GENE EXPRESSIONS IN THE INCIDENCE OF NON-SYNDROMIC CLEFT LIP AND PALATE

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Abstract

The cleft lip and aisle (CL/P) are a craniofacial malformation caused by genetic mutations, environmental factors, or an interaction between the two. The development of the lip and auricle involves morphogenesis, molecular signaling pathways, mesenchymal-epithelial interactions, and auricle fusion. CL/P events involve initiation, growth, morphogenesis, and auctioning fusion. The Genome-Wide Association Study (GWAS) identified genes and loci associated with non-syndromic CL/P into 43 types significantly associated with single nucleotide polymorphism (SNP) near the gene. This systematic review aims to explore the genetic underpinnings of CL/P by analyzing findings from Genome-Wide Association Studies (GWAS) and identifying key genes and molecular pathways involved in palatogenesis. The most significant genes and loci in non-syndromic CL/P were Interferon regulatory factor 6 (IRF6), MAF bZIP transcription factor B (MAFB), Paired Box 7 (PAX7), Forkhead Box E1 (FOXE1), Msh Homeobox 1 (MSX1), T-box transcription factor 22 (TBX22), and Methylenetetrahydrofolate reductase (MTHFR). Genetic factors play an essential role in the pathogenesis of non-syndromic CL/P, including disruption of signaling pathways. IRF6, MAFB, PAX7, FOXE1, MSX1, TBX22, and MTHFR are genes that play a role in palatogenesis. Mutations in these genes have an impact on orofacial development.

Keywords: *Cleft Lip and Palate, Genome-Wide Association Study, Non-Syndromic*

1. INTRODUCTION

During craniofacial development, cleft lip and palate (CL/P) can happen when the fusion process of the upper lip or palate does not occur as it should (Ji et al., 2020). There are two main categories in which cleft lip and palate are divided: syndromic and non-syndromic (Won et al., 2023). It occurs unilaterally, bilaterally, completely or incompletely (Nasreddine et al., 2021). The occurrence of CL/P can differ depending on geographical location, ethnicity, and economic status, with an average of 1 in every 700 newborns or 0.5–2.6 per 1,000 live births (Ji et al., 2020). The prevalence of this congenital disorder in Asia and the Americas is reported to occur in 1 in 500 live births, in Europe 1 in 1,000 live births, and in Africa 1 in 2,500 live births (Boesoirie & Gatera, 2021). The incidence of CL/P is assumed to be higher in developing countries. According to research by the Ministry of Health in 2013, 0.08% of CL/P cases occur in Indonesia (Khamila et al., 2019). The prevalence of CL/P also differs between the sexes. Men are twice as likely to have CL/P compared to women, while women are twice as likely to have CL compared to men (Nahas et al., 2021).

Research over the past few decades has focused on identifying the genetic and environmental influences on the occurrence of CL/P. The etiology of CL/P is very

complex, and many candidate genes and loci with varying functions are involved in the occurrence of CL/P (Nasreddine et al., 2021). The growth and formation of the lips and palate take place during several processes of morphogenesis, molecular signaling pathways, mesenchymal-epithelial interactions, and palatal fusion processes (Garland et al., 2020). Signaling pathways such as Transforming Growth Factors (TGF), Bone Morphogenetic Proteins (BMP), Sonic Hedgehog (SHH), and Fibroblast Growth Factors (FGF), as well as various transcription factors such as Msh Homeobox (MSX) and T-Box gene family (TBX), have been identified as mediators of cell growth, proliferation patterns, migration, apoptosis, and epithelial-mesenchymal transition (EMT) interactions (Nasreddine et al., 2021).

This systematic review aims to examine the genetic factors underlying the pathogenesis of non-syndromic cleft lip and palate (CL/P) by analyzing findings from Genome-Wide Association Studies (GWAS). The study seeks to identify key genes and loci associated with non-syndromic CL/P, particularly focusing on IRF6, MAFB, PAX7, FOXE1, MSX1, TBX22, and MTHFR, while investigating the molecular signaling pathways critical for normal palatogenesis. Using literature from 2012 to 2023, we aim to evaluate the ethnic variability in genetic associations across different populations and provide a comprehensive understanding of the genetic landscape of non-syndromic CL/P.

2. RESEARCH METHODS

The evaluation of the gathered articles utilised a thorough technique, commencing with the collection of research publications from reputable sources encompassing PubMed. The search strategy utilised targeted keywords ("(Cleft lip and palate (Non-syndromic)) and (Genome-Wide Association Study)") and complied with defined inclusion criteria: articles published in English and Bahasa Indonesia from 2012 to 2023, focussing on authentic research, encompassing both qualitative and quantitative studies. A preliminary plagiarism assessment was performed to verify authenticity, subsequently examining 90 articles following the removal of duplicates. Sixty papers were removed. A thorough examination of 30 articles ensued, assessing their entirety and leading to the deletion of 2 pieces that failed to satisfy the inclusion criteria. Finally, the 28 surviving articles were subjected to a comprehensive analysis for the extraction of pertinent statistical data. This systematic review methodology, which includes search strategy, inclusion criteria, plagiarism verification, multi-phase screening, and data extraction, guarantees the credibility, relevance, and quality of the gathered articles and the subsequent investigation (Figure 1).

3. RESULTS AND DISCUSSION

Studies on gene expression in cleft lip and palate are increasingly being reported, below are 29 studies analyzed regarding the influence of genes on cleft lip and palate (Figure 1).

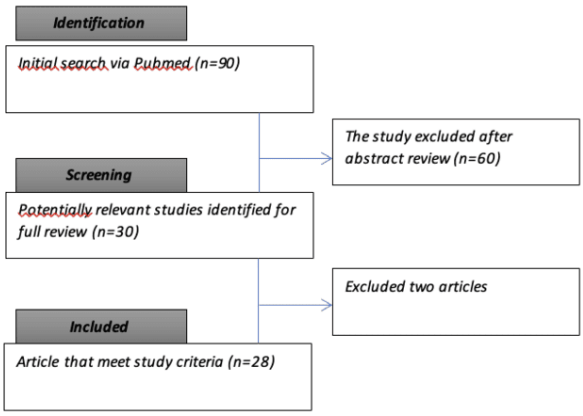

Figure 1. Data selection methodology in the literature evaluation

3.1. Embryology, Anatomy and Physiology

The process of craniofacial development involves the shaping of the lips, palate, nose, and mouth, and typically occurs between the 4th to 12th week of pregnancy (Nasreddine et al., 2021). The start of craniofacial growth involves the movement of neural crest cells from the top part of the brain tube to the pharyngeal arch or branchial arch (Ji et al., 2020). Neural critter cells are the first to arrive at the initial pharyngeal arch, while the remaining critters move in a front-to-back direction to create the remaining four pharyngeal arches. The pharyngeal arch is a temporary structure formed from 4 germ layers. The outermost part is the ectoderm, and the middle part is the endoderm, divided by the mesoderm and mesenchyma, originating from the neural crista cells (Roth et al., 2021).

