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REVIEW ARTICLE

Role of Epigenetics, Environmental and Chemical Agents in Nonsyndromic Orofacial Clefts

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ABSTRACT: Nonsyndromic orofacial clefts (nsOFCs) are the most prevalent congenital craniofacial abnormalities, significantly impacting an individual's overall health. They are clinically presented as an incomplete separation of the lips, palate, or both. NsOFC occurs as a separate entity and is not associated with any other syndromic abnormalities. Epigenetics has been regarded as a key factor in increasing the prevalence of orofacial clefts. Studies in the past have aimed to explain the involvement of epigenetics in the formation of nsOFCs. Researchers have also well-investigated and documented certain environmental factors and chemical agents that could influence the occurrence of orofacial clefts. Understanding the role and effect of the etiological factors will directly support postulating preventative measures to minimize the occurrence of clefts. This review article will offer a comprehensive overview of the epigenetics, environmental factors, and chemical agents linked to developing nsOFCs. The article will also focus on the promising epigenetic biomarkers that can potentially detect clefts early.

KEYWORDS: Cellular and Molecular Biology; Molecular Biology; Nonsyndromic Orofacial Clefts; Epigenetics; Environmental agents; Chemical agents.

Introduction

Various molecular and signaling pathways control the formation of the nasal and oral regions. Any misregulation of these specific pathways: FGF, BMP, TGP-beta, SHH, WNT, PVRL1, FOXE1, MTHFR, and FGFR1 can result in the insufficient development of the nasal and oral regions.[1, 2] In simpler terms, the birth defects caused by a failure of tissue fusion during the development of the upper lip, palate, or both are known as orofacial clefts.

Many classifications exist for orofacial clefts. Based on the anatomical involvement, orofacial clefts can be classified into clefts involving just the lip, the palate, or both. CL/P (cleft lip/palate) can be incomplete, complete, unilateral, or bilateral. CPO (cleft palate only) can be classified into submucosal and complete clefts. Broadly, clefts can also be classified into two more categories: syndromic and non-syndromic. Syndromic clefts occur in association with other abnormalities such as autism and Down syndrome, while non-syndromic clefts occur without other anomalies.[3] Recently two sonographic classifications have been introduced based on the fetus's intrauterine features. They are the Nyberg and Maarse classifications. The Maarse classification is considered more efficient than the Nyberg classification, as the latter does not distinguish between unilateral and bilateral isolated cleft lips. Furthermore, Maarse classification aids in diagnosing all types of common clefts involving the lip, alveolus, and hard and soft palates using biomarkers during the first trimester. This, in turn, facilitates communication with obstetricians and pediatric management teams to devise appropriate therapies.[4]

A recent systematic review and meta-analysis have well documented the prevalence of orofacial clefts: in every 1000 live births, the occurrence of cleft lip is 0.3, cleft palate is 0.33, and cleft lip and palate is 0.45.[5] This data suggests a high prevalence rate of orofacial clefts. Patients affected by clefts not only experience poor quality of life but are also prone to complications such as aspiration pneumonia and bronchopneumonia.[6, 7] To improve the quality of life of such patients, a comprehensive care plan is required, which can be quite expensive, ranging from 2,900 to 109,000 USD.[8]

The prevalence rate of clefts and its high treatment cost and poor quality of life warrant an intensive preventive protocol. To devise such a protocol, it is essential to understand the role of the etiological factors thoroughly. Hence, this review article aims to provide an overview of the development of the lip and palate regions during embryogenesis while discussing specific environmental and chemical factors that are responsible for the misregulation of signaling and molecular pathways. In addition to those mentioned above, the article will provide insight into the epigenetics involved in developing nsOFCs. Though there is limited information on the clinical application of miRNA biomarkers employed in early cleft detection, this article will also briefly discuss it.

