Whole-exome sequencing in syndromic craniosynostosis increases diagnostic yield and identifies candidate genes in osteogenic signaling pathways

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Running title: Whole-exome sequencing in syndromic craniosynostosis

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Abstract

Craniosynostosis (CS) is a common congenital anomaly defined by premature fusion of one or more cranial sutures. Syndromic CS involves additional organ anomalies or neurocognitive deficits and accounts for 25-30% of the cases. In a recent population-based study by our group, 84% of the syndromic CS cases had a genetically verified diagnosis after targeted analyses. A number of different genetic causes were detected, confirming that syndromic CS is highly heterogeneous. In this study, we performed whole-exome sequencing of 10 children and parents from the same cohort where previous genetic results were negative. We detected pathogenic, or likely pathogenic, variants in four additional genes (*NFIA, EXTL3, POLR2A,* and *FOXP2)* associated with rare conditions. In two of these (*POLR2A* and *FOXP2*), CS has not previously been reported. We further detected a rare predicted damaging variant in *SH3BP4*, which has not previously been related to human disease. All findings were clustered in genes involved in the pathways of osteogenesis and suture patency.

We conclude that whole-exome sequencing expands the list of genes associated with syndromic CS, and provides new candidate genes in osteogenic signaling pathways.

Introduction

Craniosynostosis (CS) is a common inborn anomaly, defined by the premature closure of one or more cranial sutures, affecting 1/1600 - 1/1800 live births (Kweldam, van der Vlugt, & van der Meulen, 2011; Tønne, Due-Tønnessen, Wiig, et al., 2020). Surgical treatment is performed in order to avoid skull deformation or compromised intracranial pressure (Eide, Helseth, Due-Tonnessen, & Lundar, 2002; Proctor & Meara, 2019). Syndromic CS is defined by the presence of additional anomalies (e.g. limb malformations, cardiac anomalies), developmental delay, intellectual disability or other major findings. Approximately 25-30% of CS cases are syndromic (Lattanzi, Barba, Di Pietro, & Boyadjiev, 2017; Tønne, Due-Tønnessen, Mero, et al., 2020; Wilkie, Johnson, & Wall, 2017).

CS is genetically heterogeneous, and pathogenic variants in more than 80 different genes have been identified (Goos & Mathijssen, 2019; Tønne, Due-Tønnessen, Mero, et al., 2020). Multiple monogenetic causes have been recognized for nonsyndromic CS (Calpena et al., 2020; Timberlake et al., 2017). Nevertheless, syndromic CS is by far the most genetically diverse group, and the incidence of CS seems to vary greatly between different syndromes. In the well-described CS syndromes, including Pfeiffer, Apert, Crouzon and Muenke, CS is usually present. These syndromes are caused by activating pathogenic variants in the *FGFR1*, *FGFR2* and *FGFR3* genes, acting in the fibroblast growth factor (FGF) pathway. In a number of other CS-associated syndromes, only a proportion of the individuals present with CS and the mechanism is often loss-of-function (partial or complete) (Connerney & Spicer; Goos & Mathijssen, 2019; Twigg & Wilkie, 2015).

The cranial sutures provide flexibility to the skull and act as growth sites for the calvarial bones. The balance of cranial growth and patency of the sutures are maintained through activation and inactivation of several signaling pathways, regulated by a number of different genes (Connerney & Spicer; Katsianou, Adamopoulos, Vastardis, & Basdra, 2016; Roth et al.,

1996; Twigg & Wilkie, 2015). Syndromic CS is often caused by pathogenic variants in genes participating in the pathways of osteogenesis and suture patency (Goos & Mathijssen, 2019; Timberlake et al., 2019; Twigg & Wilkie, 2015). Central pathways are the FGFs, transforming growth factors β (TGFβ), wingless-type integration site (Wnt), Hedgehog (Hh), Eph/ephrin and bone morphogenetic protein (BMP) signaling pathways. They control the key target genes (*RUNX2, MSX2* and *TCF/LEF*) that promote osteoblast differentiation and are markers of osteogenic differentiation (Katsianou et al., 2016) (Figure 1).