The proliferation and migration of neural crista cells contribute to forming facial protrusion or facial process (FP) (Roth et al., 2021). Migrating neural crystal cells merge with mesoderm cells to form facial primordia consisting of five distinct protrusions (Khan et al., 2020). Cells that migrate towards the most anterior part of development form the Frontonasal process (FNP). The first pharyngeal arch grows into a pair of Maxillary Processes (MxP) and Mandible Processes (MP) (Roth et al., 2021).

The Frontonasal Process (FNP) consists of medial and lateral components. The medial component develops into the nasal septum, midline structures, philtrum, premaxilla, and four incisors. Meanwhile, the lateral component, positioned on the outer sides of the nostrils, gives rise to the nasal turbinates and nasal alae. The upper lip, near the midline, upper jawbone, and palate, is formed by the fusion of the Maxillary Process (MxP) and the Frontonasal Process (FNP). In contrast, the mandibular arch develops from the fusion of the Mandibular Processes (MP) (Figure 2) (Roth et al., 2021).

The palate is crucial for various functions like respiration, communication, and digestion as it serves as a divider between the throat and the nasal passage (Roth et al., 2021). Primary and secondary processes in palate development have distinct embryological origins, but share common cellular components (Primasari, 2018). The development of the primary palate takes place during the 5th to 12th week of pregnancy. The most crucial period is from the sixth to the ninth week of pregnancy, with the primary palate being created in the seventh week of pregnancy (Nasreddine et al., 2021).

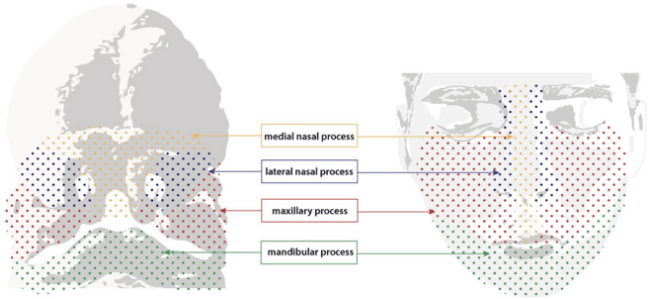

Figure 2. The structure of the face protrusion at the embryo's beginning. Graphic representation of a 5-week-old human embryo (left): medial nasal process (yellow), lateral nasal process (blue), maxillary process (red), and mandible process (green). The structures derived from these protrusions are indicated by their respective colors on the adult face (right) (Roth et al., 2021)

The growth and development of the palate occur through several stages; during the fifth week of pregnancy, the primary palate begins to be formed by the intermaxillary segment (fusion of the MNP) that develops towards the medial and caudal direction. The intermaxillary segment is an internal wedge-shaped mass that extends and descends inferiorly to the nasal pit in the inner part of the stomodeum and develops to the base of the nostrils and the nasal septum. Although the primary palate originates from the intermaxillary segment, the central part of the palate remains formed by two outward growths from MxP. By the sixth week of pregnancy, these two protrusions form the primary palate, the rice septum, the premaxilla (the upper jaw bone supporting teeth 21 22), and the filtrum (Primasari, 2018).

During the 6th to 7th week of pregnancy, the secondary palate merging takes place in a series of three stages. (1) two palatal plates form symmetrically near the tongue and increase in size vertically until they can touch each other when rotated horizontally. (Figure 3A), (2) by the 6th week of pregnancy, the two plates on the palate rotate to a level position above the tongue, coming together to form a connection (Figure 3B), (3) during human development, the medial edge epithelium (MEE) located at the palatal midline comes together and eventually vanishes, marking the conclusion of palate fusion typically occurring around weeks 12-13 (Figure 3C). Thus, MEE is vital in secondary palatal fusion during palate development (Nakajima et al., 2018).

The usual palate includes both the hard palate and the soft palate, separating the back of the oropharynx and nasopharynx. The prominent palatine veins arise bilaterally from the palatine canal through the foramen palatine major—the molle palate functions as a velopharyngeal sphincter. The muscles in the palate elevate the sphincter toward the back wall of the pharynx, creating a division between the nose and the mouth. These functions are essential for phonation, breathing, swallowing, and blowing (Tarr et al., 2018).

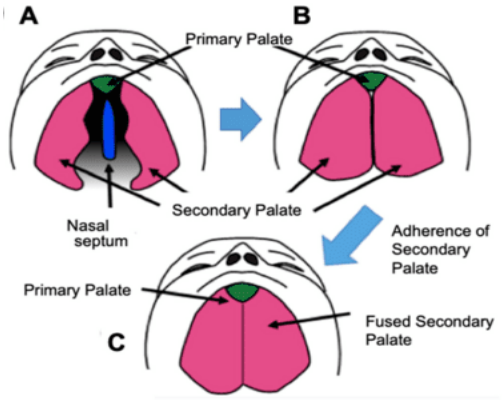

Figure 3. (A) At the beginning of palatogenesis, the secondary palate plates grow vertically on both sides of the tongue, with a gap between the secondary palate, nasal septum, and primary palate. (B) After the tongue descends, the secondary palate plates are raised and reoriented horizontally (along with the x-axis and medial-lateral axis), thus allowing contact between the palate plates and initiating fusion. (C) In the 12-13 weeks of pregnancy, The fusion of the palate is complete (Nakajima et al., 2018)

The velopharyngeal sphincter consists of five pairs of muscles, namely the levator veli palatine muscle, the tensor veli palatine muscle, the uvula muscle, the palatopharyngeal muscle, and the palatoglossus muscle. The muscles of the palate come from the fourth pharyngeal arch and receive nerve signals from the vagus nerve's pharyngeal branch. However, the tensor muscle veli palatini is an exception as it comes from the first pharyngeal arch and receives nerve signals from the trigeminal nerve's mandibular branch. The eustachian tube and the levator veli palatini originate from the tensor muscle. Together, they regulate the eustachian tube openings, facilitating air circulation in the middle ear. The palatoglossus and palatopharyngeal muscles work to constrict the oropharyngeal opening. During the closure of the velopharynx (e.g., swallowing), the tightening of two upper pharyngeal muscles leads to the forward shift of Passavant's ridge towards the back of the throat, eventually making contact with the rear soft palate (Tarr et al., 2018).