Discussion

Embryology of Orofacial Clefts:

Nasal placodes are protrusions that form on either side of the frontonasal prominence by the end of the 4th week of embryonic development. During week 6, upon hollowing, the nasal placodes form nasal pits, and lateral and medial nasal processes develop on either side. During week 7, the widening of the maxillary prominences pushes the nasal processes (nasal placodes) together in the middle. At around 8 weeks of gestation, the intermaxillary segment is formed by fusing medial nasal processes in the midline and forming the philtrum, the upper teeth, and the primary palate. An upper lip develops from the

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philtrum and maxillary prominences.[9] The process described here is clearly illustrated in Figure 1.

A cleft lip can occur in three forms: unilateral cleft lip, bilateral cleft lip, and median cleft lip. The failed fusion of maxillary and medial nasal prominences causes a unilateral cleft lip.[10] The bilateral cleft lip is caused by the failed fusion of maxillary and intermaxillary processes and the interfered migration of cranial neural crest cells. Due to this disturbed migration, a shortage of myoblasts and myocytes occurs, resulting in the insufficient development of philtrum tissues.[11] The failed fusion of frontonasal and maxillary processes causes the median cleft lip.[12]

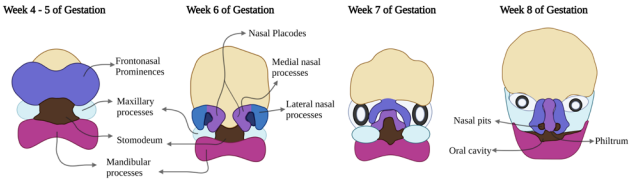

Figure 1: This figure displays the stages of orofacial development. At the end of week 4, the nasal placodes begin to take shape. In week 6, the nasal placodes hollow forming the lateral and medial nasal processes on either side. During week 7, the maxillary processes get bigger to push the nasal placodes together. During week 8, Stomodeum forms the oral cavity and the hollowing of nasal placodes creates the nasal pits. The philtrum is formed by the fusion of both medial nasal processes, and the upper lip begins to form from the philtrum and maxillary prominences. (*Created with BioRender.com*)

The palate's development occurs between the 6th and the 12th weeks of gestation. A triangle-shaped primary palate is formed on the nasal medial processes during the 7th or beginning of the 8th week, extending from the intermaxillary processes to reach the incisive foramen. By week 8, the palatal shelves begin to fuse when the lower mandible descends, bringing the tongue downward. Primary and secondary palates fuse between week 9 and week 12 along the midline as the shelves elevate. This failure of shelf elevation and fusion results in cleft palates.[13,14] This process is thoroughly depicted in Figure 2.

Environmental and Chemical agents implicated in non-syndromic orofacial clefts

Stress:

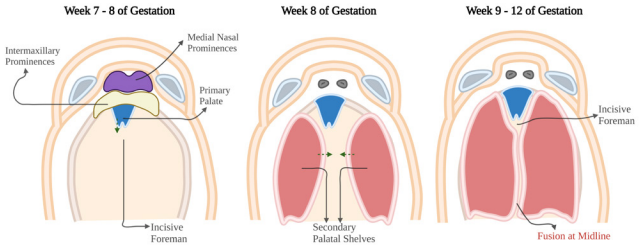

Figure 2: Palatal development commences at the end of week 7 or the beginning of week 8. First, the primary palate begins to grow from the intermaxillary prominences until they reach the incisive foramen, and then the medial nasal prominences start to hollow. They finish hollowing in week 8, where the secondary palatal shelves start growing from the maxillary prominences. Complete fusion of all three palatal shelves in the midline occurs at weeks 9–12. (*Created with BioRender.com*)

Clefts are not proven to be solely hereditary; the presentation of clefts requires external agents to activate and modify the fetus's genome.[15] One environmental agent that leads to the progression of nsOFCs is maternal stress. Stress is a well-known causation of the 'flight or fight' response; this response demands a large amount of blood from surrounding muscles to be diverted to the maternal vital organs. As a result, there will be a lack of blood inflow to the placenta, which could harm the fetus. Furthermore, elevated stress can cause repercussions in their metabolism which contributes to a change in the fetus's environment leading to altered cell growth, duplication and development. Prolonged effects can lead to excess necrosis of cells and cause alterations in the fetus's physique.[16]

