Single-suture craniosynostosis and the epigenome: current evidence and a review of epigenetic principles

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Craniosynostosis (CS) is a congenital disease that arises due to premature ossification of single or multiple sutures, which results in skull deformities. The surgical management of single-suture CS continues to evolve and is driven by a robust body of clinical research; however, the molecular underpinnings of CS remain poorly understood. Despite longstanding hypotheses regarding the interaction of genetic predisposition and environmental factors, formal investigation of the epigenetic underpinnings of CS has been limited. In an effort to catalyze further investigation into the epigenetic basis of CS, the authors review the fundamentals of epigenetics, discuss recent studies that shed light on this emerging field, and offer hypotheses regarding the role of epigenetic mechanisms in the development of single-suture CS.

Methods

A systematic literature search was conducted using the MEDLINE/PubMed electronic database and the references of relevant articles from inception until November 1, 2020 (Fig. 1). To identify relevant articles that address epigenetics in CS, the following search phrase was applied: “(epigenome OR epigenetics OR DNA methylation OR histone modification) AND (craniosynostosis OR suture synostosis).” Preestablished exclusion and inclusion criteria were used. Additionally, relevant articles were identified through reviewing the reference lists of included articles.

Abbreviations

CS = craniosynostosis; DNMT = DNA methyltransferase; EZH2 = enhancer of zeste homolog 2; MZ = monozygotic; PCR = polymerase chain reaction; qPCR = quantitative PCR; siRNA = small interfering RNA.

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criteria were defined. Articles were included if they 1) investigated epigenetics or identified an association between epigenetics and CS; and 2) were published in English. Articles investigating the role of epigenetics in CS were retained. Articles were excluded if they 1) were narrative reviews or editorials; 2) were purely genetics articles without discussion of CS epigenetics; or 3) did not identify sufficient data.

Results
Genetics of CS
Illuminating the underlying causes of premature suture ossification remains a key area of study, with ongoing efforts aimed at investigating the genetic and molecular determinants of CS. Research has documented over 55 genetic alterations that have been associated with CS. This review focuses specifically on epigenetic alterations associated with nonsyndromic single-suture CS; however, it is important to note that different articles have reviewed the genetic bases and their implications in CS extensively. Nonsyndromic CS represents 75% of all craniosynostosis cases, with 20% attributed to chromosomal imbalances. Of all nonsyndromic CS cases, the most commonly involved sutures are sagittal, metopic, coronal, and lambdoid, respectively. The main genetic bases that underly nonsyndromic single-suture synostosis are summarized in Table 1.

Fundamentals of Epigenetics
DNA is wrapped around the primary structural unit of the human genome—the nucleosome. Each nucleosome is a building brick of chromatin, and it contains two copies of four histone proteins (H2a, H2b, H3, and H4) and a 146-base-pair DNA. Two chromatin forms have been identified, euchromatin (transcriptionally active) and heterochromatin (transcriptionally inactive). Modifications to DNA (e.g., methylation) and histones (e.g., acetylation) alter chromatin configuration and, consequently, transcriptional activity. Therefore, these modifications, in addition to noncoding RNAs, control gene expression activity without altering the DNA sequence, representing a new area of study: epigenetics. It was not until 1942 that the word “epigenetics” was introduced to the literature by Waddington. Since then, the term has evolved into the definition we know today: “The study of various intracellular factors that have an effect on the stability of developmental processes through their action on genome potentialities.”

DNA Methylation
One of the best characterized epigenetic mechanisms is DNA methylation, a chemical reaction in which DNA methyltransferases (DNMTs) add a methyl group to a cytosine nucleotide that is usually followed by a guanine (CpG). While most of CpG dinucleotides are methylated and scattered throughout the genome, 30% are unmethylated and present in clusters known as CpG islands, which are found predominantly upstream from DNA transcriptional sites (i.e., at promoter or enhancer regions). Methylation of those CpG islands can, thereby, control gene expression and cellular growth. Thus far, literature has identified different types of DMNT, including DNMT1, DNMTa3, and DNMTb3, and characterized their functions. These enzymes are critical in cellular function and are involved in several disease processes. In a recent study, Ley et al. reported an increased rate of DNMT3A mutations in patients with acute myeloid leukemia.