3.2. Cleft Lip and Palate

Cleft lip and palate (CL/P) is a condition that is present at birth and commonly impacts the facial structure (Won et al., 2023). Cleft lip occurs when the frontonasal part and the maxillary protrusion do not fully fuse, resulting in a gap in the lips, alveolus, and base of the nose of different severities. If it extends to the base of the nose, it is called an incomplete gap, while if the base of the ala and the medial labial elements are not connected, it is called complete. A cleft palate occurs when the palatal plates in the MxP region do not fuse properly, resulting in a cleft in the durum palatum or molle palatum (Vyas et al., 2020).

3.2.1. Etiology and Risk Factors

The causes of cleft lip and palate involve a combination of genetic and environmental influences, with genes and the environment interacting in a complex way.

Scientific literature evidence suggests that environmental factors such as maternal smoking and alcohol consumption, seizure medication consumption, folic acid deficiency, infections, and kinship are risk factors for cleft lip and palate (Khan et al., 2020; Nahas et al., 2021). Smoking habits in pregnant women in the first trimester are associated with CL/P; mothers who smoke before pregnancy are associated with a greater risk of developing CL.14 The association between mothers who smoke and the incidence of CL/P is not very strong, but it is significant (Vyas et al., 2020).

Drinking alcohol in the early stages of pregnancy is believed to have a connection with CL/P occurrences (Tie et al., 2022). A study showed that mothers who consumed alcohol increased their risk of CL/P by 1.5-4.7 times depending on the amount of alcohol consumed. The association between alcohol consumption and genotype on CL/P risk has not been proven (Vyas et al., 2020). The occurrence of cleft lip and palate has also been linked to taking folic acid supplements while pregnant. Taking folic acid supplements in the first trimester of pregnancy was connected to a 33% drop in the risk of cleft lip and palate. Taking one folic acid pill with a dosage of 400 grams daily before the mother's final menstrual cycle has been shown to decrease the chances of CL/P. Based on previous research, as many as 15.1% of CL/P events can be prevented with pregnant women taking folic acid supplements (Du et al., 2023).

Viral, bacterial, and protozoan infections during the first trimester of pregnancy are suspected to be risk factors for cleft lip and palate. Possible viruses that could be present are varicella, rubella, rubeola, Epstein-Barr, herpesvirus, cytomegalovirus, the common cold, and influenza. The connection between the occurrence of cleft lip and palate and influenza remains unclear. However, studies have shown that the incidence of fever-inducing hyperthermia is an indirect trigger for teratogenesis. Cytomegalovirus (CMV) is also suspected to cause cleft lip and palate. In addition to the hyperthermia caused, CMV infection can disrupt the Nuclear Factor Kappa B (NFkB) signaling pathway, increasing TGF-β inhibitors and interfering with normal orofacial development. Bacteria such as Mycoplasma pneumoniae, Chlamydia Trachomatis, Treponema pallidum, and protozoa such as Toxoplasma gondii are also thought to be risk factors for cleft lip and palate events, but further research is needed to understand the relationship and teratogenic potential (Garland et al., 2020).

In 1942, Fogh-Andersen initiated genetic research indicating that hereditary factors play a role in causing CL/P. Studies suggest that taking into account family history is crucial for anticipating the likelihood of new instances of CL/P within families (Oliver et al., 2021). Khan et al. (2020) conducted a study to determine the genotypic combination pattern of the BMP2 rs235768 A>T gene in the families of CL/P patients. Mothers and children with CL/P abnormalities were identified with a change in base A to base T, a mutation missense (Serin-Arginine or TCA-TCT). This shows that the five families have different combinations. The exciting thing about the third family, the combination of a father with a genotype AA (homozygous or standard) and a mother with a TT genotype (homozygous or uncommon), has a third child with genotype AA (homozygous or common) with CL disorder or incomplete palatoschiosis.

3.2.2. Pathogenesis of cleft lip and palate

The face stops developing around the 12th week of pregnancy. If any essential processes like cell movement and cell death are interrupted, it can cause CL/P. A lack of development in the primary palate can cause cleft lip, while a failure in the secondary

palate formation can cause cleft palate (Nasreddine et al., 2021). Palatogenesis relies on precise temporal control of genetic components such as growth factors and signaling molecules for proper development. Cleft lip and palate (CL/P) occur due to disruption of normal biomolecular processes during craniofacial development (Won et al., 2023).

Cleft lip (CB) arises due to the lack of MxP fusion and intermaxillary segments. The gap that arises can vary according to the severity of the phenotype, ranging from a slight indentation in the edge of the lip vermilion to a lip wholly separated from the filtrum and nasal cavity. Cleft palate is when the palate plate fails to fuse properly during development. Reasons for this abnormality can include lack of growth in the palate, such as when neural crest cells don't migrate properly, as well as failures in both the elevation and fusion of the palatal plate, and the further degradation process after fusion. In addition, proper palate formation requires proper mandibular growth with tongue flattening and lowering, but any deviations in this process can result in the onset of a cleft palate (Tarr et al., 2018). Many signaling pathways and essential genes differentiate the palate epithelium. In establishing mesenchymal continuity in the palate, removing the epithelium between the two adjacent palatal plates, called the Midline Epithelial Seam (MES), is necessary. Cleft lip and palate (CL/P) can occur due to disruption of the MEE differentiation process, disruption of the adhesion process, and the loss of MES (Won et al., 2023).

Clinical findings in patients with CL/P can be found, such as impaired tooth growth, speech disorders, ear infections, and eating disorders (Vyas et al., 2020). Children with chronic Eustachian Tube Dysfunction (DTE) usually suffer from various ear problems like recurring acute otitis media, otitis media effusion, or tympanic membrane retraction. (Ueharu & Mishina, 2023). The tensor veli palatini muscle and the levator veli palatini muscle cause the relationship between Eustachian tube dysfunction and the palatal cleft. Less optimal in children with cleft lip and palate (Reynolds et al., 2020).

Abramyan (2019) said that the prevalence rate of middle ear pathology in patients with CL/P is as much as 65%. Eustachian tube dysfunction results in a reduction in pressure within the middle ear, resulting in the retraction of the eardrum, thereby increasing the likelihood of cholesteatoma occurring in children. Numerous research investigations have been undertaken to examine the possible connection between cleft lip/palate and middle ear infections, with comparable results found in 56 ears belonging to 28 people. There were seven ears (12.50%) that had normal ear function with a normal tympanic membrane, 36 ears (64.28%) had intact but gloomy tympanic membranes, four ears (7.14%) showed active effusion with different levels of retraction, eight ears (14.28%) had chronic suppurative media otitis (OMSK) with central perforation and one ear (1.85%) had a cholesteatoma. Hearing loss in people with CL/P is higher than in the average population. The high incidence of hearing loss in children during growth can result in learning disorders, cognitive problems, language skills, and psychosocial disturbances (Reynolds et al., 2019).