Numerous studies have established a link between stress and cleft development. A recent research survey by Mahapure KS *et al.* demonstrates a clear link between maternal stress and clefts, but only in mothers aged 35 and above.[17] The results did not indicate any association between stress and the development of clefts when the age factor was excluded. The collection of this data was done using peer-reviewed questionnaires. A systematic review and meta-analysis by Talal AlSharif M *et al.* revealed that there could be an increased risk of nsOFCs with increased maternal stress, with an odds ratio of 1.17.[18] However, the authors of the systematic review also identified a lack of distinct inclusion of stress as a sole etiological factor of nsOFC in multiple studies, which in turn reduces the reliability of those studies. In many studies, stress levels were assessed numerically depending on how individuals responded, which strongly depended on their coping skills. This could limit the credibility of the study results. Objective metrics like physiological markers or external observations can be used to reduce the dependency on human emotions and coping abilities in stress measurement. Integrating numerical information with subjective self-reports can result in a more thorough and trustworthy evaluation of stress levels and eliminate possible bias. Most research investigations provided mothers with a yes-or-no questionnaire on stress, which could compromise the accuracy of the collected data. Yes-or-no questions in research may oversimplify replies and obscure complexities. Respondents might find it challenging to represent varying degrees of exposure appropriately. This binary method reduces information quality, which may contribute to data bias and impede a complete evaluation of variables impacting the study groups. Furthermore, the possibility of multiple biases, such as recall and reporter bias, was not excluded, which questions the validity of their results.

Though the available study results have provided indirect links between age, stress, and the development of clefts, a strong correlation has not been established. To truly comprehend the effects of stress on the occurrence of clefts, more studies need to be conducted investigating only stress as a causative agent in the development of clefts. Therefore, future studies could identify the precise relationship between stress and cleft formation.

Vitamin A toxicity:

Vitamin A (retinol) is another environmental factor contributing to the development of cleft lips in fetuses. As a lipophilic molecule, retinol is well known for controlling tissue patterning and cell fate specification in embryonic development.[19] To maintain homeostasis, the mother's retinoid metabolism must carry out various functions controlled by metabolic enzymes,

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carry out various functions controlled by metabolic enzymes, binding proteins, and transporters, further mediated by Retinoic Acid signaling. The retinol in the cell is metabolized into Retinoic Acid (RA), and then Cellular Retinoic Acid Binding Proteins (CRABPs) transfer RA into the nucleus. Upon entering the nucleus, the RAR-RXR (retinoid X receptor) heterodimers regulate specific gene expression via the RARE gene. Corepressor proteins are engaged when RAR-RXR heterodimers are unbound from RA. In response to RA binding, they change conformation and engage co-activator proteins to activate transcription. It has been shown that maternal exposure to all-trans retinoic acid (ATRAs) can result in disturbed development, resulting from concentration-dependent changes in gene expression and the unnecessary activation of RA signaling. It can severely impair Tgf-β, Wnt, and Hedgehog (Shh) signaling during embryonic palatogenesis. ATRA exposure can also modulate Notch signaling in mesenchymal and epithelial palatal cells, causing p21 signaling in both tissues.[20] This process is illustrated in Figure 3.

Orofacial malformations are also closely linked with the polymorphism of these specific genes: RBP4 (retinol-binding protein 4), RARA (retinoic acid receptor alpha), and Rdh10 (retinol dehydrogenase 10). The role of RBP4 (retinol-binding protein 4) is to transfer retinol to the corresponding cells; polymorphisms in this gene can lead to an increased progression in clefts.[19] The role of RARA is to regulate gene expression through the fusion of RA with a protein it codes for; polymorphisms in this gene can significantly increase the risk of clefts.[21] Finally, Rdh10 (retinol dehydrogenase 10) changes retinol to RA by coding an enzyme; any polymorphisms in this gene may result in a higher risk of clefts.[22]

Hozyasz K *et al*. researched different groups of mothers with and without cleft children.[23] Samples of their plasma retinol were subjected to high-performance liquid chromatography. The results showed that out of 132 mothers with cleft babies, 52 of them had retinol levels above normal, thus linking retinol or vitamin A toxicity to an increased risk of clefts. Another population study by Johansen AMW *et al*. has revealed that there was a minor co-relation between vitamin A intake and the occurrence of cleft lip in the raw data. However, there is a lack of significant proof of the same across the adjusted data.[24]

Analyzing the results of numerous research investigations, it is evident that an association exist between vitamin A toxicity and nsOFC. However, the threshold for toxicity is large and can rarely be exceeded through diet alone.

