surgical reconstruction. For control samples, calvarial bone was obtained from surgeries or autopsies. Bone fragments were used to establish osteoblast cell lines as described [Park et al., 2015].

RNA isolation

Cell lines were thawed and cultured to sub-confluence in T25 flasks and passaged at a density of 175,000 cells per 25 cm^2. At 75% confluence, cells were trypsinized, washed in cold 1X PBS, and RNA was isolated using the Roche High Pure miRNA Isolation Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and only samples with RIN scores above 8.6 were used for RNA Sequencing.

TruSeq Stranded mRNA Preparation

Next-generation sequencing libraries were prepared from 1.25 μg of total RNA in a high-throughput format using the TruSeq Stranded mRNA kit (Illumina, San Diego, CA). All the steps required for sequence library construction were automated and performed on a Sciclone NGSx Workstation (Perkin Elmer, Waltham, MA). During library construction, rRNA was depleted by means of a poly-A enrichment and first and second strand cDNA syntheses were performed. Each library was uniquely barcoded using Illumina adapters and amplified by PCR. After amplification and cleanup, library concentrations were quantified using the Quant-it dsDNA Assay (Life Technologies, Carlsbad, CA). Final libraries were normalized and pooled based on Agilent 2100 Bioanalyzer results (Agilent Technologies, Santa Clara, CA) and size selected using a Pippin Prep (Sage Science, Beverly, MA). Pooled libraries were diluted to a final concentration of 2–3 nM for sequencing on a HiSeq 4000.

Read Processing and Analysis Pipeline

Samples were multiplexed and sequenced on a HiSeq 4000. Lane-level sequencing reads were base quality checked using the FASTX-toolkit and FastQC and aligned to hg19 with a reference transcriptome Ensembl v67 using TopHat2 suite [Kim et al., 2013] followed by mate-fixing. Lane-level aligned BAMs were merged with Picard MergeSamFiles tool, PCR

duplicates were marked with Picard MarkDuplicates tool, local realignment was performed around indels, and base quality score recalibration was run using GATK tools [McKenna et al., 2010] to generate sample-level BAMs. Sample BAMs were used for isoform assembly and quantitation with Cufflinks [Kim et al., 2013; Trapnell et al., 2013] and genomic features were quantitated with featureCounts [Liao et al., 2014]. Custom QC scripts generated final sample level statistics. Any transcripts with average raw counts <100 or with average FPKM scores <1.5 were considered not expressed sufficiently for reliable gene variant analysis, and were removed from further analysis.

Variant detection and genotyping were performed with the GATK Unified Genotyper version 2.6.5 [DePristo et al., 2011]. Variant calls were heuristically filtered with methods similar to SNiPR [Piskol et al., 2013]. Reads with a mapping quality score less than 20 on a Phred scale [Li et al., 2008] were filtered to remove obvious paralagous reads in pileups. Variants with allele biases of greater than 0.75 and strand biases of ≥0.1% were filtered, as were variants within 6 bp of the 5′ end of a read. Two sequence-context dependent filters further refined RNA-Seq variant calls. Variants in repeat regions were removed using a file of intervals from RepeatMasker (http://www.repeatmasker.org/), and variants in known RNA editing sites were removed if they overlapped a curated file of known RNA editing intervals [Ramaswami and Li 2014].

All variants were annotated with ANNOVAR [Wang et al., 2010] using refGene as a gene model. Variants were filtered for quality (coverage ≥5, Q≥30), frequency in the Exome Aggregation Consortium ≤ 0.01 (ExAC) [Lek et al., 2016], and in an exon, but not predicted to be synonymous. Next, variants were filtered by CADD [Kircher et al., 2014] (CADD_phred), GERP [Davydov et al., 2010] (GERP_RS++) and Polyphen2 [Adzhubei et al., 2010] (Polyphen2_HDIV) scores. If variants passed all criteria (CADD ≥15, GERP ≥3 and Polyphen2 ≥0.9), they were considered potentially damaging alleles.

Variant Identification in genes associated with craniosynostosis

Variants meeting the above criteria were filtered against a list of 61 genes [Heuze et al., 2014; Twigg and Wilkie 2015; Ye et al., 2016], 20 of which are associated with classic syndromic forms of craniosynostosis in >50% of individuals) and 41 genes associated with a syndrome in which craniosynostosis is seen as an occasional feature (<50%).

Minor Allele Frequency (MAF) scores for variants were obtained from the UCSC Genome Browser's dbSNP build 147 database [Kent et al., 2002; Speir et al., 2016], ExAC [Lek et al., 2016], and Exome Variant Server, http://evs.gs.washington.edu/EVS/ [accessed 09/19/2017]. If no allele frequencies were listed, the ExAC database was used to evaluate exome sequencing coverage, and variants in regions with coverage <30% were not assigned a MAF. If adequate coverage existed, the allele count score of the nearest variant neighbor was used as a proxy in statistical calculations. A two-tailed two-proportion z-test was used with a significance value of $p<0.05$ to determine if there were statistically valid differences between the observed allele frequencies in our sample cohort compared to the overall population frequencies from the above databases. Sequence variants were interpreted as Likely Benign, Uncertain Significance, or Likely Pathogenic according to ACMG guidelines [Richards et al., 2015].

Variant validation

RNA-Seq variants meeting criteria were validated by Sanger DNA sequencing. Proband and parental genomic DNA samples were amplified with primers (Sigma Aldrich, St. Louis, MO) designed for each variant (Supplementary Table II) using Primer3 software [Untergasser et al., 2012] and reference sequences from UCSC's Genome Browser [Kent et al., 2002; Speir et al., 2016], (GRCh37/hg19). Variant sequences were aligned to the above reference sequences using Sequencher® version 5.1 DNA (Gene Codes Corporation, Ann Arbor, MI). In addition, alignments and chromatograms were visually inspected to confirm the variants of interest. Any variants that did not confirm the RNA-Seq results (4%) were removed from further analysis.

