Pediatric Neurology 99 (2019) 7–15

Contents lists available at ScienceDirect

Pediatric Neurology

journal homepage: www.elsevier.com/locate/pnu

Topical Review

Recent Advances in Craniosynostosis

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ARTICLE INFO

Article history:
Received 30 January 2018
Accepted 24 January 2019
Available online 2 February 2019

Keywords:
Craniosynostosis
Craniofacial disorders
Genetics
Biological process
Cytogenetics
Epidemiology
Epigenetics

ABSTRACT

Craniosynostosis is a pathologic craniofacial disorder and is defined as the premature fusion of one or more cranial (calvarial) sutures. Cranial sutures are fibrous joints consisting of nonossified mesenchymal cells that play an important role in the development of healthy craniofacial skeletons. Early fusion of these sutures results in incomplete brain development that may lead to complications of several severe medical conditions including seizures, brain damage, mental delay, complex deformities, strabismus, and visual and breathing problems. As a congenital disease, craniosynostosis has a heterogeneous origin that can be affected by genetic and epigenetic alterations, teratogens, and environmental factors and make the syndrome highly complex. To date, approximately 200 syndromes have been linked to craniosynostosis. In addition to being part of a syndrome, craniosynostosis can be nonsyndromic, formed without any additional anomalies. More than 50 nuclear genes that relate to craniosynostosis have been identified. Besides genetic factors, epigenetic factors like microRNAs and mechanical forces also play important roles in suture fusion. As craniosynostosis is a multifactorial disorder, evaluating the craniosynostosis syndrome requires and depends on all the information obtained from clinical findings, genetic analysis, epigenetic or environmental factors, or gene modulators. In this review, we will focus on embryologic and genetic studies, as well as epigenetic and environmental studies. We will discuss published studies and correlate the findings with unknown aspects of craniofacial disorders.

Introduction

Craniofacial anomalies, one of the subtypes of congenital disorders, are characterized by the abnormal development of soft tissues and bones of the face and the skull that results in the formation of different head and facial features. Early closure of skull bones results in a new head shape that restricts the normal growth and development of the brain.[1] The junctions that play roles in the differentiation and interaction of the cranial or calvarial bones are termed *sutures*. In this scope, early fusion of these sutures in cranial development leads to craniosynostosis syndromes or craniofacial disorders,[2,3] which will be discussed in depth below. These congenital malformations are classified as the second most common group of craniofacial deformities.[4] The term *craniosynostosis* was first used by Otto in 1830.[5] Craniosynostosis is a genetically heterogeneous disorder that has been associated with more than 180 syndromes,[6] including Apert, Crouzon, and Saethre-Chotzen syndromes. The aim of this review is to summarize what is known about craniosynostosis from the embryologic to epigenetic levels and highlight the aspects that remain poorly understood.

Embryologic development of cranial sutures

Cranial sutures are made up of fibrous tissues and are located between cranial bones.[7,8] The sutures are flexible, and this feature leads to two main developmental processes: facilitating birth through the birth canal by means of temporary skull deformation and expanding the cranial vault during brain growth.[8,9] This morphogenic developmental process starts at an early embryonic phase (twentieth week) and ends during adulthood.[7,10] The cranium is divided into two parts: the *neurocranium* that protects the

Conflicts of interest and source of funding statement: The authors declare no conflict of interest or financial disclosures concerning the materials or methods used in this study or the findings specified in this article.

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https://doi.org/10.1016/j.pediatrneurol.2019.01.018
0887-8994/

brain and the *viscerocranium* that forms the face skeleton, which occurs through the intramembranous (IM) and endochondral (EC) bone formation, respectively.[3] In this review we will focus on IM bone formation, but EC bone formation has been extensively reviewed.[11,12]

During cranial vault development, IM ossification initiates undifferentiated mesenchymal cell condensation and surrounds the sutures.[3,13] Initially, after the condensation begins, cells start to proliferate from the osteogenic fronts.[14] The condensation of mesenchymal differentiation triggers the proliferation of chondrocytes and perichondrial cells (Fig 1A).[15,16] During this ossification process, some transcription factors play an important role, such as *RUNX2* and *OSX* in osteoblast development,[14] transforming growth factor beta (*TGF-β*) in inhibition of chondrocyte proliferation, and *MSX2* in inhibition of calvarial osteoblast differentiation.[3] However, the main regulatory pathway of this differentiation is the fibroblast growth factor (FGF) signaling pathway, which will be discussed in the next section. Excessive bone growth from the unmineralized bone matrix or early arrest of brain development might be the cause of premature suture fusion,[9] which might lead to morphologic, physiologic, and functional abnormalities,[8] like craniosynostosis.