Maternal Smoking:

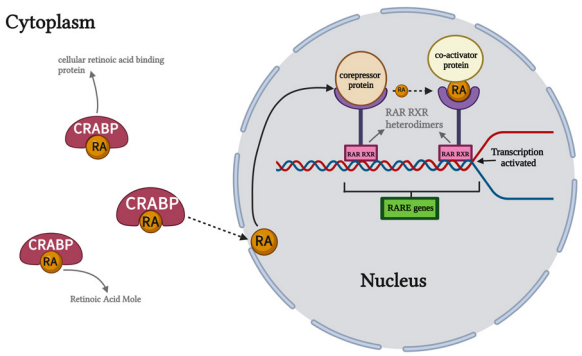

Figure 3: This diagram explains how retinoic acid begins the process of transcription in cells. First, the retinoic acid molecule is first transported into the nucleus cell membrane and then to the first RAR-RXR heterodimer. Once the retinoic acid molecule leaves the first heterodimer, it activates a corepressor protein. Upon reaching the second heterodimer, the retinoic acid molecule activates a co-activator protein, which in turn activates transcription for the RARE gene. (Created with BioRender.com)

Maternal tobacco consumption has also been identified to play a role in orofacial clefts. It is important to note that maternal smoking in most cases, only acts as a catalyst in the presence of specific genetic variants, which in turn increases the cleft risk. Infants born to mothers who smoke and have the genotype MSX1 increase their risk of clefts by 7.16 times.[25] Specifically, the gene PRL in the chromosomal region 6p22.[3] is one common single-nucleotide polymorphism (SNP), which may explain the link between clefts and tobacco consumption. The majority of the SNPs were found in the PRL gene and the minor allele C of rs35090027.[25] Based on genome-wide association studies (GWAS) using European and Asian case-parent trios and pathway-based GxE (gene-environment interactions) analyses, it has been suggested that maternal smoking could increase the incidence of clefts in fetuses bearing the genetic variant RUNX2.[26] Clefts are more likely to occur when there is a genetic variation in the TGFA, TGFB3, and MSX1 genes, as well as polymorphisms in the CYP (1A1, 2E1) and GST (M1, T1) genes.[27]

A study by Honein MA *et al*. displayed a 1.3 odds ratio associated with CLP (cleft lip and palate). However, it is more strongly associated with bilateral CLP (cleft lip and palate), with an odds ratio of 1.7.[25] Smoking more than 25 cigarettes a day can significantly increase CLP with an odds ratio of 1.8 and bilateral CLP with an odds ratio of 4.2. Another study conducted by Li Z *et al*. associated passive smoking with an odds ratio of 1.8 with CL/P (cleft lip/palate).[26] Mothers who smoked during the 2-month post-conceptual period were more likely to have an infant with CP than mothers who did not smoke. The risk of CP increased with the number of cigarettes smoked per day.[26]

Evidence indicates a robust association between maternal smoking and nsOFC, making it a widely reported risk factor. Therefore, it is crucial to counsel expectant mothers to quit smoking to prevent their infants from having nsOFC.

Folic Acid Deficiency:

Folic acid intake is crucial, as it helps produce amino acids. Insufficient intake of folic acid by pregnant women can inhibit their production of amino acids and various other cellular processes, therefore leading to orofacial clefts in infants. The MTHFR gene codes an enzyme that transforms folate into a usable form. Any mutations that occur in this gene will result in a decrease in folic acid levels, which in turn will increase the risk of clefts. These mutations of MTHFR are more commonly found in countries where folic acid fortification is optional.[28] A control study performed by Allen J Willcox *et al*. showed that folic acid intake below 400 µg per day can reduce the occurrence of clefts.[29] According to another study conducted by Kelly D *et al*., a low folate intake during the first trimester can increase the likelihood of a cleft lip by 4.36-fold compared to mothers who take folic acid.[30] The observational study by

Andrew E. Czeizel *et al*. suggests that folic acid supplements, when administered to mothers during the first four weeks of gestation, can reduce the risk of congenital abnormalities.