DAVID Functional Annotation

To identify overlapping functional domains in the 29 genes with presumed damaging variants, the gene list was uploaded using the DAVID Tool Suite [Huang da et al., 2009b]. Filtering was conducted with the application of high stringency filters and a 0.1 EASE adjustment. Enrichment scores of >1.3 and $p<0.05$ are reported for Functional Annotation Clustering, whereas scores of fold enrichment >2.0 and $p<0.05$ are reported for Functional Annotation Chart results.

Gene Burden and Variant Enrichment Analysis

Within each gene of interest we identified the number of rare exonic variants (ExAC MAF = ≤0.01) in both our proband and ExAC datasets that were predicted to be disruptive (nonsense, frameshift, or splice site) or missense variants with a CADD ≥15, GERP ≥3 and Polyphen2 ≥0.9. We analyzed these data for significant differences in the proportion of exonic variants in individual genes between our cohort and ExAC controls using a Fisher's exact test. Using the same parameters for identification of variants predicted to be disruptive or damaging we used a two-tailed Chi-square test with Yates correction to determine if damaging variants in all tested craniosynostosis genes were more common in our cohort than in the historical controls available in the ExAC database.

RESULTS

Cohort description

Three hundred and ninety-seven probands were enrolled in this study. The prevalence of each suture type and male to female ratios (Table I) is on par with previously described prevalence data [Di Rocco et al., 2009; Kolar 2011; Lajeunie et al., 1995; Lajeunie et al., 1996; Lajeunie et al., 1998; Selber et al., 2008; van der Meulen et al., 2009]. No parents were documented as affected with craniosynostosis.

Enrichment of RNA-Seq variants in craniosynostosis-related genes

Of the 397 SSC calvarial osteoblast RNA samples, six failed to give any RNA-Seq reads, and of the 87 controls, two failed; thus, 391 SSC transcripts were used for further variant analyses. When applied to our primary filtering strategies (MAF ≤ 0.01, CADD ≥15, GERP ≥3, and Polyphen2 ≥0.9), we detected a total of 11,036 non-synonymous, predicted

damaging, variants in 19,724 expressed transcripts. To assess whether or not the variants were enriched in known craniosynostosis genes, we compared the number of damaging variants in the transcripts from the genes in Table II to the number of coding variants predicted to be damaging detected in all transcripts. One hundred and two damaging variants were detected in 53 transcribed genes known to be associated with craniosynostosis. This reflects a statistically significant enrichment of damaging variants in known craniosynostosis genes ($P<0.0001$, two-tailed Chi-square test with Yates correction), and supports the hypothesis that damaging variation in known craniosynostosis genes may underlie the development of SSC in some patients.

Eight of the gene transcripts referenced in Table II had average raw or FPKM values too low for reliable variant detection: *ALX4, COLEC11, FGFR3, FREM1, GPC3, IHH, LMX1B, and PHEX*; thus potentially damaging variants in these candidates could not be ascertained. Despite adequate levels of RNA transcription, no variants met our filtering criteria for *ADAMTSL4, ASXL1, CDC45, CTSK, CYP26B1, EFNB1, ESCO2, FGFR2, GNAS, IDS, IFT43, JAG1, KRAS, MSX2, STAT3, TGFBR1, WDR19,* or *ZIC1*, although this does not rule out their potential role in craniosynostosis. Variants meeting the filtering criteria were observed in *GLI3, GNPTAB, RUNX2, TMCO1,* and *WDR35*; however, these variants did not achieve statistical significance when compared to allele frequencies in the ExAC database (Supplementary Table I).

Sixty-four of the 102 variants were significant and damaging, as defined by our stringent filtering parameters and statistical tests. These 64 variants were found in 29 genes associated with syndromic craniosynostosis (Table III and IV). Following the classification methodology of Twigg and Wilkie [2015], 15 variants were seen in eight genes considered to be core craniosynostosis genes (Table III) and 48 variants were observed in 21 genes that have been associated with syndromic disease that have craniosynostosis as an occasional feature (Table IV). The variants detected in this study were predominantly heterozygous, missense variants and were distributed nearly equally among loci associated with disorders with autosomal dominant and autosomal recessive inheritance patterns.

Parental samples were available for testing in 42% of families and transmission of damaging variants was documented in 96% of these probands, however the inheritance pattern of the remaining variants is unknown. One *de novo* variant p.(Val563Gly) was detected in *SCARF2*. Tables III–IV and Supplementary Table I display the complete set of genotypic changes, predictive *in silico* scores, and statistical calculations for the significance of the variants observed in our dataset of 391 SSC probands.

DAVID analysis

We used the DAVID tool suite [Huang da et al., 2009a; Huang da et al., 2009b] with our set of 29 genes containing rare, damaging variants to discover functionally related protein domains or motifs that may be associated in our SSC cohort. Not surprisingly, the functional chart results (Supplemental Table III) show strong associations of these genes with “disease mutation” and “craniosynostosis” ($p\leq 6.84 \times 10^{-22}$ and $p\leq 1.23 \times 10^{-18}$; respectively), since our list of 29 genes originally derived from 61 genes associated with syndromic craniosynostosis. It is notable, however, that 83% of this smaller subset (24 of 29 genes) are

involved in phosphorylation processes ($p \leq 6.33 \times 10^{-6}$), and five genes contain EGF-like domains (17.2%, $p \leq 2.87 \times 10^{-4}$), particularly *MEGF8, MASP1, FBN1, LRP5*, and *SCARF2*. Another functional group containing *TGFBR2, SKI, TCF12,* and *FLNA* is defined by Smad-binding (13.8%, $p \leq 4.32 \times 10^{-5}$), and *FGFR1, TGFBR2,* and *LRP5* are associated with the positive regulation of mesenchymal cell proliferation (10.34%, $p \leq 8.5 \times 10^{-4}$). The functional annotation analyses showed enriched clustering of genes with EGF-like domains, EGF-like calcium-binding domains, signal peptides, disulfide bonds, and kinases (Supplemental Table IV). While this result could have been predicted based on our selection of known syndromic craniosynostosis genes, the aggregation of the variants we identified in these functional domains increases the likelihood of pathogenicity.