Biological process in the formation of craniosynostosis

In general terms, craniosynostosis represents a syndromic or nonsyndromic pathologic condition and occurs as a result of premature fusion of one or more cranial sutures.[17,18] Suture patency or suture morphogenesis is an extremely important physiologic and mechanical condition for the orchestrated development of brain and skull growth.[19] Because the sutures are parts of main junctions for normal cranial development, it is important to understand the biological processes underlying suture formation.

In the embryonic period, several evolutionary conserved signaling pathways, such as FGF, TGF-β, and Wnt (previously reviewed by Katsianou and colleagues[20]), must be properly regulated for calvarial development. Moreover, cranial suture formation also requires osteoinhibitory and osteoinductive molecules, including soluble heparin-binding factors, FGF2, and TGF-β isoforms secreted by the dura mater cells.[21] These osteoinhibitory and osteoinductive molecules regulate the growth and formation of the membranous bones of the skull.

Published animal studies and computational models show that whereas TGF-β2 has an osteoinductive role, TGF-β3 has an osteoinhibitory role in suture formation.[22,23] Owing to their osteogenic roles, TGF-β*s* (NM_000660.5) are involved in the suture fusion process.[24,25] As a superfamily with more than 30 members, TGF-βs are the key players, especially for the regulation of cell proliferation, cell differentiation, and embryonic developmental processes.[26] The *TGF*-β superfamily is grouped into three classes: TGF-β1, which includes bone morphogenetic protein (BMP), TGF-β2, and TGF-β3.[27] Interestingly, we have not found a published genetic study that has identified a mutation in these genes related to known craniosynostosis syndromes.

In general, the bones of the human skull undergo IM ossification, whereby mesenchymal stem cells (MSCs) differentiate into osteoblasts to form bones.[13] We know that bone marrow is the main source of MSCs, and that their differentiation into osteogenic, chondrogenic, adipogenic, or myogenic lineages is organized via signaling pathways.[28,29] In 2001, it was shown that the hedgehog signaling pathway promotes osteogenic differentiation together with BMP pathway via SMAD and acts as a pro-osteogenic pathway.[30] In 2010, Quarto et al. reported that the enhanced activation of Wnt signaling pathway might be mediated by FGFs, and this suggested the canonical Wnt signaling pathway as the main contributor of osteogenesis and tissue regeneration of the neural crests of frontal bones.[31] Furthermore, it has been shown that whereas the activation of Wnt pathway induced osteogenic differentiation, its inhibition suppressed osteogenic differentiation[32] through Notch signaling. In 2013, Li et al. asserted that the

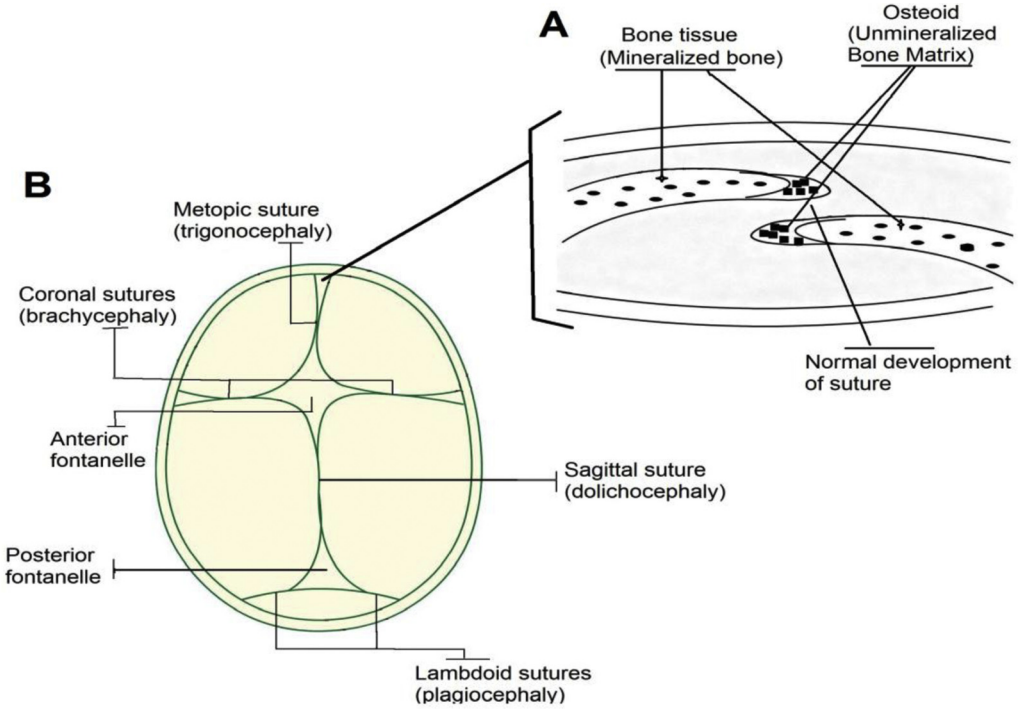

FIGURE 1. (A) Magnified view of the suture region. (B) Coronal view of the cranial suture types. The color version of this figure is available in the online edition. *Modified from Wilkie, A.O. Craniosynostosis: genes and mechanisms. Hum Mol Genet, 1997;6:1647-56, by permission of Oxford University Press.*