Studies have demonstrated that folic acid is necessary for two essential processes during embryonic development: methylation and DNA synthesis. Many studies did not document complete information on folic acid intake. Study outcomes may be impacted if self-reported measurements of folic acid use are used without providing information on formulation, dose, or time. The accuracy of relationships between folic acid intake and outcomes may be impacted by measurement errors introduced by recollection or reporting errors. The lack of clarity makes identifying the ideal dosage or timing for possible benefits difficult, thereby reducing the study's validity. Furthermore, the effect of folic acid on cleft lip and palate may be influenced by maternal nutritional variables and vitamin intake. Further research should focus on identifying the possible influence of maternal dietary variables and vitamin consumption on folic acid which in turn affects the occurrence of cleft lip/palate while minimizing the potential confounding factors.

Glucocorticoids:

Glucocorticoids are a category of corticosteroid hormones. The increased dosage of exogenous glucocorticoids can result in the formation of clefts.[32] Similar to retinol, the glucocorticoid receptor regulates the transcriptional response encoded by the gene NR3C1.[33] GR (glucocorticoid receptor) signaling also regulates palatogenesis by influencing mesenchymal proliferation and epithelial differentiation. Deviations from normal GR signaling can prevent palatal shelf formation and fusion. GR appears to modulate developmental pathways, which include Wnt, Fgf, Tgf-β, and Bmp. Misregulation of the pathways mentioned above can lead to craniofacial malformation. GR signaling alterations due to excess glucocorticoids can affect palatogenesis in several ways, which include: i) decreased extracellular matrix protein production, resulting in incomplete palatal shelf elevation and fusion.) increase in the abundance of E-cadherin, which is usually suppressed during palatal fusion iii) reduced proliferation of mesenchymal cells, leading to decreased palatal shelf elevation and fusion iv) increased apoptosis of mesenchymal cells, leading to decreased palatal shelf elevation and fusion v) decreased expression of Fgf10 (fibroblast growth factor 10), Fgfr2b, and Shh (hedgehog), which are important for palatal shelf elevation and fusion vi) disruption of the PAR complex, which is important for epithelial cell polarity and can prevent the palatal shelves from fusing.[20]

Research conducted by Pradat *et al*. utilized data from 11,150 participants, and the results indicate an association between corticosteroids and clefts.[34] One weakness of this research is the absence of information on the underlying maternal disease that motivated steroid usage, which may account for a certain amount of the greater risk. This could be avoided in future research projects by incorporating in-depth questions, evaluating medical data, conducting participant interviews, and interacting with healthcare practitioners. The meta-analysis by Xiao *et al.* also indicates that if there is any chance of facial cleft following prenatal corticosteroid exposure, it is minimal.[35] Most studies in the meta-analysis focused solely on the teratogenic characteristics of corticosteroids; they did not investigate the association between the amount and length of medication administration which could impact the study's findings. The study data of Skuladottir *et al*. suggests an inconsistent association between corticosteroids and the occurrence of clefts.[36] The shortcoming of the research originates from the requirement to categorize corticosteroids broadly because of their rare usage. This broad classification may obscure small changes in the effects or reactions associated with individual drugs, reducing the level of accuracy of the study's results. Expanding the sample size to include a wider range of corticosteroid users, collaborating with several research centers to increase the diversity of data and fostering a more thorough analysis of subgroups can aid in overcoming the constraint.

Although certain studies provide a substantial link between clefts and corticosteroids, there is not enough research targeting glucocorticoids. More research is needed to better understand the impact of glucocorticoids on clefts.