Gene Burden Test

In addition to assessing variant level differences, we used genetic burden testing to determine if these patients had significant increases in the number of predicted damaging exonic variants when compared to ExAC controls. Using our filtering strategy we identified six genes (*ATR, EFNA4, ERF, MEGF8, SCARF2* and *TGFBR2*) with a significantly increased number of predicted damaging variants between these patients and ExAC controls using the Fisher's exact test. Two of the variants observed in *EFNA4,* c.178C>T:p.(His60Tyr) and c.590T>G:p.(Leu197Arg) (Supplemental Table I), are not statistically significant when compared to allele frequencies in UCSC; however, they may confer a predisposition to SSC occurrence [Merrill et al., 2006]. Twenty individuals in our cohort were identified with predicted damaging variants in one of these six genes with significantly increased variant burden representing approximately 5% of our sample.

DISCUSSION

Although causality has not been determined, our results highlight a wide range of potentially causative variants observed in both known syndromic craniosynostosis genes and genes indirectly implicated in craniosynotosis. Beyond our identification of individual candidate genes associated with SSC, our analysis demonstrated a highly significant enrichment of predicted damaging variants in craniosynostosis genes among these patients. Our genetic burden analysis suggests that additional research into the candidates identified in this work will uncover new causes for SSC. Ideally, functional studies of key variants would be completed to establish direct proof of causation.

In order to select the genetic changes most likely to be pathogenic to the development of SSC, we restricted our analysis to non-synonymous, damaging, exonic differences in genes already known to be associated with craniosynostosis. However, there are limitations to this approach, in that there may be splicing variants, nonsense mediated decay, null alleles, large duplications or deletions, and 5′ or 3′ regulatory elements, and novel genes that were excluded from this analysis. Also, the data for the eight genes with low RNA expression in primary osteoblasts were not included and thus, additional variants may reside in those genes as well.

Craniosynostosis is rarely the result of complete loss of gene function [Patel et al., 2014; Twigg et al., 2012; Twigg and Wilkie 2015]. Therefore, we propose that heterozygous

missense changes in similar protein domains may perturb function to a degree that results in SSC, but without the other malformations associated with syndromic forms. As such, we examined conserved protein structural domains using DAVID analyses to note any commonalities in function, both within and between craniosynostosis-related genes. Damaging variants in genes with EGF-like domains (*MEGF8, SCARF2, FBN1*), and receptor protein kinase function (*IGF1R, ATR, ERF, FAM20C, TGFBR2*) were enriched in our cohort (Supplemental Tables III–VI). This work demonstrates that additional genes or gene families with related protein structures may present novel targets for SSC gene discovery, although additional studies need to be conducted to determine if these functional relationships are representative of craniosynostosis overall, or SSC in particular. Furthermore, this work demonstrates the efficacy of RNA-Seq as a viable alternative to exome sequencing to identify candidate variants in a multifactorial and multigenic disorder such as craniosynostosis. With over 95% of variant calls validated by Sanger sequencing of genomic DNA, there is great utility of this approach for studies exploring the correlations between gene expression and genetic variation.

Analysis of this cohort of highly curated participants with SSC demonstrated heterozygous missense variants in genes, which when mutated, cause dominant, recessive, and X-linked forms of syndromic craniosynostosis. We demonstrated a significant enrichment of damaging variants in the expressed 53 craniosynostosis genes in our cohort relative to all expressed transcripts ($p<0.0001$). Specific to our analyses, we identified 64 predicted damaging variants that were significantly more common in our cohort than the general population and represent 15% of the 391 patients. Although these data do not prove causality, they demonstrate that predicted damaging variants in known syndromic craniosynostosis genes are more common in patients with isolated craniosynostosis. While each of the rare, predicted damaging, variants identified in this study may increase the risk of developing craniosynostosis, the frequency of damaging variants identified in *ATR, EFNA4, ERF, MEGF8, SCARF2, and TGFBR2* suggest these genes as SSC candidates worthy of further investigation.

Upon replication of our data and clinical correlation, our study suggests that genetic evaluations of children with SSC of all suture types should be considered, not only those presenting with coronal SSC. Analysis of *FLNA* and *TCF12*, for instance, may yield additional variants in sagittal, coronal and metopic forms of SSC than previously determined. We identified transmission of the damaging variant from an unaffected parent in the vast majority of patients in which we had parental DNA. This finding is in keeping with incomplete penetrance, seen in many families with recurrence of SSC, suggesting oligogenic or gene environment interactions. In fact, we identified four patients that had two potentially damaging variants in more than one gene associated with craniosynostosis. This is an important consideration with regard to future research as well as the interpretation of increasingly common clinical sequencing data on this population.

With respect to genetic testing panels currently available, we found that 12 genes with damaging variants from our study are represented on three or more NGS craniosynostosis testing panels: *ERF, FGFR1, TCF12, IFT122, IL11RA, MASP1, MEGF8, POR, RAB23, RECQL4, SKI*, and *TGFBR2*. The remaining 17 genes, most notably *FLNA, ATR, FBN1*,

and *SCARF2*, were on ≤1 craniosynostosis gene testing panel; thus potentially causative SSC variants, such as those observed in our study, may go undetected.