canonical Wnt pathway has a main role in controlling suture patency.[33] In 2016, it was strongly emphasized that in the process of bone differentiation, several signaling pathways, such as TGF-β/BMP, Wnt, Hedgehog, Notch, and FGFs, are involved in lineage commitment of MSCs.[34] Taken together, in the process of skull forming, *TWIST1* gene is required for the early migration and survival of MSCs. Recently, it has been shown that TWIST protein has a pivotal role in the regulation and differentiation of sutural mesenchymal cells to form the skull.[20,35] Moreover, one of the recent studies published by Maruyama et al. reported the first evidence for *Axin2* to play an important role, and its high expression by a specific stem cell population, in midline sutures (sagittal and metopic).[36] This unique cell population is shown to have long-term self-renewing, clonal expansion and differentiation during calvarial development[36] and might serve as a therapeutic tool to repair the craniofacial system by using their self-renewal features.

Besides the foregoing, signaling pathways have also a major role both in IM and EC ossification processes that regulate proliferation, differentiation, and cell migration in the embryonic period.[37,38] FGF family has 23 members that bind to five different receptors (FGFR1–4 and FGFRL) to induce cellular responses.[39] Among these receptors, whereas FGFR1 signaling regulates osteogenic differentiation, FGFR2 signaling orchestrates stem cell proliferation.[40] In syndromic craniosynostosis, mutations are found mostly in *FGFR2* receptors compared with other receptors.

The repressors of the FGF signaling pathways are also important players for cranial development. Most detected mutations in FGFRs as a result of craniosynostosis have gain-of-function properties, whereas mutations in repressors such as TWIST1 show loss-of-function effects. Recently, it has been shown that TWIST1 heterodimerizes with TCF12 in the nucleus and represses the RUNX2 (runt-related transcription factor 2; NM_001015051.3) activation.[20] The *RUNX2* gene encodes RUNX2 protein, which is an essential transcription factor for the development of bone and cartilage.[41] It is thought that *RUNX2* gene has an impact on osteoblasts for bone development. In 2010, a genetic study showed that 6p21 duplication (1.1 Mb), which included the *RUNX2* gene, has been detected in first-degree cousins diagnosed with metopic synostosis. However, no significant evidence has been identified in the protein expression for *RUNX2* gene so far.[42]

In 2013, Komatsu et al. showed that BMP signaling via BMP type 1 receptor (*BMPR1A*; NM_004329.2) pathway in cranial neural crest cells causes premature suture fusion in mouse models.[43] As a member of TGF-β family, BMPs are one of the main contributors to bone- and cartilage-forming processes. In the process of cranial development, SMAD-dependent BMP pathway regulates the transcription of MSX2 and RUNX2.[20] Komatsu and colleagues have shown that the increased activity of SMAD-dependent BMP pathway could cause syndromic craniosynostosis in mice. They also showed the rescue of the craniosynostotic phenotypes[43] in mutant embryos when they used chemical receptor inhibitors such as LDN-193189. As a result they suggested BMP to be a strategic developmental protein for the early intervention of craniofacial syndromes.[43] Recently, digenic inheritance of craniosynostosis via rare SMAD6 and common BMP2 alleles has been shown as a cause of nonsyndromic midline craniosynostosis.[44]

In addition to the identified genes mentioned above, some candidate genes are also identified using model organisms. One of these candidate nuclear genes is *NELL1* (NEL-like protein 1; NM_001288713.1) located on chromosome 11p15. As an extracellular protein, NELL1 induces osteogenic differentiation and bone formation by activating the MAPK pathway.[45] It has been shown that overexpression of *NELL1* gene in a transgenic mouse model resulted in the overgrowth of cranial sutures, similar to humans. Taken together, it has been suggested that *NELL1* gene, which promotes osteoblast cell differentiation and terminal mineralization,[46,47] might have an important role in bone development by acting through *RUNX2*.

Clinical approach to craniosynostosis

Craniosynostosis was first described by Otto in 1830 as a premature suture closure. In 1851, Virchow, proposed that the growth of the skull is restricted to the fused suture perpendicularly and this leads to enhancement of growth along nonfused sutures to compensate the development of brain (a concept known as Virchow's law).[48] According to the type of fused suture, different head shapes occur, such as scaphocephaly and brachycephaly, which we will discuss later in the article. On the other hand, early fusion of the suture not only causes skull deformities but also creates characteristic facial deformities as a result of change in the shape of the skull.