Histone Modifications
Histones function to arrange DNA within the nucleus...
and are critical constituents of nucleosomes. By modifying the N-terminal tails of histone proteins, histone modification is another method to influence gene transcriptions. Of all histone modifications that have been characterized, only four have been well understood: methylation, acetylation, ubiquitination, and phosphorylation. Each type of modification is catalyzed by different enzymes and leads to different cellular consequences. Various diseases have been associated with defected histone modification enzymes, and different clinical applications relevant to these enzymes have been reported. For example, disturbances to the normal methylation of multiple histones (H3K4me1, H3K4me2, H3K4me3, and H3K9me1) have been associated with several T-cell malignancies. Recent studies reported promising evidence that histone acetyltransferase inhibitors and activators, as well as histone deacetylase activators, can advance breast cancer therapy and improve patient outcomes.

Noncoding RNAs

Additionally, noncoding RNAs, which are another important element of epigenetic regulation, control expression at the gene and chromosomal levels and include four main types: small interfering RNA (siRNA), microRNA (miRNA), piwi-interacting RNA (piRNA), and long noncoding RNA (IncRNA). Although all types are involved in epigenetic machinery, each one functions differently. miRNA is a single-strand RNA that mainly regulates gene expression at the posttranscriptional level and restricts histone-modifying enzymes. Unlike miRNA, which is expressed in mature mammalian cells, piRNAs regulate stem cell and embryonic tissue differentiation. Clinically, noncoding RNAs, especially miRNA, have been used as diagnostic and prognostic biomarkers for diseases such as type 2 diabetes and CNS injuries where miRNA expression profiles are altered.

Tools and Techniques to Study Epigenetics

Various tools and techniques are used to integrate the epigenome and analyze protein/DNA interplay qualitatively and quantitatively. DNA methylation analysis is a common technique that studies epigenetics using sodium

| TABLE 1. Overview of the main genes underlying nonsyndromic single-suture synostosis formation |
|---|---|---|
| Gene | Function & Summary of Previous Reports | Sutures Involved |
| SMAD6 | BMP & TGF-ß/activin-signaling pathways, & mutations have been implicated in syndromic & nonsyndromic sagittal CS. | Sagittal, metopic |
| IL11RA | IL11RA is a constituent of the interleukin 11 receptor & involved in extracellular signal transduction. | Sagittal, metopic |
| TCF12 | TCF12 is found in various intracellular signaling processes and acts as an SMAD cofactor & heterodimerizes w/ TWIST. | Coronal |
| ZIC1 | ZIC1 encodes for 1 member of the zinc family that functions as a transcriptional activator & plays a significant role in CNS & craniofacial development. | Coronal |
| EFNA4 | EFNA4 is attached to the cell membrane by glycosylphosphatidylinositol. EFNA4 protein is a multidomain protein that encodes & interacts w/ tyrosine kinase receptors. | Coronal |

BMP = bone morphogenetic protein; TGF = transforming growth factor.