Other Agents:

Other external epigenetic agents that increase the risk of clefts in the fetus were recorded by Regina Altoé S *et al*. [37] Their results concluded that gender, ethnicity, age and education of the parents influenced the probability of occurrence of clefts in their children. Other factors include diabetes, hypertension, infections, obesity, analgesics use, antibiotic intake, anti-inflammatory intake, anticonvulsant intake, benzodiazepínes intake, X-ray exposure and consanguinity.[38] Maternal consumption of alcohol, even at a low level of once a day per week, can cause a 1.6 – 2.1 times higher risk of CLP occurrence in infants.[39] Furthermore, maternal exposure to PM2.5, PM10, O_3, and CO during the first 3 months of gestation could also contribute to the formation of CLP and CPO.[40] Intensified research efforts are essential to comprehensively identify and understand all established and emerging risk factors for cleft lip and palate, ultimately aiming to minimize its occurrence and improve health outcomes for affected individuals.

Recommendation:

To reduce recollection bias when identifying causal components in retrospective investigations, blinding measures, utilization of shorter recall periods, standardized questionnaires, cross-validation of replies with external records, and using memory aids could enhance accuracy and dependability, lowering the role of biased recollections. To avoid biases associated with the use of binary questionnaires, established and standardized scales for measuring the etiological factor can be used. Using a range of questions to elicit multiple elements of stress experiences reduces the dependence on binary replies. Additionally, objective indicators, such as physiological markers, can supplement self-reports and improve the accuracy of stress evaluations. Furthermore, concealing particular facts or the research hypothesis from reporters and participants could help avoid bias. This blinding eliminates preconceived assumptions and promotes objective reporting, hence increasing the dependability of the study's results. Sample matching, clear selection, and community engagement can be considered to

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lower bias in selection resulting from differential participation. Using various recruiting strategies, maintaining anonymity, providing incentives, and clearly conveying study objectives will help reduce selection bias arising from unequal participation. Inclusive sampling, community participation, multicenter trials, and transparency in reporting increase the study results' generalizability. These methods improve data reliability while rendering findings more relevant to various groups. Employing stratification, matched sampling, and randomization to distribute participant groups evenly improves representativeness and reduces bias for case-control studies. Targeted recruiting tactics to assure varied representation could reduce the influence of disparate involvement of participants of different socioeconomic groups on outcomes. Communicating positive aspects of the research clearly will increase inclusion while lowering the danger of biased outcomes owing to unequal involvement.

The survey should be designed to include more thorough questions on factors that could impact the study results. This thorough technique collects important variables, lowering the likelihood of missing confounding factors in the study. Evaluating medical data, conducting participant interviews, and interacting with healthcare practitioners will minimize bias and promote the validity of the collected data.

Furthermore, future studies should adopt a rigorous methodology, considering the confounding variables. This could be achieved by restricting the research population to acceptable estimations of confounding factors, consequently decreasing their unpredictability and influence on outcomes. Ensuring that cases and controls are comparable, and matching them based on important criteria such as age, sexual orientation, and socioeconomic position will also increase the study's validity. Confining research only to particular locations may reduce the ability to generalize its results since localized features may not reflect more widespread populations.

Role of Epigenetics in Nonsyndromic Orofacial Clefts

DNA methylation:

Khan MFJ *et al.* provide evidence showing higher methylation on the medial side of the lip tissue in patients with cleft lips.[41] Furthermore, a study by Sharp GC *et al.* found that the methylation level can change over time.[42] Patients with cleft lip and palate (CL/P) had higher levels of methylation in their lip tissue than patients with cleft palate only (CPO). This suggests that the methylation level at the time of birth may be more reflective of the true embryological methylation in orofacial clefts. Alterations in DNA methylation are known to cause the occurrence of CL/P. DNA methylation involves adding a methyl group to the C5 position on the cytosine ring in DNA. A group of DNA methyl transferase catalyzes the whole process.[43] This process can be influenced by environmental agents to cause hypo or hypermethylation. A summary of all the genes has been provided in Table 1.