From the clinical standpoint, particularly for the syndromic patients,[18] additional dysmorphisms such as orbital abnormalities and asymmetry of ears as well as the characteristic head shape can be detected in physical examination in addition to neurological and ophthalmic problems[49] owing to early closure of the sutures. During the prenatal period, detailed ultrasound findings may help to identify the initial anomalies of craniosynostosis, like abnormal head and extremity shapes. On the other hand, in the postnatal period, besides the physical observations, three-dimensional computed tomography is commonly used for the confirmation of synostosis.

Craniosynostosis cases are typically divided into syndromic and nonsyndromic groups. More than 70% of the patients have been diagnosed with nonsyndromic craniosynostosis (nsCRN).[50] Although autosomal dominant inheritance is responsible for most of the syndromic cases, nearly half of the patients result from *de novo* mutations.[49] In syndromic cases, other or additional accompanying findings of craniosynostosis can be observed, including the anomalies of extremities, heart, and central nervous system. Especially in one-third of the cases with coronal synostosis, single-gene mutations are detected more frequently.[51,52] Coronal synostosis is the most commonly observed type in Saethre-Chotzen,[53] Muenke, and craniofrontonasal syndromes. These syndromes can be distinguished by the presence or absence of hand and foot abnormalities, cleft lip palate, and nose anomalies. Unfortunately, in patients diagnosed with craniosynostosis, the restricted brain growth might lead to complications related to several severe medical conditions such as seizures, brain damage, loss of eyesight, cognitive and mental impairment, as well as complex deformities and breathing problems in infants. On the other hand, it should be noted that the potential brain effects mostly relate to syndromic cases, rather than nonsyndromic single-suture cases.

Epidemiology and genetics of syndromic craniosynostosis

The prevalence of craniosynostosis has been estimated to be one in 2500, worldwide.[25,54,55] In addition to phenotypic variations, the specific identify of the closed suture (e.g., coronal or lambdoid) and whether the closure is unilateral or bilateral are also used to specify the form of craniosynostosis. Mainly, the syndrome can be divided into two subgroups, primary and secondary craniosynostosis, as shown in a basic schema (Fig 2). According to Aviv et al., primary craniosynostosis is also divided into two subgroups.[17] Secondary craniosynostosis, which is more frequent than the primary ones, is a condition that occurs as a result of several causes, such as metabolic disorders, malformations, mucopolysaccharidosis, and some environmental factors to which the fetus has been exposed.[1,17]

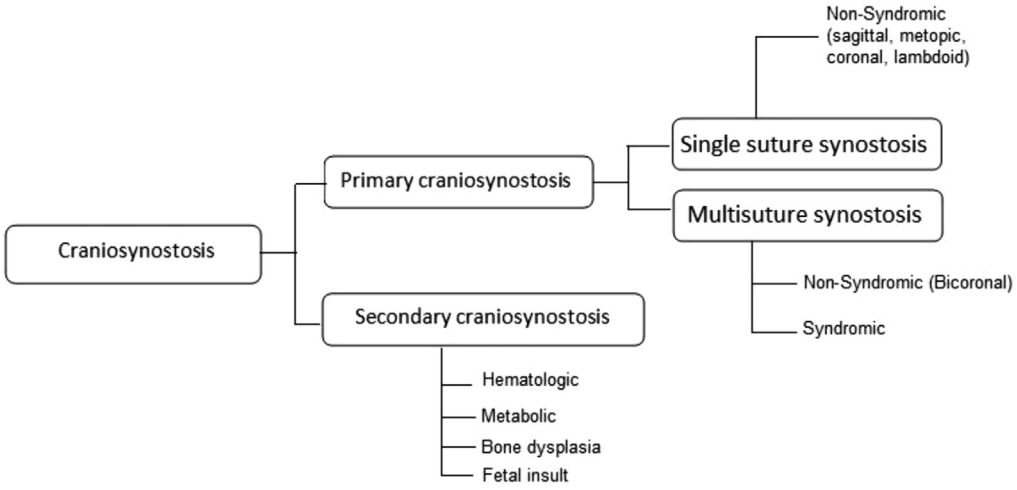

FIGURE 2. Classification of craniosynostosis syndromes.

The syndromic craniosynostosis group forms 25% of all craniosynostosis cases[56] and is inclusive of variable deformities of the body along with various systems such as cardiac, genitourinary, and musculoskeletal systems.[1] Syndromic craniosynostoses are mostly defined as autosomal dominant disorders. More than 180 syndromes that comprise syndromic craniosynostosis have been identified.[51] The major ones are Apert (OMIM #101200), Crouzon (OMIM #123500), Pfeiffer (OMIM #101600), Saethre-Chotzen (OMIM #101400), Jackson-Weiss (OMIM #123150), Beare-Stevenson (OMIM #123790), and Antley-Bixler (OMIM #207410) syndromes.[57,58] Although the most common forms of syndromic craniosynostosis are inherited as autosomal dominant, recessive inheritance, incomplete penetrance, and variable expressivity can also be observed in patients.[6,59,60] On the other hand, mosaicism can also be the cause of craniosynostosis. A mosaic individual has been identified with an *FGFR2* (NM_001144916.1) gene mutation,[61] and the level of mosaicism has been estimated at 19% for the *EFNB1* (NM_004429.4) gene mutations. Therefore it has become an issue suggesting further and careful investigation of patients with unsolved molecular diagnosis.[18,62]