3.2.3. Classification

Cleft lip and palate (CL/P) are categorized into two groups: syndromic and non-syndromic. Syndromic cleft lip and palate (CL/P) refer to a facial abnormality that is linked with other deformities. Usually, it is caused by a single gene disorder (monogenic or Mendelian) and is associated with genetic mutations or chromosomal abnormalities.

Cleft lip and palate (CL/P) syndromic are associated with various syndromes: Pierre Robin, Apert, Down, Marfan, Crouzon, and Nager. Non-syndromic CL/P, conversely, it is thought to have multiple causes and frequently includes a genetic predisposition to external influences (gene-environment interactions) (Garland et al., 2020; Vyas et al., 2020). Non-syndromic cleft lip and/or palate (CL/P) is found in 60%-70% of cases, while syndromic CL/P conditions are found in 30%-40% of cases (Oliver et al., 2021).

Generally, orofacial cleft is categorized as either cleft lip with or without cleft palate (CL/P) or cleft palate alone (CL) (Garland et al., 2020). There are several classifications to describe cleft lip and palate in humans (Figure 4) (Ji et al., 2020). The classification of unilateral cleft lips (CB) is determined by both the width of the cleft as well as its position within the lip. This results in the identification of three distinct subtypes (Ji et al., 2020).

1) Incomplete cleft lip (CB): A cleft or gap in the lip that is smaller than a complete cleft lip. The size and position of the cleft may vary.
2) Complete cleft lip (CB): A condition where the cleft spans the full width of the upper lip, extending from the base of the nose to the boundary between the lip and adjacent skin.
3) Median cleft lip (CB): A rare type of unilateral cleft lip that divides the center of the upper lip into two distinct sections.
4) Bilateral cleft lip (CB): A cleft affecting both sides of the upper lip, which occurs less frequently than a unilateral cleft lip (Won et al., 2023).

Cleft palate (CL) can be complete involving the durum palate and molle palate or incomplete involving one of them. Unilateral cleft palate (CL) has three subtypes: (Won et al., 2023)

1) Complete cleft palate (CL): involves the durum palate and molle palate
2) Incomplete cleft palate (CL): involves the durum palate or molle palate
3) Submucosal cleft palate (CL): a small molle palate hole with the mucous membrane remaining intact. This makes it more challenging to diagnose than other forms of CL. So, the diagnosis may be delayed until the patient can speak or hear.

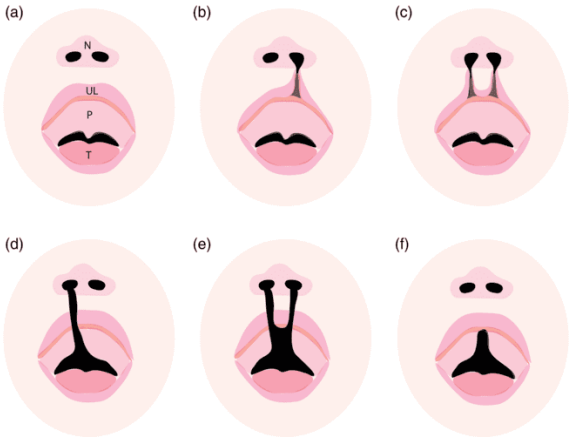

Figure 4. (a) Normal lip and palate structure (b) Unilateral cleft lip (can be left or right with varying severity) (c) Bilateral cleft lip (d) Unilateral cleft lip with cleft palate (e) Bilateral cleft lip with cleft palate (F) Cleft palate only. N, nose or nose; P, palate or palate; T, tongue or tongue; UL, upper lip or upper lip (Ji et al., 2020)

3.3. Craniofacial Formation Signaling Pathway

Some of the commonly researched molecular pathways consist of pathways related to extracellular signaling factors, transcription factors, and cell adhesion molecules (Deshpande & Goudy, 2019). In understanding neural crystal cells along maxillofacial development, several crucial developmental signaling pathways must be understood. There are four signaling pathways that cannot be separated from the neural crystal cells (Roth et al., 2021).

Once the palatal fusion is complete, the anterior two-thirds will undergo mineralization through intramembrane ossification into the durum palatum, and the posterior third will form the palate fibromuscular tissue into the molle palatum. The signaling pathways involved in the development of the palate have been the subject of thorough research, including Transforming Growth Factors β (TGF-β), Bone Morphogenetic Proteins (BMP), Sonic Hedgehog (SHH), Fibroblast Growth Factors (FGF), and Wnt/β-catenin which are responsible for regulating all palate-forming processes between the embryonic oral epithelium and the palate mesenchyma, as well as the regulation of transcription factors. This pathway dysregulation can give rise to genetic variations that are thought to be closely related to CL/P events (Nasreddine et al., 2021).

This pathway involves ligands secreted in various affinities to extracellular matrix molecules. Maintaining ligands within the extracellular matrix or binding them to their inhibitors enables accurate regulation of the timing and location of their effects. Despite being commonly discussed as separate entities, these signaling pathways actually intersect either directly through a central signaling hub within cells or indirectly via cross-communication between different pathways, which work together to provide the necessary instructions for craniofacial development to be well coordinated (Roth et al., 2021).

3.3.1. Transforming Growth Factors (TGF)

Transforming Growth Factors (TGF) is a cytokine that controls various cellular responses during embryology and tissue homeostasis (Tie et al., 2022). Growth Factors-β (TGF-β) has three isoforms, including TGF-β1, TGF-β2, and TGF-β3. All three TGF-β isoform receptors are expressed in the MEE; type 1 receptors (TβR1) and type 2 receptors (TβR2) are serine or threonine kinase receptors, and the third is type 3 accessory receptors (TβR3, βglycans). The TGF-β signaling is allegedly regulated by the Smad-dependent and non-Smad-dependent signaling lines (Nakajima et al., 2018). The cell releases this growth factor and it is then attached to nearby receptors, which in turn send signals internally using cytoplasmic proteins from the Smad family (Deshpande & Goudy, 2019). Based on their structure and function, Smads are divided into three primary categories: Receptor-Smad (R-Smad), Common mediator-Smad (Co-Smad), and Inhibitor-Smad (I-Smad). R-Smads are further subdivided into two groups: Smad 2/3 and Smad 1/5/8 (Du et al., 2023).