A recent population-based study from our group detected a genetic cause in 84% of the syndromic CS cases. The individuals had been investigated by comparative genomic hybridization (aCGH) and exome-based high-throughput sequencing (HTS), filtering for 1570 clinically relevant genes (Tønne, Due-Tønnessen, Mero, et al., 2020). We suspected that some genetic causes were not detected by these methods. In this study, we used WES in order to detect possible unidentified syndromes in addition to novel CS genes.

Methods

Editorial Policies and Ethical Considerations

The study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics (REK_2018/797) and by Oslo University Hospital (permit number P360:18/05374). A signed informed consent form was collected from all included individuals. All families received genetic counselling before and after whole-exome sequencing (WES).

Since 2001, all individuals in Norway with CS have been diagnosed, treated and received follow-up from the Norwegian National Unit for Craniofacial Surgery at Oslo University Hospital. The result of genetic testing of an 18-year cohort of syndromic CS cases (n=104), retrieved from the unit`s prospective registry, were recently published by our group, including

clinical criteria for syndromic CS (Tønne, Due-Tønnessen, Mero, et al., 2020). In 15 individuals, the genetic results came back negative. Of these, 10 were available and consented to further genetic analysis. All parents were healthy, except one mother who had a similar phenotype as the child (individual 4).

Collection of EDTA blood, exome based HTS and bioinformatic handling were performed and described in our previous study (Tønne, Due-Tønnessen, Mero, et al., 2020). We reanalyzed the sequencing data from the previous study using our research pipeline. Downstream filtering and analysis were done with Filtus (Vigeland, Gjotterud, & Selmer, 2016) on the variants within coding regions and predicted splice sites of close to the entire exome (approximately 18 500 genes). Untranslated regions and non-coding RNA were not included. A few genes associated with severe late onset disease (e.g. *BRCA1/2*) were excluded. We filtered for *de novo*, homozygous, compound heterozygous and hemizygous variants. For individual 4 we also included all maternally transmitted loss-of function variants and missense variants with a Combined Annotation Dependent Depletion (CADD) score of 20 or more (meaning that the variant is predicted to be among the 1% of most deleterious substitutions in the human genome (Rentzsch, Witten, Cooper, Shendure, & Kircher, 2019). Variants reported with a frequency exceeding 0.0001 in gnomAD, or exceeding 0.05 in our in-house database were excluded, as well as synonymous variants (all located outside predicted splice-sites). The remaining variants were classified in accordance with the American College of Medical Genetics and Genomics (ACMG) and the Association for Clinical Genomics Science (ACGS) criteria (Ellard S, 2020; Richards et al., 2015). Variants were thereafter investigated by a literature search with regard to their effect on gene function in addition to the respective gene`s biological function, acting and interacting pathways. Databases used included GeneCards (Stelzer et al., 2016), Uniprot (Consortium, 2020), Alamut ("Alamut Visual version 2.11," 2018), UCSC Genome browser (Kent et al., 2002),

HGMD (Stenson et al., 2020), gnomAD (Karczewski et al., 2020), ClinVar (Landrum et al., 2020), OMIM (Amberger & Hamosh, 2017), Pubmed (Sayers et al., 2021) and Google Scholar. Sanger sequencing confirmed all selected and discussed variants (Supportive information Figure 1). The variants in *EXTL3* and *SH3BP4* were submitted to GeneMatcher (Sobreira, Schiettecatte, Valle, & Hamosh, 2015).

Results

WES revealed likely or possible genetic causes in five of the 10 analyzed individuals (individuals 1-5) (Table 1). For the remainder there were no likely causes detected. Of the individuals without a detected cause, three had a clinical diagnosis. Clinical information for all individuals is presented in Table 1. All detected rare *de novo* and homozygous/compound heterozygous/hemizygous variants are presented in Supporting information Table 1.