Mutations associated with syndromic craniosynostosis have been documented in several nuclear genes such as *FGFR1, FGFR2, FGFR3, TWIST1, MSX2,* and *EFNB1.*[49,63] Primary target genes and mutations for different types of syndromic cases are shown in Table. Atleast 112 mutations have been identified in the *FGFR2* (NM_001144916.1) gene (www.hgmd.cf.ac.uk/ac/gene.php?gene=FGFR2). The hotspot exonic regions in *FGFR2* gene are (exon 7) IIIa and (exon 9) IIIc (Fig 3). It has been shown that the mutation prevalence in patients with Apert and Crouzon syndromes varied between 31% and 100% in different populations worldwide, including studies from Japan, Canada, Germany, France, and the United States.[64–74] In the Turkish population, these ratios are 91.6% for Apert syndrome, 57% for Crouzon syndrome, and 83% for Pfeiffer syndrome.[75]

As mentioned in the previous section, the FGF receptor signaling cascade involving ERK1/2, p38 MAPK, SAPK/JNK, PKC, and PI3K pathways has been shown to play important roles in regulating MSC differentiation.[38,76] The FGF members exert different effects on osteogenic differentiation of MSCs. After the binding of FGF, FGF receptors dimerize and set off the downstream signaling cascade. Therefore the *FGFR2* is the first gene that comes into prominence for the molecular diagnosis of syndromic craniosynostosis during routine genetic screening. When craniosynostosis is suspected, owing to its high mutation rate, the *FGFR2* gene analysis will expedite the genetic diagnosis of the syndrome through routine molecular diagnostic testing.[77] The most frequently mutated genes and common genetic variations in the different subtypes of syndromic craniosynostosis are summarized in Table.

Another nuclear gene related with syndromic craniosynostosis, particularly for Saethre-Chotzen syndrome, is the *TWIST* (NM_000474.3) gene. It has been suggested that TWIST protein expression, a basic helix-loop-helix transcription factor in osteoprogenitor cells' sagittal and coronal sutures, is one of the participants in the differentiation and proliferation of osteoblasts.[20,78] In 2000, it was reported that the activation of TWIST is coordinated with FGF signaling, and haploinsufficiency of TWIST resulted in Saethre-Chotzen syndrome.[79]

Recently Xu and colleagues identified the genetic variants of nine unrelated probands with syndromic craniosynostosis.[80] As a result of whole-exome sequencing analysis, they identified several pathologic variants in *TWIST1*, *FGFR2*, *IFT122* (as compound heterozygous), and *SMC1A* genes among which five were previously reported, two were novel, and two were rarely seen with uncertain significance. The study has shown for the first time that *SMC1A* gene variants are mostly related with the Cornelia de Lange syndrome (OMIM #122470) and is related with craniosynostosis.[80]

Published studies suggest that not only molecular alterations but also cytogenetic chromosomal aberrations, especially deletions and duplications, occur in all the chromosomes related with the craniosynostosis except for chromosomes 16 and 19. Most of these chromosomal aberrations are associated with midline synostosis.[37] Passos-Bueno et al. in 2011 observed that both chromosomal deletions (~60%) and duplications (~50%) (range in size from 124 to 128 Mb) occur most often in individuals with metopic craniosynostosis. However, the ratios in cases with sagittal and lambdoid sutures have been shown as 20% and 40%, respectively. In coronal synostosis cases, the most frequent aberration has been observed as deletions (~20%).[81] Approximately 16% of syndromic craniosynostosis patients had these chromosomal aberrations.[81] In another study of 45 patients with syndromic craniosynostosis, the prevalence of submicroscopic chromosomal rearrangements (ranged in size from 0.2 to 5 Mb) was approximately 22%. This finding clearly highlights the importance of gene dosage effect on syndromic craniosynostosis.[82] Interestingly, in at least two cases, it has been shown with multiple chromosomal aberrations that both had deletions in chromosomes 7p21 (ranging in size from 3 to 12 Mb)[83,84] and 22q11.2.[37,85,86] All these genetic and cytogenetic factors make craniosynostosis a highly complex condition.