Initially, ligands (TGF-β1, TGF-β2 or TGF-β3) bind to TβR2, forming complex bonds and then phosphorylating and activating TβR3. Once TβR3 is active, TβR3 and TβR2 will form complex bonds, phosphorylating and activating the Smad-dependent signaling pathway. The complex binding of TβR3 in the Smad-dependent signaling pathway will stimulate the dissociation of R-Smad so that Smad2/3 will bind to Smad4 and translocate this bond to the nucleus, thereby mediating the activation of TGF-β target

gene expression. Alternatively, TGF-β can also activate non-Smad-dependent signaling pathways, namely through Mitogen-Activated Protein Kinase (MAPK) signaling (Extracellular-Regulated Kinase/ERK, Transforming Growth Factor β -Activated Kinase 1/TAK1, p38, c-Jun N-Terminal Kinase/JNK), Phosphoinositide 3-Kinases/PI3K signaling and Rho Associated-Protein Kinase (RhoA-ROCK) signaling. These Smad-dependent and non-Smad-dependent signaling pathways will stimulate the EMT process. This signaling is essential for palatal plate fusion as the final target of the Smad-dependent and non-Smad dependent signaling pathway (Figure 5) (Nakajima et al., 2018).

3.3.2. Bone Morphogenetic Proteins (BMP)

Bone Morphogenetic Proteins (BMP) are a multi-functional growth factor structurally belonging to the TGF-β subfamily (Oliver et al., 2021). There are more than 20 ligand families of BMPs, but only BMP2 and BMP4 have been shown to play a role in palate development (Tarr et al., 2018). BMP4 is a signaling molecule essential in forming cartilage, bones, teeth, and facial development (Khan et al., 2020).

After the BMP ligand binds to the BMP receptor to form a hetero-multimer consisting of BMP receptors type 2 (BMPR2) and BMP receptors type 1 (BMPR1), then the signaling is transduced through the Smad-dependent pathway (Smad 1/5/9) or the non-Smad dependent pathway (TAK1-p38). Through the Smad pathway, BMPR1 phosphorylates the Smad1/5/9 proteins that transduce signals to the nucleus via Smad4 to alter the expression of target genes, e.g., Msh homeobox (Msx2) and Dickkopf Wnt Signaling Pathway inhibitor 1 (Dkk1). Smad 6/7 inhibits phosphorylation and transition of Smad1/5/9. Noggin is an extracellular antagonist for the BMP ligand that suppresses the BMP signal (Ueharu & Mishina, 2023).

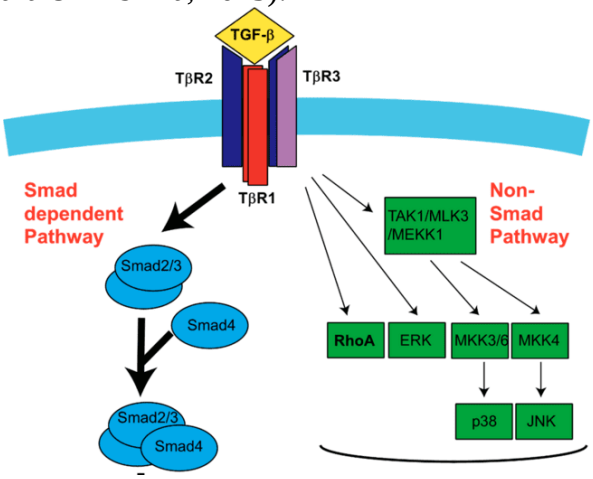

Figure 5. The pencil line is TGF-β (11)

The presence of BMP2 and BMP4, along with the gene Msx1, has been observed in the development of the palate (Nakajima et al., 2018). The transcription of BMP4 in the front region of the palate is controlled by Msx1 (Tarr et al., 2018). Mutations in BMP4 are associated with non-syndromic CL/P events (Khan et al., 2020). BMP2 mutations in neural crista cells expressing Wnt1 cause CL due to elevation failure in the palatal plate. The function of BMP2 in the roof of the mouth does not play a role in causing the rise, as issues with bone formation and cartilage development in the jaw can also lead to a change

in the position of craniofacial structures that inhibit the movement of the palate plate, causing the occurrence of CL/P (Reynolds et al., 2020).

3.3.3. Sonic Hedgehog (SHH)

There are three homologous hedgehogs, including the Desert hedgehog (DHH), the Indian hedgehog (IHH), and the Sonic hedgehog (SHH). Among the three hedgehogs homologous, SHH regulates craniofacial developmental processes ranging from neural crystal cell migration to craniofacial fusion prominence (Abramyan, 2019). SHH is detected in the facial ectoderm and plays a role in controlling the growth of nerve cells in the face (Reynolds et al., 2020). The SHH pathways are categorized into canonical and non-canonical types. The canonical pathway involves three key components: the Patched-1 receptor (PTCH1), the Smoothened receptor (SMO), and the Glioma-associated Oncogene Homolog (GLI) transcription factors, which include GLI1, GLI2, and GLI3. The SHH protein activates the SHH signaling pathway by binding to PTCH1, causing SMO to be inhibited. Intracellular GLI2 will enter the nucleus and bind to DNA to transcribe the SHH target gene. If there is no SHH protein, SMO will be directly activated intracellularly and then activate Protein Kinase A (PKA) to bind to GLI2. The suppressor of fused (SUFU) is the downstream of the SHH signaling pathway by binding to GLI2 so that GLI2 cannot enter the nucleus. SUFU bound to GLI3 will be degraded by PKA so that GLI3 cannot enter the nucleus. When the SHH signaling pathway does not run properly, the expression of the SHH gene will not occur and will cause disturbances in morphogenesis (Abramyan, 2019). FOXF2 is a direct target of SHH signaling in the development of the primary lip and palate. Besides FOXF2, the expression of several other FOX genes in the facial primordial mesenchyme, originating from neural crest cells, depends on SHH signaling. These include Forkhead Box Protein C2 (FOXC2), Forkhead Box Protein D1 (FOXD1), Forkhead Box Protein D2 (FOXD2), and Forkhead Box Protein F1 (FOXF1). FOXF1 and FOXF2 play a crucial role in the regulatory interaction between SHH and FGF in the secondary palate, where SHH-dependent FOXF1/2 activates FGF18, which in turn provides feedback to modulate SHH expression (Reynolds et al., 2020).