Individual 1 is a 15-year-old boy with healthy non-consanguineous parents and two healthy siblings. At birth, his weight was 3660 gr (75th centile), his length 49 cm (25th centile) and his head circumference 39 cm (1 cm > 97.5th centile). A metopic CS was surgically treated at the age of 10 months. At the age of 2 years, he developed hydrocephalus and MRI revealed a thin corpus callosum. He exhibited a mild developmental delay, autism, macrocephaly (97.5th centile), supernumerary teeth and reduced vision (not related to his hydrocephalus). His facial features included a long face, hypotelorism, short nose, long philtrum, micrognathia and simple low-set ears. Ultrasound of kidneys and urinary tract were normal.

Two *de novo* variants inducing premature stop codons were detected *in cis* in exon 2 of the Nuclear factor I/A (*NFIA*) gene, c.124A>T, p.(Lys42*) and c.250C>T, p.(Arg84*). The variants are not reported in gnomAD and are expected to individually result in degradation of NFIA mRNA due to nonsense-mediated mRNA decay. The variant c.124A>T is considered to

be the causal variant as the two variants are located *in cis*, hence c.124A>T will result in degradation of NFIA mRNA. *NFIA* has a high probability of loss-of-function intolerance (pLi =1). Heterozygous pathogenic loss-of-function variants in *NFIA* are associated with BRain Malformations with or without Urinary Tract Defects (BRMUTD) (MIM#613735). The described phenotype is variable and associated features include corpus callosum abnormalities, urinary tract anomalies, developmental delay, macrocephaly and nonspecific dysmorphic features (Senaratne & Quintero-Rivera, 1993). CS has previously been reported in one single case and in one family with three out of four affected individuals, all associated with microdeletions involving solely *NFIA* (Nyboe, Kreiborg, Kirchhoff, & Hove, 2015; Rao et al., 2014).

Individual 2 is a 13-year-old boy of consanguineous parents, with two healthy siblings. His birthweight was 2600 gr (3th centile). A metopic CS was surgically treated abroad at the age of 5 months. He has short stature (6 cm < 3th centile), microcephaly (2 cm < 3th centile), kyphosis, hip dysplasia and delayed skeletal age. He also has mild intellectual disability, hypotonia, eating difficulties, reflux and recurrent and long-lasting infections. Immunological investigations revealed a low number of T-cells and reduced fraction of CD8+ late effector/memory T-cells. His facial features include coarse facies, deep-set eyes, hypotelorism, prominent nose and a broad nasal tip.

A homozygous missense variant in the Exostosin-like glycosyltransferase 3 (*EXTL3*) gene was detected, c.2392G>A, p.(Val798Met). The variant has not been reported in gnomAD and changes an amino acid that is highly conserved between species and received a CADD score of 32. All other pathogenic or likely pathogenic variants in *EXTL3* reported in the literature are missense variants. The variant is located in a predicted Pfam domain (glycosyl transferase family 64 domain, amino acids 663-904) catalyzing the transfer reaction of N-acetylglucosamine (GlcNac) and N-acetylgalactosamine essential for formation of heparin

sulphate (HS) chains. Pathological biallelic missense variants in *EXTL3* are associated with Immunoskeletal dysplasia with neurodevelopmental abnormalities (ISDNA) (MIM#617425). Variable skeletal abnormalities and neurodevelopmental defects are associated with the condition, in addition to immunodeficiency restricted T-cell deficiency. CS has previously been reported in two siblings with ISDNA (Volpi et al., 2017). There has been one likely pathogenic missense variant reported in the same domain as detected in individual 2, hypothesized to cause the condition by disrupting the GlcNac transferase activity (Oud et al., 2017). The variant detected in individual 2 is interpreted to be likely pathogenic.