Hypomethylation:

The hypo methylated genes that cause nsCL/P are as follows: MYC found on chromosome 8, FAT1 found on chromosome 4, Chr1 found on chromosome 1, WHSC1 found on chromosome 4, VAX1 found on chromosome 19, NTN1 found on chromosome 1, BICC1 found on chromosome 10, SMOC1, PVRL1, CCL2 which is specific for cleft lip, MTHFR c.677C > T, UQCC2, GLI2, and RPS29.[41-45] GWASs' have shown that the MYC locus is strongly associated with nsCLP.[44] Cleft lip patients with variant c.677C > T of the MTHFR gene have higher methylation levels on the medial side of their lips. A hypofunctional MTHFR gene results in lower SAM and consequently hypomethylation. In this context, polymorphisms in MTHFR c.677C > T may increase the risk of non-syndromic cleft lip and palate.[41] From the available literature it is evident that hypomethylation causes the aberrant expression of genes that are involved in craniofacial development. This expression could result in the development of a cleft. Recognizing the role of DNA hypomethylation in nsOFC will help to develop novel genetic strategies for early diagnosis leading to better prognosis and developing preventive methods to reduce the occurrence of clefts.

Hypermethylation:

Hypermethylated genes associated with clefts are LINE-1 and IRF-6, LOC146880, CLASRP (found on chromosome 9), TBX1, COL11A2, HOXA2, CRB2, PDGFRA, and CRISPLD2 (which is specific for cleft palate), WNT3A, GLI2, PIK3C2G, SOX2, AMOT, PITX2, and SLC25A5.[41, 42, 44-47] The role of IRF-6 is to promote epithelial-mesenchymal transition in lip and palatal fusion. Thus, high methylation levels on this gene can lead to nsCL/P (non-syndromic cleft lip/palate), characterized by the stunted growth of the palate and upper. A study by Li *et al.* found that the methylation of the gene TBX1 was significantly higher in people with cleft palates than in people without cleft palates.[46] This suggests that TBX1 may be a potential biomarker for cleft palate. However, the study concludes that further research needs to be conducted in this domain to substantiate their results. The hypermethylation of LOC146880 can lead to nsCL/P through a mediation pathway that begins with an SNP caused by abnormal DNA methylation.[45]

Table 1: The table provides a comprehensive list of the methylated genes associated with nsOFCs.

Hypermethylated Genes		Hypomethylated Genes	
LINE-1	CRB2	MYC	SMO1
IRF-6	PDGFRA	FAT1	PVRL1
LOC146880	CRISPLD2	Chr1	CCL2
CLASRP	UQCC2	WHSC1	GLI2
TBX1		VAX1	SOX2
COL11A2		NTN1	AMOT
HOXA2		BICC1	PITX2
GLI2		SLC25A5	MTHFR c.677C > T

MiRNA Biomarkers and their use in cleft early identification:

In many developmental and pathological processes, noncoding RNA (ncRNA) plays an important role in gene expression. A microRNA (miRNA) is a smaller molecule of ncRNAs that can regulate gene expression post-transcriptionally by binding to mRNAs, inhibiting their translation, and/or decreasing their half-life. Normally, miRNAs regulate gene expression through the complementarity between their strands and mRNA target strands, but in mammals, there is an imperfect complementarity. As a result of this mechanism, one miRNA inhibits multiple mRNA targets in a cell, thereby regulating various functions such as cell migration, adhesion, differentiation, apoptosis, and epithelial-mesenchymal transitions (EMTs). Multiple critical processes can be affected by miRNA dysfunction during orofacial development.[48, 49]

As epigenetics and miRNAs have recently been investigated as etiological factors in clefts, their role in cleft identification is limited. However, studies have shown that unusual expression of miRNAs in tissues can be monitored through biological liquids, plasma, and palate fibroblasts. Mendes SMDA *et al*., in their systematic review, aimed to identify all the miRNA biomarkers associated with all the subtypes of orofacial clefts that were expressed in plasma. 100 different molecules showed strong connections with clefts, of which 14 were significantly associated with CP and CL/P, 9 with only CP, 44 with CL/P, and one correlated with CL/P and CL.[50] The Vincenzo Grassia *et al*. study successfully identified 131 miRNAs in salivary fluids that correlate with CL/P.[48] They had chosen to use saliva for their investigatory procedure as a non-invasive method that could be employed for sample collection from the patient. Considering the ease of sample collection, this method can also be employed in regular clinical practice for identifying certain miRNA biomarkers in bodily fluids, which will significantly aid the cleft care team in formulating an early diagnosis.