TABLE.
Primarily Targeted Genes and Common Genetic Mutations for Different Types of Syndromic Craniosynostosis (www.omim.org)

Syndromic Craniosynostosis					
Subtype	OMIM #	Affected Suture	Inheritance Pattern	Affected Gene, Mutation Rate (%)	Specific Mutations or Exonic Regions
Crouzon	123500	Multisuture, coronal, sagittal	AD	*FGFR2* (50%)	Exon IIIa Exon IIIc
Apert	101200	Coronal, multisuture	AD	*FGFR2* (97%)	p.Ser252Trp p.Pro253Arg
Pfeiffer	101600	Multisuture	AD	*FGFR1; FGFR2* (67%)	p.Pro252Arg p.Thr290Cys
Saethre-Chotzen	101400	Coronal	AD	*TWIST1* (71%)	Exon 1
Muenke	602849	Coronal	AD	*FGFR3*	p.Pro250Arg

Abbreviation:
AD = Autosomal dominant

Epidemiology and genetics of nonsyndromic craniosynostosis

Another subgroup of primary craniosynostosis is the most common craniosynostosis form called *nonsyndromic craniosynostosis,* which is also known as *isolated craniosynostosis,* shown in Fig 2.[17,25,87,88] In general terms, 8% of the cases are familial.[89]

nsCRN has a heterogeneous pattern that shows both clinical and genetic multifactorial features. In nsCRN, there are four main sutures, sagittal, metopic, coronal, and lambdoid, and are shown in Fig 1B. As a result of early fusion of these calvarial sutures, abnormal head shapes occur and are called trigonocephaly, brachycephaly, plagiocephaly, and scaphocephaly (dolichocephaly) (Fig 1).[55]

In spite of the fact that the prevalence of nsCRN is around 0.4 to 1 in 1000, recent studies show that the observed prevalence is increasing in different subtypes of nsCRN reported by different clinical centers.[90–92] As a result of retrospective studies performed in patients diagnosed with metopic craniosynostosis, it has been reported that the incidence of the syndrome has increased in the United States from 3.9%[93] to 4.6%[94] and in France from 13.8%[95] to 15.6%.[90,96] Between 2008 and 2013, a research performed in Netherlands involving 759 cases determined the prevalence as 7.2 in 10,000. They found that the incidence of total, sagittal, and metopic craniosynostosis has increased significantly for unknown reasons.[97]

All forms of single-suture synostosis have been observed with different rates in the general population. The most common form with a prevalence of 45% to 50% of all nsCRN is sagittal synostosis.[6,54,98] Although it has been known that coronal synostosis is the second most common form, recent epidemiologic studies have shown that approximately 25% of all patients with nsCRN have metopic synostosis.[19,99] Coronal synostosis can be diagnosed as unilateral or bilateral form and comprises nearly 17% of all nsCRN cases.[100] The least common form with a prevalence of 1% to 5% is lambdoid craniosynostosis[6,98] and has been rarely diagnosed during genetic testing.[54] In addition, multiple forms of suture synostosis also occur approximately in 8% of all nsCRN cases and are mostly observed as sagittal and lambdoid synostosis.[100]

The genetic cause of nsCRN syndrome is mostly unknown. Common gene mutations, mostly missense and nonsense variations, and chromosomal alterations associated with craniosynostosis are rarely found in nonsyndromic cases.[37] In 2016, Ye et al. performed a genetic study in over 93 patients diagnosed with sagittal craniosynostosis, which is the most common form of nsCRN, and have screened *FGFR1-3*, *TWIST1*, *RAB23,* and *BMP2* genes. In their cohort, the variations in these genes were determined as approximately 1%. Only a few numbers of the cases have sagittal craniosynostosis as a result of mutations in these genes.[101] Hitherto, at least 57 nuclear gene mutations located in different chromosomes associated with craniosynostosis have been identified (www.omim.org).

On the other hand, there is not much information on the phenotypic characterization of nsCRN, and its underlying reasons still remain unknown.[89,102] Based on the literature, it has been suggested that both genetic and environmental factors play roles in nsCRN.[49,89,103] Hitherto, only a small portion of cases can be diagnosed with rare mutations in *FGFR2*, *TWIST1*, *FREM1*, *LRIT3*, *EFNA4,* and *RUNX2* genes.[104] In recent years, *TCF12* gene has also become an important causative gene especially responsible for coronal nsCRN.[105] This gene, *TCF12* (NM_207036.1), encodes the basic helix-loop-helix partner of *TWIST1* gene, which is associated with Saethre-Chotzen syndrome.[105,106] A study performed in 182 Spanish patients diagnosed with craniosynostosis has suggested that the *TCF12* gene testing is a necessity when the affected suture is coronal,[106] and if the cases were negative for *TWIST1* gene mutations. Therefore with the development of high-throughput sequencing technologies, the number of genes playing roles in the formation of craniosynostosis is expected to be identified significantly more in the near future.[107,108] Moreover, genome-wide association studies also suggest a high potential approach in the identification of new genomic regions that might be related with craniosynostosis.