3.3.4. Fibroblast Growth Factors (FGF)

Fibroblast Growth Factors (FGF) mediate mesenchymal-epithelial interactions during palate development (Reynolds et al., 2020). At the moment, there are 22 types of human FGF ligands categorized into seven subgroups according to how they work and their ability to bind with 5 recognized FGF receptors. FGF receptors (FGFRs) are tyrosine kinase receptors, as in BMP and TGF-β signaling. The FGF signaling pathway plays a role in developing the lips and palate (Nakajima et al., 2018). FGF receptors comprise 3 immunoglobulin-like (Ig) domains. The Ig I domain is thought to inhibit ligand binding, while Ig II and Ig III regulate ligand specificity. FGFR1–3 plays the most crucial role in the developing palate, while FGFR4 and FGFR5 are not explicitly examined. FGFR 1-3 mutations are identified as causative factors for syndromic and non-syndromic CL/P (Tarr et al., 2018). FGF10 gene expression has been detected in the anterior palatal mesenchyma, which affects the SHH signaling pathway and regulates BMP2 expression. Meanwhile, FGF2 is expressed in the epithelium and the posterior palate, and FGF8

induces the expression of Paired Box 9 (PAX9) in the posterior region of the palatal mesenchymal (Nakajima et al., 2018).

3.3.5. Wingless-Related Integration Site (Wnt) /β-catenin

During the process of craniofacial development, the Wnt signaling pathway engages with several signaling regulators, and certain parts of this pathway also play a role in the development of orofacial clefts (Reynolds et al., 2020). The canonical Wnt signal results in the accumulation of free β-catenin, which acts as a transcription factor and controls the expression of the target gene in the cytoplasm. After binding of the Wnt ligand to the Frizzled receptor (Fzd), Fzd binds to Dishevelled (Dlv), and the Lipoprotein Receptor-related Protein (LRP) co-receptor binds to Axin, which inhibits degradation. β-catenin will bind to the T-cell factor/Lymphoid Enhancer Factor (TCF/LEF) transcription factor in the cytoplasm to regulate the transcriptional activation of essential Wnt target genes in various cells/tissues, such as the orofacial slit-associated gene Msx1/Msx2 in the orofacial primordia (Reynolds et al., 2019).

In the canonical pathway where Wnt is absent, intracellular β-catenin will be constantly phosphorylated to degrade Glycogen Synthase Kinase (GSK-3β), Axin protein, Casein Kinase (CK1) and Ademato us Polyposis Coli (APC). Disruptions in both canonical and non-canonical Wnt signaling pathways can result in reduced epithelial proliferation and increased cell death in the mesenchyme and palatal epithelium. This can cause defects in the frontonasal. The mammalian genome contains 19 Wnt ligands, some of which have been linked to orofacial clefts in human patients, including both syndromic and non-syndromic cases. One study of non-syndromic CL/P associations using SNPs found evidence of an association of Wnt5A, Wnt7A, Wnt8A, and Wnt11 in different populations (Reynolds et al., 2020).

3.4. Non- Syndromic Ceft Lip and Palate- Related Genes

In both human and experimental animal studies, researchers have identified over 300 genes that play a role in the fusion of the palate. It has been found that a mutation in a single gene has the potential to lead to clefts in the orofacial region (Deshpande & Goudy, 2019). In recent decades, genetic risk factors for non-syndromic craniosynostosis have been successfully identified through various methods, including gene candidates and chromosomal regions as well as the Genome-Wide Association System (GWAS) in a variety of ethnic groups (Wu-Chou et al., 2019). The first GWAS performed on non-syndromic CL/P events showed that the vulnerability loci occurred on chromosome 8q24, which was subsequently replicated in several independent GWAS examinations. GWAS identified 24 specific significant genes. Certain genes showed a strong connection to a heightened likelihood of CL/P and could potentially be the cause of Mendel's disorder. The Genome-Wide Association System (GWAS) identified 43 genes or loci significantly associated with Single Nucleotide Polymorphism (SNP) near genes involved in craniofacial development (Nasreddine et al., 2021).

Several genes have been identified as increasing the risk of non-syndromic CL/P, including those encoding growth factors like TGFα and TGF-β3, as well as transcription factors such as Msh Homeobox (MSX1) and T-Box Transcription Factor 22 (TBX22), genes involved in xenobiotic metabolism such as Cytochrome P450 Family 1 Subfamily A member 1 (CYP1A1), Glutathione S-transferase (GSTM1) and N-Acetyltransferase 2 (NAT2), uptake of genes in nutrient metabolism such as Methylenetetrahydrofolate

reductase (MTHFR) and Retinoic Acid Receptor Alpha (RARA), and genes involved in immune response namely PVLR1 and Interferon regulatory factor 6 (IRF6). Based on gene sequences and loci, the most significant in non-syndromic CL/P events were IRF6, MAF bZIP Transcription Factor B (MAFB), Paired Box 7 (PAX7), Forkhead Box E1 (FOXE1), Msh Homeobox 1 (MSX1), TBX22 and MTHFR (Nasreddine et al., 2021).

3.4.1. Interferon regulatory factor 6 (IRF6)

Interferon regulatory factor (IRF) is a homologous protein that regulates interferon transcription (IFN) and is an expression of IFN-induced genes (Antonczyk et al., 2019). IRF6 is a protein that plays a role in controlling how genes are expressed by attaching to certain parts of DNA (Deshpande & Goudy, 2019). IRF6 is found on chromosome 1q32.2 and is linked to both syndromic and non-syndromic forms of CL/P. The IRF6 gene produces proteins with a DNA-binding domain at the N-terminal and a SMIR domain at the C-terminal for regulating interferon. Research shows the IRF6 allele is associated with non-syndromic CL/P in different populations (Gurramkonda et al., 2018). Similar to TGF-β3, IRF6 is also expressed along the MEE and is involved in the TGF-β signaling pathway (Deshpande & Goudy, 2019). Interferon regulatory factor 6 (IRF6) plays a vital role in the formation and maintenance of oral periderm, palate adhesion, and palate fusion (Figure 6) (Gurramkonda et al., 2018; Martinelli et al., 2020; Won et al., 2023).

The IRF6 genetic variant is strongly associated with non-syndromic CL/P incidence among different populations (Nasreddine et al., 2021). Past studies have indicated that out of all the genes believed to play a role in cleft lip and palate, IRF6 stands out as the most consistent candidate. The relationship between SNP IRF6 and susceptibility to non-syndromic cleft lip and palate was found to be consistent in Central European and Han Chinese populations, while the Brazilian population exhibited varied levels of susceptibility in the analyses. No vulnerabilities were seen in the non-Hispanic white and Swedish group studies. This may still be due to differences in the origin of patients and recruitment criteria (Wu-Chou et al., 2019). The association between IRF6 rs642961 polymorphism and non-syndromic CL/P was dominant in Asian populations (Nasreddine et al., 2021). In a study conducted by Soleymani et al. (2022) obtained from 105 children with non-syndromic CL/P and 180 genotype controls IRF6 rs2013162 and rs2235375 using Polymerase Chain Reaction-Restriction Fragment Long Polymorphism (PCR-RFLP). IRF6 polymorphism is associated with the incidence of non-syndromic CL/P in populations in Iran.