Recommendation

Residual confounding is the chance that unmeasured or insufficiently controlled factors will still impact study outcomes. Even after controlling recognized confounding factors, if additional substantial factors are not taken into account, there is a probability that the relationships will be impacted through these confounding factors, thereby leading to bias or misunderstanding. To minimize residual confounding, researchers should systematically identify and quantify potential confounders, conduct sensitivity assessments, disclose the results openly and implement proper measures to reduce the effect of confounding elements and improve the study's reliability.

Future studies that combine DNA methylation, histone modification, and miRNA investigations are essential in providing a broader comprehension of the regulation of genes. This is crucial, as exploring the interactions between these factors can provide a comprehensive understanding of the complicated mechanisms that control gene expression.

Knowledge of the association of miRNA biomarkers with nsOFCs has been derived from primarily animal studies. Human research is crucial, even if animal studies offer insightful information on the processes behind cleft lip and palate. By considering the genetic and environmental elements that are specific to people, human research guarantees direct applicability to human biology. Further experimental validation is essential to translating research findings into therapeutic applications and improving our comprehension of miRNA's function in cleft lip and palate. This guarantees that the data are reliable and applicable, opening the path for future clinical applications and advances in treating this ailment.

Conclusion

In conclusion, the etiology of nonsyndromic orofacial clefts is greatly influenced by the complex interactions among epigenetic variables, environmental factors, and chemical agents. The analysis highlights the complex interplay between several factors influencing this congenital deformity and emphasizes the need to comprehend the molecular processes regulating embryonic development. DNA methylation is an example of epigenetic change essential for controlling the expression of genes during key phases of craniofacial development. Numerous environmental variables, including maternal nutrition and lifestyle choices, might have an impact on these alterations. Determining how epigenetic changes occur in response to environment stimuli gives a significant understanding of the intricate dynamics that cause orofacial clefts.

Factors related to the environment, such as maternal smoking and maternal medication usage, have been recognized as potential indicators of risk for non-syndromic orofacial clefts. Hence, it is critical to advocate the significance of public health initiatives and awareness programs that educate pregnant women about the benefits of living a healthy life throughout pregnancy. Additionally, the effects associated with chemical agents should not be disregarded. Thorough risk evaluation and remedial actions are required to reduce possible exposures and avoid negative consequences.

Integrating epigenetic and environmental information will help us recognize high-risk individuals and design tailored preventative and early intervention strategies. Collaboration among researchers, physicians, and policymakers is critical for enhancing the understanding of nonsyndromic orofacial clefts and optimizing the treatment outcomes for the affected. Furthermore, it will also help shape public health campaigns that advocate the awareness of clefts and personalized medical techniques, promoting a holistic approach to address the intricate, multifaceted etiology of orofacial clefts.

This review includes contemporary research data to provide readers with up-to-date information on epigenetic risk factors and environmental and chemical agents involved in developing nsOFCs. However, further research is needed on some of the epigenetic agents discussed in this review to establish a definitive association between these agents and clefts. Specifically, extensive research needs to be conducted on epigenetic biomarkers, as they are one of the few non-invasive early detection methods for the patient. Studies aiming to thoroughly analyze the influence of environmental and chemical agents on the methylation of certain genes should be conducted to aid in a better understanding of their association. A comprehensive list of miRNA biomarkers expressed in nsOFCs must be developed in the future, supported by multiple empirical studies.

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The role of miRNA biomarkers in the early detection of clefts is pivotal; hence it is vital to devote more attention to this area. Furthermore, case-control studies in different demographic regions are necessary to fully comprehend etiological variables' influence on oral clefts. The outcomes of the demographic studies will determine whether the present research findings are consistent or if there are regional variations in the patterns of association between various etiological factors and nsOFCs. This should be followed by the development of tailor-made, comprehensive cleft management protocols based on the patient's individual needs.

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