One of the keys to understanding disease causation is the elucidation of the links between genetic variants and their functional consequences. This will require close collaborations between basic scientists, clinicians and genome scientists [Manolio et al., 2017]. To that end, future work on this cohort will include traditional differential gene expression analyses as well as correlation analysis of patient specific variants and expression data from osteoblast cell lines derived from affected individuals. These data will serve to guide additional research into the pathogenesis of single suture craniosynostosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We express our gratitude to all of the participants and their families for providing samples for this study. We thank Linda Peters and Deanna Mercer for recruitment and facilitating collections for these families and institutions. This research was supported by NIH/NIDCR DE018227-10 (MLC).

References

Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. Nat Methods. 2010; 7(4): 248–249. [PubMed: 20354512]

Boulet SL, Rasmussen SA, Honein MA. A population-based study of craniosynostosis in metropolitan Atlanta, 1989–2003. Am J Med Genet A. 2008; 146A(8):984–991. [PubMed: 18344207]

Boyadjiev SA. Genetic analysis of non-syndromic craniosynostosis. Orthod Craniofac Res. 2007; 10(3):129–137. [PubMed: 17651129]

Cohen, MM., MacLean, RE. Craniosynostosis : diagnosis, evaluation, and management. New York: Oxford University Press; 2000. p. xxp. 454

Davydov EV, Goode DL, Sirota M, Cooper GM, Sidow A, Batzoglou S. Identifying a high fraction of the human genome to be under selective constraint using GERP++ PLoS Comput Biol. 2010; 6(12):e1001025. [PubMed: 21152010]

DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, Philippakis AA, del Angel G, Rivas MA, Hanna M, McKenna A, Fennell TJ, Kernytsky AM, Sivachenko AY, Cibulskis K, Gabriel SB, Altshuler D, Daly MJ. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet. 2011; 43(5):491–498. [PubMed: 21478889]

Di Rocco F, Arnaud E, Renier D. Evolution in the frequency of nonsyndromic craniosynostosis. J Neurosurg Pediatr. 2009; 4(1):21–25. [PubMed: 19569905]

Heuze Y, Holmes G, Peter I, Richtsmeier JT, Jabs EW. Closing the Gap: Genetic and Genomic Continuum from Syndromic to Nonsyndromic Craniosynostoses. Curr Genet Med Rep. 2014; 2(3): 135–145. [PubMed: 26146596]

Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009a; 37(1):1–13. [PubMed: 19033363]

Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009b; 4(1):44–57. [PubMed: 19131956]

Johnson D, Wilkie AO. Craniosynostosis. Eur J Hum Genet. 2011; 19(4):369–376. [PubMed: 21248745]

Kabbani H, Raghuveer TS. Craniosynostosis. Am Fam Physician. 2004; 69(12):2863–2870. [PubMed: 15222651]

Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D. The human genome browser at UCSC. Genome Res. 2002; 12(6):996–1006. [PubMed: 12045153]

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 2013; 14(4):R36. [PubMed: 23618408]

Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. Nat Genet. 2014; 46(3):310–315. [PubMed: 24487276]

Kolar JC. An epidemiological study of nonsyndromal craniosynostoses. J Craniofac Surg. 2011; 22(1): 47–49. [PubMed: 21187784]

Lajeunie E, Le Merrer M, Bonaiti-Pellie C, Marchac D, Renier D. Genetic study of nonsyndromic coronal craniosynostosis. Am J Med Genet. 1995; 55(4):500–504. [PubMed: 7762595]

Lajeunie E, Le Merrer M, Bonaiti-Pellie C, Marchac D, Renier D. Genetic study of scaphocephaly. Am J Med Genet. 1996; 62(3):282–285. [PubMed: 8882788]

Lajeunie E, Le Merrer M, Marchac D, Renier D. Syndromal and nonsyndromal primary trigonocephaly: analysis of a series of 237 patients. Am J Med Genet. 1998; 75(2):211–215. [PubMed: 9450889]

Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, Tukiainen T, Birnbaum DP, Kosmicki JA, Duncan LE, Estrada K, Zhao F, Zou J, Pierce-Hoffman E, Berghout J, Cooper DN, Deflaux N, DePristo M, Do R, Flannick J, Fromer M, Gauthier L, Goldstein J, Gupta N, Howrigan D, Kiezun A, Kurki MI, Moonshine AL, Natarajan P, Orozco L, Peloso GM, Poplin R, Rivas MA, Ruano-Rubio V, Rose SA, Ruderfer DM, Shakir K, Stenson PD, Stevens C, Thomas BP, Tiao G, Tusie-Luna MT, Weisburd B, Won HH, Yu D, Altshuler DM, Ardissino D, Boehnke M, Danesh J, Donnelly S, Elosua R, Florez JC, Gabriel SB, Getz G, Glatt SJ, Hultman CM, Kathiresan S, Laakso M, McCarroll S, McCarthy MI, McGovern D, McPherson R, Neale BM, Palotie A, Purcell SM, Saleheen D, Scharf JM, Sklar P, Sullivan PF, Tuomilehto J, Tsuang MT, Watkins HC, Wilson JG, Daly MJ, MacArthur DG. Exome Aggregation C. Analysis of protein-coding genetic variation in 60,706 humans. Nature. 2016; 536(7616):285–291. [PubMed: 27535533]

Li H, Ruan J, Durbin R. Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Res. 2008; 18(11):1851–1858. [PubMed: 18714091]

Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics. 2014; 30(7):923–930. [PubMed: 24227677]