| TABLE 2. Overview of the main studies investigating the role of epigenetics in CS formation |
| Authors & Year | Study Type | Summary |
| Farooq et al., 2020 | Case series | 4 sets of twins were investigated w/ CS; 2 twin sets were MZ & developed different phenotypes w/ variable severity. |
| Magge et al., 2017 | Case report | An MZ twin pair w/ a discordant presentation; 1 newborn w/ metopic CS. |
| Lakin et al., 2012 | Systematic review | This article showed that different findings support the involvement of epigenetics in CS: 62% of twin pairs in the study presented w/ discordant phenotypic features such as different sutures involved & variable severity; 2-fold increased risk in males; as phenotypic variability among MZ twins. |
| Gill et al., 2012 | Case-control | The authors report a 2-fold risk increase of CS w/ advanced maternal age (≥40 yrs). |
| Sanchez-Lara et al., 2010 | Case-control | This article identified 4 maternal & fetal factors related to CS, including plurality, preterm delivery, nulliparity, macrosomia (birth weight >4000 g), & low birth weight. |
| Oppenheimer et al., 2009 | In vivo experimental | Using animal models, the authors associated head compression w/ sagittal CS after observing histological ossification in mice exposed to head compression. |
| Oppenheimer et al., 2012 | In vivo experimental | Using a similar methodology, the authors documented histological ossification in 4 of 6 subjects. |
| Barreto et al., 2017 | Basic since research | This study indicates gene expression variations btwn fused & patent sutures, & proposed that extracellular environment stiffening could accelerate ossifications & lead to premature skull bone fusion. |
| Dudakovic et al., 2015 | Basic since research | Using chemical inhibition & knockout models, the authors reported enhancement of osteogenic commitment in human progenitor mesenchymal cells after inhibiting H3K27 methyltransferase (EZH2) & documented development of CSs. |
Epigenetics of CS

Although relatively little is known about the epigenetic basis in CS, recent studies have begun to investigate this relationship (Table 2). Detailed epigenetic mechanisms have been established in several diseases such as cancer, cardiovascular disease, obesity, and diabetes. Considering these findings, and in light of recent supporting evidence, several authors have posited the hypothesis of an epigenetic basis in the development of CS. Importantly, only 20% of CS cases are attributed to genetic causes. Recent studies have associated DNA methylation in ovum and sperm with advanced age, partially explaining the high incidence of fetal abnormalities in late pregnancies. Additionally, a small number of experimental, animal model, and twin studies have shown some evidence of epigenetic modifications in CS pathogenesis.

Barring a postzygotic mutation resulting in mosaicism, monozygotic (MZ) twins have virtually identical genomes. Therefore, differences in phenotypes between MZ twins suggests the involvement of epigenetic causes. Several studies report phenotypic discordance among MZ twins, raising questions about the interplay of genetics and environmental factors. One study found DNA methylation and histone acetylation in one-third of MZ twin pairs with discordant presentations. Furthermore, among the general population, an increased incidence rate of CS among twins compared to healthy twins has been recognized. To evaluate phenotypic concordance among twins with CS, Farooq et al. conducted a case series investigating four sets of twins with CS. Three twin pairs were MZ; two developed variable phenotypes with different levels of severity and management plans. Similarly, Magge et al. reported discordance in a genetically identical twin pair. One newborn was healthy, whereas the other newborn developed metopic craniosynostosis. A systematic review by Lakin et al. showed that 62% of twin pairs with CS in the study presented with discordant phenotypic features such as different sutures involved or variable severity. While this study showed a higher concordance rate among MZ twins than dizygotic twins, confirming the genetic role of craniosynostosis, MZ twins were incompletely concordant, supporting the hypothesis of epigenetics. The study also reported high male predominance (65.3%) and indicated that twinning was 2.62 times greater in patients with craniosynostosis than unaffected controls of the general population. Together, these results further support the hypothesis of epigenetics involvement in CS formation.

Different maternal, paternal, pregnancy-related, and environmental factors have been established to contribute to CS formation mediated by epigenetic machinery. Prior research has associated CS with maternal vitamin D deficiency, cigarette smoking, and teratogens such as phenytoin and fluconazole. While bisulfite PCR, MS-PCR, and MethyLight assay interrogate epigenetics in a gene-specific approach, other techniques such as microarray, mass spectrometry, and next-generation sequencing focus on genome-wide analysis.