Nasroen et al. (2018) researched the effect of changes in IRF6 polymorphism rs2235373 in patients with non-syndromic cleft lip and palate. This study uses a laboratory analysis method by isolating DNA from venous blood and RNA isolation from the palate mucosal epithelium swabbed 15-20 times. There are several reasons why this study uses the allanite mucosal epithelium to evaluate mRNA changes. First, because IRF6 is the most critical gene in epithelial proliferation. Based on previous research on animal models, IRF6 plays an essential role in forming the oral periderm during fetal time. Second, IRF6 is strongly expressed in several organs of the body, including the epithelium of the oral mucosa.

3.4.2. MAF bZIP Transcription Factor B (MAFB)

MAF bZIP Transcription Factor B (MAFB) plays a role in regulating lineage-specific hematopoiesis. In studies in rat experimental animals, MAFB was expressed on the palate plate and MEE during palatal fusion. Several GWAS were conducted in Asia to identify the role of MAFB in non-syndromic CL/P events. It was found that the MAFB SNP rs13041247 gene was not significant in the East Asian population, while MAFB SNP rs17820943 and rs6072081 were suspected to be associated with non-syndromic CL/P.9 It was reported that there was a link between the polymorphism of MAFB SNP rs13041247, rs6065259, and rs6072081 and the incidence of non-syndromic CL/P in Vietnam (Soleymani et al., 2022).

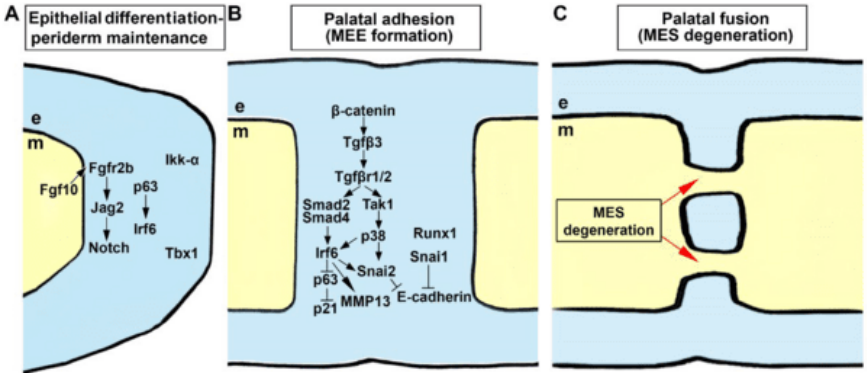

Figure 6. (A) Fgf10 as the upstream signaling pathway of Jagged Canonical Notch Ligand 2 (Jag2-Notch) in regulating the development of palatal epithelium (B) TGF-β3 activates IRF6 through the Smad-dependent signaling pathway (Smad2/4) and the non-Smad-dependent pathway (MAPK-p38). IRF6 activates Snai2, which acts as an EMT regulator; the decrease in Snai2 expression causes a delay in palatal fusion. IRF6 also activates Matrix metalloproteinase-13 (MMP13), which plays a role in peridem desquamation. Furthermore, IRF6 will induce palate adhesion and eliminate MEE, so the process will continue to be palatal fusion (C). New palatal fusion will occur if MES is degraded. MES degradation occurs when migration, apoptosis, and transition from TGF-β3 and IRF6-induced mesenchymal epithelium (Won et al., 2023)

3.4.3. Paired Box 7 (PAX7)

Many research studies have shown that the PAX gene plays a role in the formation of the face and skull by influencing how cells grow, move, and differentiate in the early stages of development (Nasroen et al., 2018). PAX7 is involved in the formation of neural crest cells. While the PAX7 gene has been linked with CL/P, it is not linked with CL (Nasroen et al., 2018). polymorphism PAX7 rs742071 was previously proposed as a non-syndromic CL/P risk factor (Tie et al., 2022).

3.4.4. Forkhead Box E1 (FOXE1)

During the process of palatal fusion, FOXE1, a type of transcription factor, is present in the epithelium of the secondary palate and controls the activities of MSX1 and TGF-β3, both of which are essential for the development of the palate (Nasreddine et al., 2021). GWAS reports a significant association between FOXE1 and non-syndromic CL/P in different populations (Soleymani et al., 2022).

3.4.5. Msh Homeobox 1 (MSX1)

Msh Homeobox 1 (MSX1), located on chromosome 4q16, is part of the Homeobox gene family, which is involved in craniofacial development, limb movement, and tumor growth suppression. MSX1 has been linked to the occurrence of non-syndromic cleft lip and cleft lip with cleft palate (CL/P) in various populations. The MSX1 genotypes rs12532-AG and rs12532-GG are thought to be associated with non-syndromic CL/P risk. Mutations in MSX1 can cause about 2% of non-syndromic CL/P cases. Mutations in MSX1 can disrupt mesenchymal cell proliferation (Nasreddine et al., 2021).

3.4.6. T-Box Transcription Factor 22 (TBX22)

The T-box 22 gene (TBX22) encodes a transcription factor essential for developmental processes, particularly in palatogenesis (Khan et al., 2020). Mutations in TBX22 can be found in patients with isolated CL (Nasreddine et al., 2021).

3.4.7. Methylenetetrahydrofolate reductase (MTHFR)

MTHFR, situated on chromosome 1q36, is responsible for producing enzymes that aid in the breakdown of folic acid (Nasreddine et al., 2021). Mutations found in this specific gene have been linked to the condition known as MTHFR deficiency. An association was reported between MTHFR gene polymorphism and non-syndromic CL/P risk (Nasroen et al., 2018). Another study reported an association between MTHFR rs1801133 polymorphism and non-syndromic CL/P incidence. However, other studies have shown that the relationship between MTHFR polymorphism with non-syndromic CL/P varies between populations (Nasreddine et al., 2021).

4. CONCLUSION

The genetic associations to non-syndromic CL/P observed in GWAS are not universal across populations. There is ethnic variability in the specific genetic loci implicated, indicating that various genetic elements could have an influence on diverse ethnic groups. While GWAS have identified numerous genetic loci, the next step is to functionally validate these findings. Understanding how specific variants influence gene expression and craniofacial development is crucial for uncovering the molecular mechanisms underlying non-syndromic CL/P. This review underscores the need for further research to elucidate the complex interplay between genetic and environmental factors in CL/P, which could inform future diagnostic and therapeutic strategies.

REFERENCES

Abramyan, J. (2019). Hedgehog signaling and embryonic craniofacial disorders. *Journal of Developmental Biology*, *7*(2), 9.