Manolio TA, Fowler DM, Starita LM, Haendel MA, MacArthur DG, Biesecker LG, Worthey E, Chisholm RL, Green ED, Jacob HJ, McLeod HL, Roden D, Rodriguez LL, Williams MS, Cooper GM, Cox NJ, Herman GE, Kingsmore S, Lo C, Lutz C, MacRae CA, Nussbaum RL, Ordovas JM, Ramos EM, Robinson PN, Rubinstein WS, Seidman C, Stranger BE, Wang H, Westerfield M, Bult C. Bedside Back to Bench: Building Bridges between Basic and Clinical Genomic Research. Cell. 2017; 169(1):6–12. [PubMed: 28340351]

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010; 20(9):1297–1303. [PubMed: 20644199]

Merrill AE, Bochukova EG, Brugger SM, Ishii M, Pilz DT, Wall SA, Lyons KM, Wilkie AO, Maxson RE Jr. Cell mixing at a neural crest-mesoderm boundary and deficient ephrin-Eph signaling in the pathogenesis of craniosynostosis. Hum Mol Genet. 2006; 15(8):1319–1328. [PubMed: 16540516]

Miller KA, Twigg SR, McGowan SJ, Phipps JM, Fenwick AL, Johnson D, Wall SA, Noons P, Rees KE, Tidey EA, Craft J, Taylor J, Taylor JC, Goos JA, Swagemakers SM, Mathijssen IM, van der Spek PJ, Lord H, Lester T, Abid N, Cilliers D, Hurst JA, Morton JE, Sweeney E, Weber A, Wilson LC, Wilkie AO. Diagnostic value of exome and whole genome sequencing in craniosynostosis. J Med Genet. 2017; 54(4):260–268. [PubMed: 27884935]

Park SS, Beyer RP, Smyth MD, Clarke CM, Timms AE, Bammler TK, Stamper BD, Mecham BH, Gustafson JA, Cunningham ML. Osteoblast differentiation profiles define sex specific gene expression patterns in craniosynostosis. Bone. 2015; 76:169–176. [PubMed: 25753363]

Patel N, Salih MA, Alshammari MJ, Abdulwahhab F, Adly N, Alzahrani F, Elgamal EA, Elkhashab HY, Al-Qattan M, Alkuraya FS. Expanding the clinical spectrum and allelic heterogeneity in van den Ende-Gupta syndrome. Clin Genet. 2014; 85(5):492–494. [PubMed: 23808541]

Piskol R, Ramaswami G, Li JB. Reliable identification of genomic variants from RNA-seq data. Am J Hum Genet. 2013; 93(4):641–651. [PubMed: 24075185]

Ramaswami G, Li JB. RADAR: a rigorously annotated database of A-to-I RNA editing. Nucleic Acids Res. 2014; 42(Database issue):D109–113. [PubMed: 24163250]

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, Committee ALQA. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015; 17(5): 405–424. [PubMed: 25741868]

Selber J, Reid RR, Chike-Obi CJ, Sutton LN, Zackai EH, McDonald-McGinn D, Sonnad SS, Whitaker LA, Bartlett SP. The changing epidemiologic spectrum of single-suture synostoses. Plast Reconstr Surg. 2008; 122(2):527–533. [PubMed: 18626371]

Speir ML, Zweig AS, Rosenbloom KR, Raney BJ, Paten B, Nejad P, Lee BT, Learned K, Karolchik D, Hinrichs AS, Heitner S, Harte RA, Haeussler M, Guruvadoo L, Fujita PA, Eisenhart C, Diekhans M, Clawson H, Casper J, Barber GP, Haussler D, Kuhn RM, Kent WJ. The UCSC Genome Browser database: 2016 update. Nucleic Acids Res. 2016; 44(D1):D717–725. [PubMed: 26590259]

Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat Biotechnol. 2013; 31(1):46–53. [PubMed: 23222703]

Twigg SR, Lloyd D, Jenkins D, Elcioglu NE, Cooper CD, Al-Sannaa N, Annagur A, Gillessen-Kaesbach G, Huning I, Knight SJ, Goodship JA, Keavney BD, Beales PL, Gileadi O, McGowan SJ, Wilkie AO. Mutations in multidomain protein MEGF8 identify a Carpenter syndrome subtype associated with defective lateralization. Am J Hum Genet. 2012; 91(5):897–905. [PubMed: 23063620]

Twigg SR, Wilkie AO. A Genetic-Pathophysiological Framework for Craniosynostosis. Am J Hum Genet. 2015; 97(3):359–377. [PubMed: 26340332]

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. Primer3--new capabilities and interfaces. Nucleic Acids Res. 2012; 40(15):e115. [PubMed: 22730293]

van der Meulen J, van der Hulst R, van Adrichem L, Arnaud E, Chin-Shong D, Duncan C, Habets E, Hinojosa J, Mathijssen I, May P, Morritt D, Nishikawa H, Noons P, Richardson D, Wall S, van der Vlugt J, Renier D. The increase of metopic synostosis: a pan-European observation. J Craniofac Surg. 2009; 20(2):283–286. [PubMed: 19326483]

Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010; 38(16):e164. [PubMed: 20601685]

Wilkie AO, Byren JC, Hurst JA, Jayamohan J, Johnson D, Knight SJ, Lester T, Richards PG, Twigg SR, Wall SA. Prevalence and complications of single-gene and chromosomal disorders in craniosynostosis. Pediatrics. 2010; 126(2):e391–400. [PubMed: 20643727]

Ye X, Guilmatre A, Reva B, Peter I, Heuze Y, Richtsmeier JT, Fox DJ, Goedken RJ, Jabs EW, Romitti PA. Mutation Screening of Candidate Genes in Patients with Nonsyndromic Sagittal Craniosynostosis. Plast Reconstr Surg. 2016; 137(3):952–961. [PubMed: 26910679]