Individual 3 is a 5-year-old boy with healthy non-consanguineous parents. A thickened nuchal fold was detected during pregnancy. He was born at term with weight 3425 gr (50th centile), length 50 cm (25th centile) and head circumferences 37 cm (90th centile). Metopic CS was treated at the age of 2 months. As an infant, he had severe hypotonia and needed tube feeding. Hypospadia was detected shortly after birth. Newborn screening indicated congenital adrenal hyperplasia (CAH), which was confirmed by the detection of a homozygous deletion within the *CYP21A2* gene (exon 1-3). He received extensive physical therapy due to hypotonia from 3 months of age, and at the age of 14 months, he walked independently. He is motorically clumsy, runs slowly and gets tired easily. He has facial tics, midface hypoplasia, hypotelorism, broad nasal root, dysplastic ears, single palmar fissure and 2.-3. syndactyly bilaterally.

A *de novo* heterozygous variant was detected in Polymerase 2 RNA Subunit A (*POLR2A*), c.4329_4330delinsAA, p.(Ala1444Thr). The variant has not been reported in gnomAD and changes a highly conserved amino acid. Pathogenic variants in *POLR2A* are associated with Neurodevelopmental disorder with hypotonia and variable intellectual and behavioral abnormalities (NEDHIB) (MIM#618603). The condition has a variable phenotype with varying severity. There are 27 individuals reported and common features are delayed

development (mild to severe), hypotonia, ataxia, epilepsy, feeding difficulties, sleep disturbances and behavior abnormalities. There are no other individuals reported with CS, but four individuals are reported to have brachyplagiocephaly (Haijes et al., 2019) and two are reported to have prominent supraorbital ridges (Hansen et al., 2021). Missense variants seem to be associated with a more severe phenotype, presumably due to a dominant-negative effect. Four reported cases have in-frame deletions in *POLR2A*, predicted to alter the protein structure (Haijes et al., 2019; Hansen et al., 2021). Individual 3`s phenotype is in concordance with the described features, however slightly milder. The variant detected is considered to be likely pathogenic.

Individual 4 is a 6-year-old boy with non-consanguineous parents. He was born at term with weight 3320 gr (25th centile), length 51 cm (50th centile) and head circumference 35 cm (25th centile). A sagittal CS was surgically treated at the age of 3 months. He has severe verbal dyspraxia, confirmed by a speech-language pathologist (SLP) and experiences social difficulties. He has reduced vision. His mother was surgically treated for sagittal CS and has verbal dyspraxia with great difficulties in complex speech production, confirmed by the same SLP. He has one healthy sister (9 years old) without CS. The sister had delayed language development until the age of 6. At present, she has normal speech, in particular no verbal dyspraxia or articulation difficulties (confirmed by the same SLP). She is however in need of some special education for reading and writing.

A maternally inherited heterozygous frameshift variant in Forkhead box P2 (*FOXP2*) was detected, c.484del, p.(Gln162Asnfs*100). *FOXP2* has a high pLi of 1. The variant has not been reported in gnomAD and induces a premature stop codon. Both missense and nonsense variants in *FOXP2* are known to cause Speech-language disorder-1/Developmental verbal dyspraxia (MIM#605317). The condition is characterized by severe orofacial dyspraxia, affecting both speech and expressive language (Morgan, Fisher, Scheffer, & Hildebrand,

2016). Both mother and son have persisting symptoms of Speech-language disorder, confirmed by a SLP. The variant is considered to be likely pathogenic. The variant was also detected in the healthy sister, with delayed speech up until the age of 6, suggesting variable expressivity. Although *FOXP2* has not previously been associated with syndromic CS, altered facial skeletal morphology has been reported, and the *FOXP2* transcription factor interacts with several pathways involved in osteogenesis (see discussion). No other maternally inherited loss of function variants in genes predicted to be involved in cranial development or which interfere with bone metabolism were detected. A few rare maternally inherited missense variants with CADD > 20 were identified, in which four have been demonstrated to be involved in the pathways of osteogenesis and suture patency (*RHOB, CTNND2, COL27A1,* and *PDILT*). Missense variants are less likely to interfere with the pathways concerning CS, and there were no reports of involvement in cranial development for these genes. All maternally inherited rare variants with CADD > 20 are presented in Supporting information Table 1.