Antonczyk, A., Krist, B., Sajek, M., Michalska, A., Piaszyk-Borychowska, A., Plens-Galaska, M., Wesoly, J., & Bluyssen, H. A. R. (2019). Direct inhibition of IRF-dependent transcriptional regulatory mechanisms associated with disease. *Frontiers*

in Immunology, *10*, 1176.

Boesoirie, S. F., & Gatera, V. (2021). Hubungan Derajat Celah Langit-Langit dengan Keadaan Telinga Tengah Berdasarkan Timpanogram pada Pasien Celah Langit-Langit. *Farmasi*, *10*(2).

Deshpande, A. S., & Goudy, S. L. (2019). Cellular and molecular mechanisms of cleft palate development. *Laryngoscope Investigative Otolaryngology*, *4*(1), 160–164.

Du, X., Cai, L., Xie, J., & Zhou, X. (2023). The role of TGF-beta3 in cartilage development and osteoarthritis. *Bone Research*, *11*(1), 2.

Garland, M. A., Reynolds, K., & Zhou, C. J. (2020). Environmental mechanisms of orofacial clefts. *Birth Defects Research*, *112*(19), 1660–1698.

Gurramkonda, V. B., Syed, A. H., Murthy, J., & Lakkakula, B. V. K. S. (2018). IRF6 rs2235375 single nucleotide polymorphism is associated with isolated non-syndromic cleft palate but not with cleft lip with or without palate in South Indian population. *Brazilian Journal of Otorhinolaryngology*, *84*(4), 473–477.

Ji, Y., Garland, M. A., Sun, B., Zhang, S., Reynolds, K., McMahon, M., Rajakumar, R., Islam, M. S., Liu, Y., Chen, Y. P., & Zhou, C. J. (2020). Cellular and developmental basis of orofacial clefts. *Birth Defects Research*, *June*, 1–30. https://doi.org/10.1002/bdr2.1768

Khamila, N., Nurwiadh, A., & Putri, F. A. (2019). Characteristic of cleft lip and palate at cleft center of padjadjaran university dental Hospital: 2 years retrospective study. *METHODS*, *2020*.

Khan, M. I., Prashanth, C. S., & Srinath, N. M. (2020). Genetic factors in nonsyndromic orofacial clefts. *Global Medical Genetics*, *7*(04), 101–108.

Martinelli, M., Palmieri, A., Carinci, F., & Scapoli, L. (2020). Non-syndromic cleft palate: an overview on human genetic and environmental risk factors. *Frontiers in Cell and Developmental Biology*, *8*, 592271.

Nahas, L. D., Alzamel, O., Dali, M. Y., Alsawah, R., Hamsho, A., Sulman, R., Alzamel, M., & Omar, A. (2021). Distribution and risk factors of cleft lip and palate on patients from a sample of Damascus hospitals-A case-control study. *Heliyon*, *7*(9).

Nakajima, A., F. Shuler, C., Gulka, A. O. D., & Hanai, J. (2018). TGF-β signaling and the epithelial-mesenchymal transition during palatal fusion. *International Journal of Molecular Sciences*, *19*(11), 3638.

Nasreddine, G., El Hajj, J., & Ghassibe-Sabbagh, M. (2021). Orofacial clefts embryology, classification, epidemiology, and genetics. *Mutation Research/Reviews in Mutation Research*, *787*, 108373.

Nasroen, S. L., Maskoen, A. M., Soedjana, H., Soemantri, E. S. S., & Hilmanto, D. (2018). The effects of IRF6 rs2235373 polymorphism on mRNA expression changes in non-syndromic cleft lip and palate with various phenotypes. *Padjadjaran Journal of Dentistry*, *30*(3), 221–230.

Oliver, J. D., Jia, S., Halpern, L. R., Graham, E. M., Turner, E. C., Colombo, J. S., Grainger, D. W., & D'Souza, R. N. (2021). Innovative molecular and cellular therapeutics in cleft palate tissue engineering. *Tissue Engineering Part B: Reviews*, *27*(3), 215–237.

Primasari, A. (2018). *Embriologi dan tumbuh kembang rongga mulut*.

Reynolds, K., Kumari, P., Sepulveda Rincon, L., Gu, R., Ji, Y., Kumar, S., & Zhou, C. J. (2019). Wnt signaling in orofacial clefts: crosstalk, pathogenesis and models. *Disease Models & Mechanisms*, *12*(2), dmm037051.

Reynolds, K., Zhang, S., Sun, B., Garland, M. A., Ji, Y., & Zhou, C. J. (2020). Genetics and signaling mechanisms of orofacial clefts. *Birth Defects Research*, *112*(19), 1588–1634.

Roth, D. M., Bayona, F., Baddam, P., & Graf, D. (2021). Craniofacial Development: Neural Crest in Molecular Embryology. *Head and Neck Pathology*, *15*(1), 1–15. https://doi.org/10.1007/s12105-021-01301-z

Soleymani, M., Ebadifar, A., Khosravi, M., Esmaeilzadeh, E., & Khorshid, H. R. K. (2022). Association of rs2013162 and rs2235375 polymorphisms in IRF6 gene with susceptibility to non-syndromic cleft lip and palate. *Avicenna Journal of Medical Biotechnology*, *14*(2), 181.

Tarr, J. T., Lambi, A. G., Bradley, J. P., Barbe, M. F., & Popoff, S. N. (2018). Development of normal and cleft palate: A central role for connective tissue growth factor (CTGF)/CCN2. *Journal of Developmental Biology*, *6*(3), 18.

Tie, Y., Tang, F., Peng, D., Zhang, Y., & Shi, H. (2022). TGF-beta signal transduction: biology, function and therapy for diseases. *Molecular Biomedicine*, *3*(1), 45.

Ueharu, H., & Mishina, Y. (2023). BMP signaling during craniofacial development: new insights into pathological mechanisms leading to craniofacial anomalies. *Frontiers in Physiology*, *14*, 1170511.

Vyas, T., Gupta, P., Kumar, S., Gupta, R., Gupta, T., & Singh, H. P. (2020). Cleft of lip and palate: A review. *Journal of Family Medicine and Primary Care*, *9*(6), 2621–2625.

Won, H. J., Kim, J. W., Won, H. S., & Shin, J. O. (2023). Gene Regulatory Networks and Signaling Pathways in Palatogenesis and Cleft Palate: A Comprehensive Review. *Cells*, *12*(15), 1–17. https://doi.org/10.3390/cells12151954

Wu-Chou, Y.-H., Lu, Y.-C., Chen, K.-T. P., Chang, H.-F., Lin, Y.-T., & Lo, L.-J. (2019). Association studies between regulatory regions of IRF6/TP63 genes and nonsyndromic oral clefts. *The Cleft Palate-Craniofacial Journal*, *56*(6), 778–785.

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