Table I

Single Suture Cases - Sex and Suture Type distribution

Suture	N	% Total Cases	M	F	Ratio (M:F)
Sagittal	201	50.6	152	49	3.1:1
Metopic	94	23.7	74	20	3.7:1
Coronal	81	20.4	26	55	0.47:1
Lambdoid	21	5.3	15	6	2.5:1
Total Cases	**397**	**100%**	**267**	**130**	**2.1:1**

Table II

List of 61 genes associated with syndromic forms of craniosynostosis

Gene	Disease	Inheritance	OMIM # (Gene)
ADAMTSL4	Ectopia lentis	AR	610113
ALPL	Hypophosphatasia	AR/AD	171760
ALX4	Frontonasal dysplasia 2, Parietal foramina 2, susceptibility to Craniosynostosis 5	AR/AD	605420
ASXL1	Bohring-Opitz syndrome	AD	612990
ATR	Seckel syndrome 1	AR	601215
CDC45	Meier-Gorlin syndrome 7	AR	603465
COLEC11	3MC syndrome 2	AR	612502
CTSK	Pycnodysostosis	AR	601105
CYP26B1	CS with radiohumeral fusions and other skeletal and craniofacial anomalies	AR	605207
EFNA4	———	AD	601380
EFNB1	Craniofrontonasal dysplasia	XLD	300035
ERF	Craniosynostosis 4, Chitayat syndrome	AD	611888
ESCO2	Roberts syndrome, SC phocomelia syndrome	AR	609353
FAM20C	Raine syndrome	AR	611061
FBN1	Marfan syndrome, Weill-Marchesani syndrome 2	AD	134797
FGFR1	Pfeiffer syndrome, Osteoglophonic dysplasia, Trigonocephaly 1	AD	136350
FGFR2	Apert syndrome, Crouzon syndrome, Beare-Stevenson syndrome, Bent bone dysplasia	AD	176943
FGFR3	Muenke syndrome, Crouzon syndrome with acanthosis nigricans	AD	134934
FLNA	Otopalatodigital syndrome, type I and II; frontometaphyseal dysplasia	XLR/XLD	300017
FREM1	Trigonocephaly 2	AD	608944
GLI3	Greig cephalopolysyndactyly syndrome	AD	165240
GNAS	Osseous heteroplasia, pseudohypoparathyroidism	AD	139320
GNPTAB	Mucolipidosis IIIA/B	AR	607840
GPC3	Simpson-Golabi-Behmel syndrome, type 1	XLR	300037
HUWE1	Intellectual disability, X-linked syndromic, Turner type	XLR	300697
IDS	Mucopolysaccharidosis II	XLR	300823
IDUA	Hurler, Scheie, and Hurler/Scheie syndromes	AR	252800

Gene	Disease	Inheritance	OMIM # (Gene)
IFT122	Cranioectodermal dysplasia 1	AR	606045
IFT43	Cranioectodermal dysplasia 3	AR	614068
IGF1R	———	unk	147370
IHH	Acrocapitofemoral dysplasia, Brachydactyly, type A1	AR/AD	600726
IL11RA	Craniosynostosis and dental anomalies	AR	600939
IRX5	Hamamy syndrome	AR	606195
JAG1	Alagille syndrome 1	AD	601920
KAT6A	Intellectual disability, autosomal dominant 32	AD	601408
KMT2D	Kabuki syndrome 1	AD	602113
KRAS	Cardiofaciocutaneous syndrome 2, Noonan syndrome 3	AD	190070
LMX1B	Nail-patella syndrome	AD	602575
LRP5	Osteopetrosis, autosomal dominant 1; Osteosclerosis	AD	603506
MASP1	3MC syndrome 1	AR	600521
MEGF8	Carpenter syndrome 2	AR	604267
MSX2	Craniosynostosis 2, Parietal foramina 1, Parietal foramina with cleidocranial dysplasia	AD	123101
PHEX	Hypophosphatemic rickets, X-linked dominant	XLD	300550
POR	Antley-Bixler syndrome with genital anomalies and disordered steroidogenesis	AR	124015
RAB23	Carpenter syndrome	AR	606144
RECQL4	Baller-Gerold syndrome, RAPADILINO syndrome, Rothmund-Thomson syndrome	AR	603780
RUNX2	Cleidocranial dysplasia	AD	600211
SCARF2	Van den Ende-Gupta syndrome	AR	613619
SH3PXD2B	Frank-ter Haar syndrome	AR	613293
SKI	Shprintzen-Goldberg syndrome	AD	164780
SPECC1L	Opitz GBBB syndrome, type II	AD	614140
STAT3	Hyper-IgE recurrent infection syndrome	AD	102582
TCF12	Craniosynostosis 3	AD	600480
TGFBR1	Loeys-Dietz syndrome 1	AD	190181
TGFBR2	Loeys-Dietz syndrome 2	AD	190182
TMCO1	Craniofacial dysmorphism, skeletal anomalies, and Intellectual disability syndrome	AR	614123

Gene	Disease	Inheritance	OMIM # (Gene)
TWIST1	Craniosynostosis 1, Saethre-Chotzen syndrome	AD	601622
WDR19	Sensenbrenner syndrome	AR	614378
WDR35	Cranioectodermal dysplasia 2	AR	613602
ZEB2	Mowat-Wilson syndrome	AD	605802
ZIC1	Craniosynostosis 6	AD	600470

Online Mendelian Inheritance in Man, (OMIM®) https://www.omim.org/

Table III

Summary details of significant variants in core genes associated with craniosynostosis