Individual 5 is a 9-year-old boy of healthy non-consanguineous parents. He was born at term, with weight 2900 gram (5th centile) and length 48 cm (10th centile). Between the age of 5 and 18 months, he had severe eating difficulties and a significant growth stagnation. Pansynostosis was detected at 18 months. He had no sign of intracranial hypertension or restricted intracranial volume and was treated conservatively. He has had one epileptic seizure and MRI revealed a Chiari I malformation. He has normal growth parameters, except microcephaly (2 cm < 2.5th centile). His facial features include midface hypoplasia with exophtalmos, hypertelorism, long palpebral fissures and broad arched eyebrows with lateral sparing, a depressed nasal tip and widely spaced teeth. He has persistent fetal pads. His psychomotor development is normal.

Homozygosity for a missense variant in the SH3 domain-binding protein 4 (*SH3BP4*) was detected, c.128C>A, p.(Pro43His). The variant changes a highly conserved amino acid, is not reported in homozygous state in gnomAD, and received a CADD score of 33. *SH3BP4* has not been previously associated with human disease. The variant is considered to be of uncertain significance.

Compound heterozygosity for two rare missense variants in the Lysine-specific methyltransferase 2D (*KMT2D*) gene were also detected, c.11599C>A, p.(Gln3867Lys) and c.7182C>A, p.(Ser2394Arg). Heterozygosity for *KMT2D* pathogenic variants is associated with Kabuki syndrome (MIM#147929). The mechanism is usually loss-of function, but missense variants are reported (15-20% of the cases), however with a slightly different phenotype (Baldridge et al., 2020). Individual 5 has typical facial features of Kabuki syndrome (long palpebral fissures, arched eyebrows with lateral sparing). Synostosis of multiple sutures, fetal pads and early eating difficulties are also in concordance with the described phenotype (Topa, Samuelsson, Lovmar, Stenman, & Kölby, 2017). However, he has a normal psychomotor development, which is uncommon in Kabuki syndrome. The parents have no dysmorphic features. Both variants are considered to be of uncertain significance. The variant c.11599C>A is reported in ClinVar as a VUS for Kabuki syndrome in two other individuals.

Discussion

We performed WES in 10 children clinically diagnosed with syndromic CS, where previous genetic analysis did not reveal any causative variants. We detected six likely or possible genetic causes in five of the children. In four of the children (individuals 1-4), a pathogenic or likely pathogenic variant was detected in a gene associated with a condition related to their respective phenotype. This increased the diagnostic yield in the cohort. All findings were in

genes participating in one or more pathways known to be involved in osteogenesis or suture patency (Figure 1).

Known genetic causes of syndromic CS are rapidly accumulating, primarily due to an increased use of HTS. This sometimes leaves the genetic findings in the transition between research and diagnostics. For the conditions BRMUTD (*NFIA*) and ISDNA (*EXTL3*), these are the fifth and third cases respectively, which include CS, and confirm CS as a feature in these two rare conditions. Both *NFIA* and *EXTL3* have been demonstrated to be involved in the pathways of osteogenesis and suture patency. *NFIA* is a transcription factor important for normal development of several organ systems (Lu et al., 2007). The protein participates in osteoblast differentiation by interacting with the Wnt and Ihh pathways (Singh et al., 2018; Xie, Zhang, Gao, Ge, & Tang, 2015) (Figure 1).

EXTL3 is essential during early development and knockout of *EXTL3* in mice results in lethality (Takahashi et al., 2009). EXTL3 is a key protein in the regulation of heparan sulphates (HS) biosynthesis, and thereby the formation of heparan sulphate proteoglycans (HSPGs) known to be important for normal skeletal development (Oud et al., 2017). Extensive studies have shown HSPGs to play an important role in regulating the Wnt, Hh, BMP and FGFs activity and signaling pathways (Norton, Ledin, Grandel, & Neumann, 2005). Functional studies performed in zebrafish have shown *EXTL3* to be involved in the regulation of the FGF, Wnt and Hh signaling pathways in particular (Venero Galanternik, Kramer, & Piotrowski, 2015; Venero Galanternik, Lush, & Piotrowski, 2016) (Figure 1).