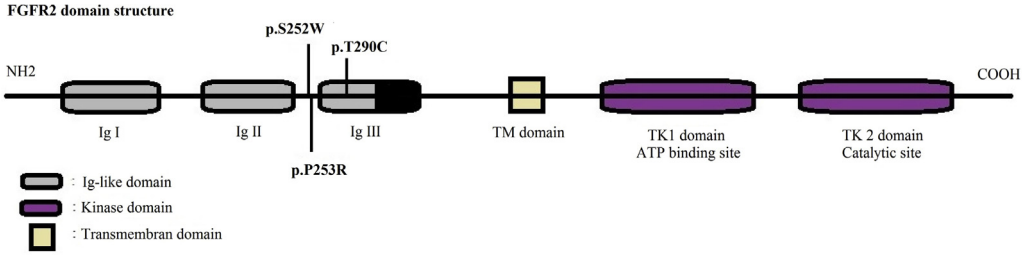

FIGURE 3. Protein structure and the most frequent mutations in *FGFR2* gene. The color version of this figure is available in the online edition.

In recent years, with the development of next-generation sequencing technologies, digenic inheritance began to get attention, especially in Mendelian diseases, such as facioscapulahumeral muscular dystrophy type 2[109] and craniosynostosis. Besides, epistatic interactions between genetic and allelic patterns with increased number of mutations result in complex phenotypic changes.[109] On the other hand, the incomplete penetrance of *MSX2*, *IGFR1*, *ALX4,* and *ERF* nuclear genes might be the main causes of craniosynostosis[110–112] and has been observed in approximately 10% of the patients with sagittal nsCRN. One can clearly understand why craniosynostosis is called an oligogenic, multifactorial, and heterogeneous disorder.[54] Although it has been very well known that different phenotypes and incomplete penetrance are the main causes of craniosynostosis, especially for the *TWIST1* and *FGFR* gene-related forms,[113] the two-loci inheritance pattern that includes a common *BMP2* variant in combination with rare *SMAD6* gene mutation has been shown to explain the complex phenotypic changes in a recent study.[44] In 2012, a strong association between sagittal synostosis and the *BBS9* intronic sequence (a region of 167 kb) on the 7p14 chromosomal region and *BMP2* gene on chromosome 20 has been reported.[104] Moreover, a recent study has shown an allelic difference in the regulatory functions of *BMP2* gene at the location of rs1884302 region (g.7125642 T>C).[114] Interestingly, based on zebrafish transgenesis assay models, it has been reported that *BMP2* gene is overexpressed when the allele is (C) instead of (T), which suggested a potential role for the cause of sagittal nsCRN.[114] These studies also support the idea of an altered BMP signaling pathway that might be the reason for the unsolved craniosynostosis/craniofacial disorders.

We believe that with the development of novel technologies on sequencing of the genome, with the help of bioinformatic tools and functional studies, not only common single nucleotide polymorphisms but also rare or *de novo* variants or microdeletions can be defined much more accurately. On the other hand, the overall success rate of current technologies, such as targeted or whole-exome sequencing, in genetic diagnosis is not higher than 25% to 30%.[115,116] Recent studies using targeted exome sequencing have reported that these rates are approximately 38% for craniosynostosis and 47% for other skeletal syndromes,[108,117] and unfortunately, we still do not know the real underlying reasons for the other 50%, whether they are genetic or not.

Epigenetics of craniosynostosis

Epigenetics is defined as heritability with or without temporary changes in gene expression without any alterations in DNA sequence.[118] Epigenetic modifications can be categorized as DNA methylation of CpG dinucleotides, histone modifications (acetylations or deacetylations), nucleosome positioning, and noncoding-RNA-based silencing, which are comprehensively reviewed by several researchers.[119–121] Epigenetic modifications are of great importance for the normal development and biological processes. These modifications are the key regulators of many cellular processes such as gene and microRNA (miRNA) expressions, cellular differentiation, and embryogenesis.[120] It is a matter of vital importance that epigenetic marks, like methylation or acetylations, should be tagged at the right genomic location at the right time,[120,122] otherwise many human diseases such as cancer and craniosynostosis may occur as a result of these dysregulations of epigenetic modifications. It is now very well known that these epigenetic regulations can be altered by environmental conditions and products like dietary components, chemicals, and pollutants[121] by influencing the activity of modifier enzymes. In addition, recent studies have shown that advanced maternal or paternal age may also trigger the DNA methylation abnormalities in ovum and sperm.[123,124]

In the development of craniosynostosis, not only genetic factors but also epigenetic factors play important roles and are mainly divided into two subgroups: mechanical forces and external environmental interactions.[125] In addition to the intrauterine compression caused by multiple pregnancies, high birth weight, low pelvic station, and oligohydramnios types of mechanical forces are the effects of craniosynostosis induced by the gene expression patterns in specific signaling pathways.[126] The role of suture microenvironment, cell mechanics, and mechanotransduction processes on suture formation have previously been reviewed by Al-Rekabi and others.[9] In 2017, a recently published study by Barreto and colleagues demonstrated that signaling mechanisms of fused sutures differ from those of the patent sutures and that these differences are triggered by extracellular environmental stiffer substrates.[127] As a result of the alterations in gene expression profiles, upregulations of *IGF1*, *TSHZ2*, *MMP9, IL1β*, *WIF1*, *BMP6,* and *NOX1* genes involved in BMP6 pathway are stiffness dependent in cells of fused sutures. This study has enlightened the effects of abnormal extracellular environment on gene regulations that might be the causes of early osteogenesis and suture ossification.[127]