Gene	Inheritance Pattern	Proband	Sex	SSC[a]	Variant	ExAC MAF	GO-ESP All MAF	Inh	Class[b]
ERF	AD	95681†	F	C	NM_006494.3:c.1103C>T; p.(Ser368Phe)	***	***	na	US
FGFR1	AD	95655	M	M	NM_023110.2:c.181G>A; p.(Val61Met)	8.6E-06	***	mat	US
IL11RA	AR	95536	M	S	NM_001142784.2:c.388T>A; p.(Cys130Ser)	***	***	na	US
MEGF8	AR	95509	F	C	NM_001410.2:c.1282G>A; p.(Val428Met)	2.5E-05	***	na	US
		95459	M	C	NM_001410.2:c.2167C>T; p.(Arg723Cys)	3.4E-05	***	na	US
		95639	F	M	NM_001410.2:c.3224C>T; p.(Thr1075Ile)	***	***	na	US
		163666	F	S	NM_001410.2:c.3685G>A; p.(Gly1229Arg)	1.9E-05	***	pat	US
		95677	M	C	NM_001410.2:c.4532G>A; p.(Arg1511His)	1.6E-04	***	na	US
		163575‡	F	M	NM_001410.2:c.5507A>T; p.(Asp1836Val)	3.7E-05	***	na	US
POR	AR	95681†	F	C	NM_000941.2:c.1358G>A; p.(Arg453His)	5.0E-05	2.0E-04	na	US
RAB23	AR	95644	F	C	NM_183227.2:c.25G>A; p.(Ala9Thr)	1.1E-04	2.0E-04	na	US
SKI	AD	163646	F	C	NM_003036.3:c.463G>T; p.(Ala155Ser)	***	***	pat	US
TCF12	AD	163548	M	C	NM_207036.1:c.1831C>T; p.(Arg611Cys)	8.2E-06	***	pat	LP
		95628	F	M	NM_207036.1:c.1907A>G; p.(Lys636Arg)	***	***	na	LP
		163717	M	S	NM_207036.1:c.2024A>C; p.(Glu675Ala)	***	***	mat	US

[a] Type of Single Suture Craniosynostosis (SSC): C, coronal; S, sagittal; M, metopic; L, Lambdoid

[b] Variants classified according to ACMG Guidelines (see methods). P, Pathogenic; LP, Likely Pathogenic; US, Uncertain Significance; LB, Likely Benign.

† Proband 95681 has two potentially damaging variants on Table III: *ERF* p.(Ser368Phe) and *POR* p.(Arg453His).

‡ Proband 163575 has two potentially damaging variants on Tables III and IV: *MEGF8* p.(Asp1836Val) and *MASP1* p.(Arg119Gln)

*** not listed

Inh= Inherited allele: paternal (pat); maternal (mat); na, neither parent available for testing.

Am J Med Genet A. Author manuscript; available in PMC 2019 February 01.

Table IV

Summary details of significant variants in genes associated with occasional craniosynostosis

Gene	Inheritance Pattern	Proband	Sex	SSC[a]	Variant	ExAC MAF	GO-ESP All MAF	Inh	Class[b]
ALPL	AD/AR	95547	F	C	NM_000478.5:c.359G>T; p.(Gly120Val)	***	***	na	US[c]
		95564	M	S	NM_000478.5:c.1112C>T; p.(Thr371Ile)	8.2E-06	***	pat	LP[d]
ATR	AR	95609	M	C	NM_001184.3:c.326G>A; p.(Arg109Gln)	8.5E-06	***	na	US
		163643	M	M	NM_001184.3:c.347G>C; p.(Cys116Ser)	***	***	pat	US
		95559	M	M	NM_001184.3:c.5427A>T; p.(Leu1809Phe)	8.3E-05	***	na	US
		163681	M	M	NM_001184.3:c.5476G>A; p.(Val1826Met)	***	***	pat	US
		95563	M	M	NM_001184.3:c.7375C>T; p.(Arg2459Cys)	1.7E-05	***	pat	US
EFNA4	AD	163511	M	M	NM_005227.2:c.550C>T; p.(Leu184Phe)	***	8.0E-05	mat	US
FAM20C	AR	163503[¥]	M	M	NM_020223.3:c.1228T>A; p.(Ser410Thr) NM_020223.3:c.1228T>A; p.(Ser410Thr)	4.8E-03	6.1E-03	mat; pat	LP
FBN1	AD	163553	M	M	NM_000138.4:c.1169C>T; p.(Ser390Phe) NM_000138.4:c.8149G>A; p.(Glu2717Lys)	4.0E-05 1.6E-04	*** 1.5E-04	na	US
		95587	M	S	NM_000138.4:c.7661G>A; p.(Arg2554Gln)	1.6E-04	8.0E-05	na	LB
FLNA	XLR/D	163560	F	S	NM_001456.3:c.842C>T; p.(Pro281Leu)	***	***	na	US
		163714	M	S	NM_001456.3:c.3348C>A; p.(Asp1116Glu)	***	***	mat	US
		163606	M	S	NM_001456.3:c.3755C>T; p.(Ala1252Val)	1.2E-05	***	mat	US
		95479	M	S	NM_001456.3:c.4625C>T; p.(Thr1542Ile)	3.5E-05	***	na	US
		163641	M	S	NM_001456.3c.4897C>T; p.(Arg1633Cys)	***	***	mat	US
		163603	F	C	NM_001456.3:c.7798C>T; p.(His2600Tyr)	2.0E-04	***	mat	US
HUWE1	XLR/D	95566	F	L	NM_031407.6:c.6293A>T; p.(Gln2098Leu)	2.3E-05	***	na	US
		95694	F	S	NM_031407.6:c.9800G>A; p.(Arg3267His)	4.5E-05	***	na	US
IDUA	AR	163644	F	S	NM_000203.4:c.686C>G; p.(Pro229Arg)	***	***	mat	US
IFT122	AR	163547	M	S	NM_052985.3:c.3624+1G>A	***	***	mat	LP
IGF1R	AD	95490[§]	F	C	NM_000875.4:c.3737G>A; p.(Arg1246His)	8.2E-06	1.5E-04	na	US
		163699	M	S	NM_000875.4:c.4058G>A; p.(Arg1353His)	2.0E-04	7.7E-04	na	US
IRX5	AR	95490[§]	F	C	NM_005853.5:c.1025C>T; p.(Thr342Ile)	***	***	na	US
KAT6A	AD	95526	M	M	NM_006766.4:c.101A>G; p.(Asn34Ser)	2.5E-05	***	na	US