NEDHIB (*POLR2A*), detected in individual 3, has not previously been described to cause CS, but brachyplagiocephaly has been reported in several individuals. Brachyplagiocephaly is in some cases the consequence of CS (uni-or bicoronal CS). *POLR2A* encodes the largest subunit (RPB1) of RNA polymerase II and its function is regulated by methylation and

acetylation of particular residues in its C-terminal domain, which again is essential for the regulation of growth factor induced genes (Mita et al., 1995; Schröder et al., 2013). RNA polymerase II is a protein complex responsible for mRNA-synthesis of all human protein-encoding genes and shown to regulate transcription through a number of different mechanisms and interactions, where RPB1 is important for both transcription initiation, elongation and termination (Haijes et al., 2019; Kecman et al., 2018; X. Liu, Bushnell, & Kornberg, 2013). *POLR2A* has been demonstrated to be involved in several different pathways, including the Wnt pathway in a study of meningiomas, where mutant *POLR2A* was shown to affect the expression of *WNT6*/*WNT10*, known to be involved in controlling neural crest cell development (Clark et al., 2016). *POLR2A* has also been predicted to be indirectly involved in alternate splicing of FGFR2 through Epithelial splicing regulatory protein 2 (ESRP2) (Schoch et al., 2020) (Figure 1) and directly involved in the regulation of osteoclast genesis by interacting with CREB1 (C. Liu et al., 2021).

Speech-language disorder-1 (*FOXP2*) detected in individual 4 and his affected mother, has not previously been described to include CS. However, several individuals are reported to have dysmorphic features involving the facial skeleton (e.g. prominent forehead) (Cesario, Almaidhan, & Jeong, 2016). In addition, *FOXP2*`s important roles in regulating bone morphogenic genes and ensuring normal cranial development have been demonstrated in several functional studies (Cesario et al., 2016; Xu et al., 2018; Zhao et al., 2015). This supports our hypothesis that the loss-of function variant detected in *FOXP2* may also cause CS in these two family members. The *FOXP2* gene encodes the FOXP2 transcription factor, one of the most highly conserved proteins in mammals. It is involved in a number of developmental pathways (Richter et al., 2020), one of which is involved in craniofacial development, where FOXP2 has been shown to play an important role in skull shaping and

bone remodeling (Cesario et al., 2016; Xu et al., 2018). FOXP2 participates in the Wnt signaling pathway where it negatively regulates several bone morphogenic genes (e.g. *RUNX2, AXIN2, BMP4, FGF9)* involved in ossification in cranial mesenchyme and CS (Govindarajan & Overbeek, 2006; Katsianou et al., 2016; Richter et al., 2020) (Figure 1). Importantly, *FOXP2* has been shown to be one of the suppressors of *RUNX2* in several studies (Cesario et al., 2016; Zhao et al., 2015). RUNX2 is one of the key transcription factors for bone formation, including the cranium (Benson & Opperman; Katsianou et al., 2016; Long & Ornitz, 2013). Loss-of-function variants in another RUNX2 suppressor, *TWIST1*, are associated with coronal CS in Saethre-Chotzen syndrome, presumably due to increased osteogenesis, as TWIST1 normally binds to RUNX2 in the coronal suture, acting as a negative regulator of osteogenic differentiation (Bialek et al., 2004; el Ghouzzi et al., 1997; Wu & Gu, 2019). *FOXP2* has also been shown to be significantly downregulated in the sutures of sagittal CS (Potter, Rhodes, Vega, Ridder, & Shiang, 2015). In a study of FOXP2`s role in cranial base development, *Foxp2* conditional knock-out demonstrated a significant impact on the lambdoid sutures in a dose-dependent manner in mice (Xu et al., 2018). However, the knockout mice exhibited decreased expression of Runx2 with attenuated synostosis of the lambdoid sutures as a result. These different observations might be explained by the large number of cofactors involved in promoting osteoblast differentiation by stimulating *RUNX2* expression (Zhao et al., 2015). In addition, the *FOXP* genes are hypothesized to have context-and time-dependent functions in bone development, in the sense that their impact may vary in time, tissue and developmental stages (Zhao et al., 2015). Another example on how *FOXP2* affects skeletal development is its role in endochondral ossification in long bones. In a functional study performed in mice, overexpression of *FOXP2* was shown to inhibit endochondral ossification, and compound deficiency of *FOXP1/2* resulted in advanced osteoblast maturation and increased proliferation (Zhao et al., 2015). The