Twin studies, especially monozygotic twins as valuable biological sources, have shown the effects of environmental factors.[128–130] Moreover, for monozygotic twins, intrauterine maternal biological and exogenic factors are eliminated. Thus these features allow us to measure the epigenetic influences on phenotypic differences. Although most environmental factors in the uterus are the same for multiple and various births, some physical fetal head constraints such as intrauterine growth restrictions might be the cause of early suture fusions.[131] However, a recent study of identical twins noted a structural discordance in metopic craniosynostosis.[132] One of the identical twins had metopic nsCRN without any other suture and bone abnormalities, whereas the other twin did not have these abnormalities. This might be due to differences during settling positions *in utero* and exposure to various pressure intensities. In 2003, Borke et al. showed in rat and murine animal models that in the presence of tensile induction, *Tbx2* gene expression level increased in cranial sutures, which led to altered bone formation, whereas *Cx43* gene expression decreased.[133] Taken together, these two studies suggest that as a result of multiple births, intrauterine constraints might play a role in nsCRN pathogenesis through force-induced gene expression alterations.

Several maternal factors can stimulate craniosynostosis during the embryonal stage of the prenatal period. Some of these factors related with craniosynostosis could be the vitamin D deficiency,[134,135] hyperthyroidism,[135,136] cigarette smoking during pregnancy,[137,138] and exposure to teratogens like retinoic acids, phenytoin, and the antifungal fluconazole.[125,135,139,140] As an example, in 2010, it has been shown *in vitro* that increased retinoic acid level enhances osteogenic differentiation in suture-derived mesenchymal cells by increasing BMP expression.[141] In recent years, besides the effects of maternal origin, it has been well studied that paternal age is also associated with an increased risk for congenital craniofacial and other malformations.[142,143] Owing to the increase in *de novo* mutation rate in sperm correlated with aging, older age has a higher risk for several monogenic disorders. This situation is collectively termed *paternal age effect* (PAE) disorders.[144] These findings resulted in the *selfish spermatogonial selection* hypothesis that was suggested to understand the mechanism of gonadal selection.[142] One of the most convincing results obtained from the studies on understanding the PAE were performed by Maher et al. in 2016.[145] Following the isolation of pathogenic mutations directly from normal human testes, the

marks of *de novo* pathogenic mutations in tissues have been shown for the first time and explained the selfish spermatogonial selection hypothesis.[145] One of the very well-studied model for the PAE is Apert syndrome, which occurs as a result of two transversions (c.755C>G or c.758C>G) in 99% of the cases. The c.755C>G mutation occurs at a CpG dinucleotide repeat in 66% cases, whereas the c.758C>G transversion occurs in non-CpG dinucleotide in 33% cases.[142,146] One of the possible reasons of this high mutation rate in CpG dinucleotide can be the escape from the methylation process. When considering the relationship between advanced paternal age and arising methylation abnormalities,[123] the possible cause of high *de novo* mutation rates in sperms may be epigenetic changes. This leads to the selfish selection of sperms and congenital malformations.

Another potential factor in the development of craniosynostosis is the lack of information in the area of small noncoding RNA molecules called miRNAs. Based on the studies done in well-known molecular signaling pathways, miRNAs are involved in lineage commitment of MSCs. It is found that various miRNAs are related with the regulation of differentiation of MSCs. A few studies have suggested that some miRNAs such as *Mir-133b* play a role especially in FGF signaling pathway, which may also regulate cranial suture formation.[147] According to the Potter and Rhodes study, 31 overexpressed and nine underexpressed miRNAs were identified in fused sutures ($n = 7$) when compared with patent sutures ($n = 7$) and they indicated that patent suture's miRNA expression levels are different than fused sutures.[148] Taken together, we believe that the mystery of cranial suture formation parameters will be solved in time.

In conclusion, as a multifactorial and clinically heterogeneous disorder, craniosynostosis should be evaluated by multidisciplinary experts. Today, in addition to the clinical evaluation, molecular genetic testing is the main step of genetic counseling. To fully understand the underlying mechanisms of craniosynostosis and provide appropriate genetic counseling, both clinical and molecular diagnostic features of the condition should be addressed.

This study was funded by the Research Funds Office of Akdeniz University, Antalya, Turkey (Grant #TDK-2015-933). We thank Martha Knight and Özge Alper, PhD, for editing the manuscript.

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