Gene	Inheritance Pattern	Proband	Sex	SSC[a]	Variant	ExAC MAF	GO-ESP All MAF	Inh	Class[b]
		95638	M	M	NM_006766.4:c.2533C>T; p.(Arg845Cys)	3.2E-04	2.0E-04	na	US
		95682	F	C	NM_006766.4:c.2533C>T; p.(Arg845Cys)	3.2E-04	2.0E-04	na	US
KMT2D	AD	163541	F	S	NM_003482.3:c.7036G>A; p.(Gly2346Ser)	6.8E-05	***	mat	US
		163528	M	S	NM_003482.3:c.7198C>G; p.(Pro2400Ala)	7.6E-05	1.6E-04	pat	US
		95699	M	M	NM_003482.3:c.7390G>A; p.(Ala2464Thr)	***	***	na	US
		95624	F	S	NM_003482.3:c.9212G>A; p.(Arg3071Lys)	***	***	mat	US
		95670	M	S	NM_003482.3:c.13780G>C; p.(Ala4594Pro)	8.7E-05	***	pat	US
LRP5	AD	95463	M	L	NM_002335.3:c.3404G>A; p.(Arg1135His)	5.8E-05	***	na	US
		163503[¥]	M	M	NM_002335.3:c.4423C>T; p.(Arg1475Trp)	1.1E-05	***	pat	US
MASP1	AR	163575[≠]	F	M	NM_139125.3:c.356G>A; p.(Arg119Gln)	1.0E-04	4.6E-04	mat	US
RECQL4	AR	163499	M	S	NM_004260.3:c.1063C>T; p.(Arg355Trp)	1.7E-05	1.6E-04	na	US
		95562	M	S	NM_004260.3:c.3430C>T; p.(Arg1144Cys)	2.0E-04	4.6E-04	na	US
		95691	M	M	NM_004260.3:c.3430C>T; p.(Arg1144Cys)	2.0E-04	4.6E-04	na	US
SCARF2	AR	95558	M	C	NM_153334.6:c.1638G>T; p.(Trp546Cys)	5.0E-05	1.5E-04	na	US
		163579	M	S	NM_153334.6:c.1688T>G; p.(Val563Gly)	***	***	De Novo	P
		95688	M	S	NM_153334.6:c.1999A>C; p.(Lys667Gln)	***	***	mat	US
		95585	M	M	NM_153334.6:c.2593dupG; p.(Ala865Serfs*184)	***	***	pat	LP
SH3PXD2B	AR	163690	M	M	NM_001017995.2:c.1265T>C; p.(Ile422Thr)	***	***	na	US
		163632	M	S	NM_001017995.2:c.2276C>G; p.(Pro759Arg)	***	***	na	US
SPECC1L	AD	95540	M	C	NM_001145468.3:c.2056A>G; p.(Met686Val)	1.7E-05	***	na	US
TGFBR2	AD	163591	M	S	NM_003242.5:c.569G>A; p.(Arg190His)	6.6E-05	***	na	US
		95640	F	C	NM_003242.5:c.967C>G; p.(Leu323Val)	4.1E-05	***	na	US
		95578	F	S	NM_003242.5:c.1222C>A; p.(Leu408Met)	1.2E-04	***	pat	LP[c]
ZEB2	AD	163531	M	M	NM_014795.3:c.167T>C; p.(Leu56Pro)	8.2E-06	***	na	US

[a]Type of Single Suture Craniosynostosis (SSC): C, coronal; S, sagittal; M, metopic; L, Lambdoid

[b]Variants classified according to ACMG Guidelines (see methods). P, Pathogenic; LP, Likely Pathogenic; US, Uncertain Significance; LB, Likely Benign.

[c]Proband's paternal 1st cousin also has sagittal craniosynostosis.

[≠]Proband 163575 has two potentially damaging variants on Tables III and IV: *MEGF8* p.(Asp1836Val) and *MASP1* p.(Arg119Gln)

¥ Proband 163503 has two potentially damaging variants on Table IV: *FAM20C* p.(Ser410Thr) and *LRP5* p.(Arg1475Trp)

§ Proband 95490 has two potentially damaging variants on Table IV: *IGF1R* p.(Arg1246His) and *IRX5* p.(Thr342Ile)

*** not listed

Inh= Inherited allele: paternal (pat); maternal (mat); n/a, neither parent available for testing.

Table V

Craniosynostosis genes associated with increased variant burden

Gene	Case Exonic Variants	Case Damaging Variants	ExAC Exonic Variants	ExAC Damaging Variants	MAF	P Value
ATR	13	5	3780	504	0.001	0.011
EFNA4	4	4	1618	484	0.01	0.011
ERF	6	3	1336	169	0.001	0.033
MEGF8	19	10	5517	1585	0.001	0.040
SCARF2	17	3	2036	207	0.01	0.039
TGFBR2	3	3	925	163	0.001	0.007