study also demonstrated attenuated and reduced growth of the mice skulls when *FOXP2* was both overexpressed and when deficient. Lastly, *FOXP2* has been shown to induce growth arrest in osteosarcoma (Gascoyne et al., 2015).

In individual 5, a presumably damaging variant in a gene of unknown significance, *SH3BP4* was detected. *SH3BP4* forms a complex together with FGFR2b and phosphatidylinositol-3-kinase (FGFR2b-P13K-SH3BP4), that ensures FGFR2b recycling and controls cell migration and epithelial branching (Francavilla et al., 2013; Ornitz & Itoh, 2015). Fibroblast growth factors (FGFs) are positive regulators of osteogenic differentiation and function by activating the FGFRs and through several steps and downstream pathways stimulate osteoblast proliferation and differentiation. The FGFRs have however two different isoforms (IIIb and IIIc) with different affinity to ligands and localization. The FGFR2c isoform is the one involved in the osteocyte lineage and normal bone development, while the IIIb isoform is involved in epithelial branching (Eswarakumar, Horowitz, Locklin, Morriss-Kay, & Lonai, 2004). P13K/Akt is a major growth-signaling pathway, known to be crucial for normal bone development, and is suggested to interact with the Wnt pathway, together affecting osteoblast activity (Raucci, Bellosta, Grassi, Basilico, & Mansukhani, 2008). In addition, *SH3BP4* has been demonstrated to act as a negative regulator of the Wnt pathway (Antas et al., 2019) (Figure 1). The impact of pathogenic variants in *SH3BP4* concerning bone and cranial development is however uncertain and further studies or affected individuals would be needed in order to make such association.

The importance of the two variants in *KMT2D*, detected in the same individual, is also uncertain. Kabuki syndrome has been associated with CS in several cases. The *KMT2D* gene is known to participate in the Wnt pathway (Schwenty-Lara, Nehl, & Borchers, 2020) (Figure 1). Autosomal recessive Kabuki syndrome has not previously been reported, and the two variants might represent rare normal variants. As the presumed mechanism of Kabuki

syndrome is loss-of-function, it is however possible that compound heterozygosity for two missense variants might result in reduced production of the lysine specific methyltransferase 2D protein, and in turn reduced total activity of this enzyme, with a similar or milder phenotypic result.

By performing WES in children with syndromic CS we identified genetic causes that were previously undetected, in addition to identifying variants in genes not previously associated with CS. Recently, a similar study discovered other novel genetic causes of CS, also comprising genes involved in the pathways of osteogenesis and suture patency (Timberlake et al., 2019). The small sample sizes studied with this approach suggests that there are still undetected genetic causes of syndromic CS, presumably in genes involved in these pathways.

The ultra-rare genetic syndromes identified in the study, present with CS in only a fraction of the cases, in contradiction to the more common CS syndromes (e.g. *FGFR*s), in which nearly all individuals present with CS. This suggests different pathophysiological mechanisms. The gain-of-function variants in the FGFRs might be less influenced by other factors and interfering mechanisms than the loss-of-function variants, often detected in the rare causes of syndromic CS. Several of the genes detected in this study were also transcription factors (*NFIA*, *POLR2A*, and *FOXP2*). Transcription factors are known to be influenced by a number of different factors, which may explain some of the differences. In addition, poor brain growth and extrinsic forces applied to the skull are known to affect the development of CS (Twigg & Wilkie, 2015). Variants in other genes, environmental and epigenetic factors might also influence and contribute to the result of CS in these ultra-rare syndromes.