Analyzing the interaction between proteins (e.g., histones) and DNA is an alternative approach used to investigate the epigenome. Chromatin immunoprecipitation combined with DNA sequencing (ChIP-Seq) uses antibodies to target specific proteins, with subsequent gene expression analysis conducted using PCR, quantitative PCR (qPCR), microarray, or sequencing. This method associates certain proteins and histone modifications with specific genes and chromosomal regions. At its core, ChIP-Seq enables researchers to determine if a protein is bound to a piece of DNA and is thus a critical technology for assessing the role of transcription factors. Another key epigenomic technology is the Assay for Transposable-Accessible Chromatin using sequencing (ATAC-Seq). This ATAC-seq is an experiment that allows researchers to determine if DNA is in a region of open chromatin or closed chromatin.

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suture synostosis than specimens cocultured with the “no load” control group. Taken together, these results suggest that fetal constraint and biomechanical forces may be involved in the genesis of CS.

Although many authors have investigated the association between different epigenetic factors and CS development, few studies have looked into the molecular and mechanistic details. Barreto et al. investigated the effect of altered microenvironmental stiffness in the osteogenic commitment of suture cells and compared the genetic expression of cells from patent and fused sutures of CS. Using PCR array, variable mechanoresponses among those cells were further investigated and indicated gene upregulations caused by microenvironmental stiffness. The authors indicated an augmented expression of IFG1 and TSHZ2 genes in cells from fused sutures, and given their role in bone formation, the upregulation of these genes suggests their important role in accelerated ossification and possible involvement in CS formation. In addition to IFG1 and TSHZ2, this study also reported stiffness-induced upregulation of WIF1, NOXI, BMP6, IL1β, and MMP9 expression—WIF1, NOXI, and BMP6 control osteogenic differentiation; IL1β mediates activating inflammation; MMP9 is involved in the breakdown of extracellular matrix—using qPCR analysis. Furthermore, the authors observed a notable increase of expression in these genes when cells from fused sutures were cultured with an osteogenic medium. Highlighting the role of altered force transmission in defining the timing and magnitude of suture synostosis, this study opened opportunities to explore further the potential mechanotransductive mechanisms associated with CS.

Dudakovic et al. examined the expression of a large cohort of epigenetic regulators (>300) during osteogenic differentiation of human mesenchymal cells to determine critical epigenetic regulators involved in osteogenic commitment. Enhancer of zeste homolog 2 (EZH2) is a methyltransferase enzyme that catalyzes trimethylation of histone 3 lysine 27 (H3K27me3), and despite its pivotal role during normal skeletal development, genetic analyses indicated downregulation of this enzyme during osteogenic differentiation. Testing the hypothesis, Dudakovic et al. used a small molecule inhibitor and siRNA knockdown to evaluate the consequence of EZH2 inhibition on osteogenic differentiation. As hypothesized, blocking EZH2 in vivo stimulated osteogenic differentiation and suppressed adipogenic differentiation, suggesting that EZH2 is a crucial regulator of osteogenic commitment and skeletal development. However, in addition to identifying other epigenetic regulators, further illuminating the enzyme mechanism is warranted to better understand the process and subsequently proceed to clinical applications. The results of the studies mentioned above are of significant importance because epigenetic targets are used to treat multiple types of cancer as well as various other medical conditions including mood disorders and epilepsy, autoimmune diseases, pain, and Alzheimer’s disease. Targeting some of these enzymes and proteins (e.g., EZH2) can be leveraged to inhibit ossification and, therefore, interfere with suture-synostosis pathogeneses, opening up avenues to improve outcomes and prognosis in patients with CS.

Conclusions

As with many diseases, a comprehensive understanding of the fundamental epigenetic mechanisms underlying CS development can aid in disease prevention and treatment, and therefore improve patient outcomes. In this article, we have reviewed the current scientific literature investigating the role of epigenetics in CS. Thus far, little has been established about the epigenetics involved in CS and its interplay with genetic and environmental factors. Therefore, further research is necessary to reveal the critical molecular aspects of epigenetic regulation underlying CS development.

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