The high diagnostic yield when including our previous study, underscores the importance of identifying syndromic CS cases in the clinic, in order to provide genetic analyses to this group. We have demonstrated that the genetic causes of syndromic CS are highly

heterogeneous and that targeted analysis is not always sufficient. The most efficient test strategy for syndromic CS would probably be to perform a small targeted panel, followed by WES or whole genome sequencing (WGS) for negative cases, without the second step of an extended panel. The advantages of performing a targeted analysis initially are that this detects a large number of the genetic causes and reduce the risk of incidental findings.

The strength of this study is that we have investigated close to all human protein-coding genes, limiting the possibility of another undetected cause. Nevertheless, we were unable to detect a genetic cause in five individuals, three of which had a clinical diagnosis. In two cases with a clinical diagnosis, a lack of genetic findings may not be surprising, as these syndromes are usually not associated with a genetic cause (Oculoauriculovertebral spectrum (OAVS) and cerebral palsy). Their CS might be an isolated event, not associated with their other findings. For the remainder, an undetected genetic cause is nonetheless suspected, for instance non-coding, regulatory or small copy number alterations that our analysis did not capture.

We have not been able to detect a second individual or family to confirm the association with CS in *SH3BP4*, *FOXP2* and *POLR2A*. We attribute this to the limited number of individuals having these rare disorders. Functional studies or other affected individuals would be needed in order to confirm causation.

Conclusion

By performing WES, we identified several rare genetic causes to syndromic CS, previously undetected by panel analysis. In addition, interesting candidate genes for CS were revealed, all participating in the pathways of osteogenesis and suture patency.

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Data availability statement: The data that support the findings of this study are openly available in ClinVar at www.ncbi.nlm.nih.gov/clinvar, accession number SCV001943320 - SCV001943321 (Tønne E., 2021).

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Figure legend

Figure 1. *Signaling pathways involved in cranial growth and suture patency.* The figure illustrates the most important signal transduction pathways in the development of the cranial sutures. Disruptions in one of these pathways may lead to craniosynostosis. The figure is inspired by Figure 2 in (Katsianou et al., 2016). Compared to the original figure, the pathways have been simplified, the graphic is slightly adjusted, one pathway has been added (Hedgehog signaling pathway) and some interactions between the pathways and key target genes have been added. The genes discovered in the study are marked with a dotted line toward the pathways in which functional studies have implicated a regulatory effect. This includes studies on other tissues than the cranium. The conditions associated with the genes *NFIA*, *EXTL3*, *KMT2D* and *POLR2A* (marked with a star) are previously reported to include craniosynostosis (n=3) or brachyplagiocephaly (n=1). Abbreviations: ERF, ETS domain-containing transcription factor; ERK1/2, extracellular signal regulated kinase 1/2; *EXTL3*, Exostosin-like glycosyltransferase 3; FGF, fibroblast growth factor; *FOXP2*, Forkhead box P2; GLI, glioma-associated oncogene homolog; Ihh, Indian hedgehog; *KMT2D*, Lysine-specific methyltransferase 2D; LRP5/6, low-density lipoprotein receptor-related protein 5/6; MEK,mitogen-activated protein kinase; MSX2, Muscle segment homeobox 2; *NFIA*, Nuclear factor I/A; *POLR2A*, Polymerase 2 RNA Subunit A; RUNX2, Runt-related transcription factor 2; *SH3BP4*, SH3 domain-binding protein 4; Shh, Sonic hedgehog; SMO, Smoothened; TCF/LEF, T-cell factor/lymphoid enhancer factor; TGF/BMP, transforming growth factors β/ bone morphogenetic protein; TWIST1, twist-related protein 1; Wnt, wingless-type integration site.

Supporting information legend

Supporting information Table 1. *Filtered variants in all investigated individuals*.

The table presents all variants remaining after the filtering process described in the methods section. The table includes the frequency and CADD score for each variant.

Supporting information Figure 1. *Results from Sanger sequencing. Deleted or altered bases are highlighted.*