FACTORS AFFECTING FACIAL DEVELOPMENT AND FORMATION OF CLEFT LIP AND PALATE: A LITERATURE REVIEW

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ABSTRACT
The craniofacial region forms in a complicated developmental process regulated by multiple genes and growth factors. Disruption and dysregulation during facial development can lead to multiple congenital facial anomalies including cleft lip and palate. This literature review collects and analyses the existing information about the interaction of multiple growth factors and genes within the developing facial region and their association with facial pathology. The factors analysed in this review are DLX4, FOXE1, HOXB3, MSX2, PAX7, PAX9, RYK, SHH, SOX3, WNT3A, WNT9B and BARX1.

Keywords: cleft lip; cleft palate; genes; growth factors

INTRODUCTION
Development of the face is a complex process which involves a delicate balance between the action of genes that regulate the formation of the facial structures as well as the interaction of growth and developmental factors necessary for correct differentiation and maturation of the craniofacial region. If this balance is disrupted during the embryonic development, it can lead to multiple craniofacial anomalies, including cleft lip and palate.

This literature review is focused on analysing and collecting the existing information about the interaction of multiple genes and growth factors within the developing facial region. Some of these factors have been associated with craniofacial development abnormalities, but others have not been studied in such great detail regarding the formation of the facial pathologies. The
Factors affecting facial development

A literature review was conducted with PubMed (Medline), Google Scholar and ClinicalKey databases using different keywords: genes, gene proteins. By eliminating duplicates and checking article compatibility with the topic, 69 articles were selected and analysed. Data were collected from the last 30 years. The last database search was done on 3 July 2020.

**DLX4**

The distal-less homeobox (DLX) genes are homeodomain-containing transcription factors that are divided into three bi-gene DLX clusters. Each cluster contains two closely located gene pairs (Dlx1/Dlx2, Dlx3/Dlx4, Dlx5/Dlx6) that can be convergently transcribed and have been detected in both mice and humans [1]. The subsequent gene products DLX2, DLX3 and DLX4 proteins show high similarity in the structure of their homeodomains and composition of surrounding amino acids [2]. DLX genes belong to the homeobox gene super-family. Homeobox genes are expressed at specific time intervals and in specific regions during the embryonic development and control formation of the body axis and morphogenesis of all organ systems [3]. Dlx genes play an important role during the development in the process of neurogenesis and limb patterning. The function of Dlx4 during the development is unclear [3]. In mice, all Dlx genes have a different expression pattern in the branchial region during the process of embryogenesis [4]. Expression of murine Dlx genes has been described in the mesenchyme derived from neural crest cells within the first pharyngeal arch or jaw primordia. Dlx1 and Dlx2 genes are expressed within the precursor of the upper jaw – the maxillary arch, but Dlx3-Dlx6 are expressed within the precursor of the lower jaw – the mandibular arch [5]. Embryonic expression of DLX4 has not been well studied in humans, and Dlx4 expression is absent in most adult tissues [6]. A DLX4 sequence variant (mutation c.546_546delG, predicting p.Gln183Argfs*57) has been reported to be linked to the formation of cleft lip and palate where the pathological DLX4 variant produced bilateral cleft lip and palate in a mother and her child [1].

**FOXE1**

Forkhead box protein E1 (FOXE1) is a member of a transcription factor family that contains a DNA-binding forkhead domain and is involved in embryonic pattern formation [6]. Multiple FOXE1 mutations have been linked with the development of the cleft lip/palate [7]. FOXE1 is essential for proper MSX1 and TGFβ3 expression in the developing palate [8]. The proposed function of
FOXE1 is regulation of chondrogenesis [9]. FOXE1 gene is expressed at the point of fusion between maxillary and nasal processes during palatogenesis [7]. FOXE1 is expressed in the secondary palate epithelium of both mice [6] and human embryos at week 11 [10]. Newborn mice null for FoxE1 exhibit cleft palate and thyroid anomalies [6]. FOXE1 is expressed not only in the oral epithelium but also in the heart and thyroid [11]. Homozygous mutations of FOXE1 cause the Bamforth–Lazarus syndrome characterized by cleft palate, choanal atresia, bifid epiglottis, thyroid agenesis or dysgenesis, hypothyroidism and spikey hair [12].

**HOXB3**

Homeobox genes are regulatory genes that encode transcription factors during embryogenesis and normal development in which they regulate cell differentiation and proliferation [13]. There are more than 20 subclasses of homeobox genes; the most notable among these is the homeobox (HOX) gene family that consists of 39 genes [14]. These genes are subdivided into four groups: A, B, C and D [15]. Homeobox B3 (HOXB3) plays a role in migration of neural crest stem cells and is important for correct formation of pharyngeal organs and structures derived from the third and fourth pharyngeal arch pouches (thymus, parathyroid glands). HOXB3 together with HOXA3 and HOXD3 overlap in functions to regulate correct migration of the thymus and parathyroid glands during embryogenesis [16]. HOXB3 has recently attracted attention as its altered expression has been observed in a variety of cancer types [17].

**MSX2**

Muscle segment homeobox gene 2 (MSX2) is a member of the family of divergent homeobox-containing genes. There are three different Msx genes in mice and two in humans. Homeobox-containing genes share a well-conserved sequence of 183 bp coding for a helix-loop-helix motif of 64 amino acids. Most homeobox genes are organized in clusters (HOXA, B, C, and D genes) that control the development of the trunk spatially and temporally. However, other homeobox genes, dispersed around the genome and classified as divergent homeogenes, also include the MSX family which is crucial for the development of the head [18]. MSX1 and MSX2 gene mutations cause different cleft lip and palate phenotypes – from cleft palate to bilateral cleft lip and palate [19, 20, 21]. MSX2 is detectable in the orofacial skeleton: the mandibula and maxilla, Meckel’s cartilage and teeth germs [22]. The mutation of the MSX2 gene causes
Boston-type craniosynostosis in humans [22, 23]. MSX2 is essential for proliferation of osteoblast progenitors during normal craniofacial development [22].

**PAX7**

Paired box 7 (PAX7) is a transcription factor that is involved in neural crest development by affecting the expression of neural crest markers Slug, SOX9, and SOX10 [25]. PAX7 expression has been detected in the palatal shelves, Meckel’s cartilage, and in nasal structures like the nasal epithelium. Mice with mutant Pax7 have development malformations of the maxilla and the nose [24, 27]. PAX7 was previously associated with non-syndromic cleft lip/palate in four human populations in a candidate gene association study [24, 26]. The single SNP at 1p36 associated with cleft lip/palate is located in an intron in the PAX7 gene (encoding paired box 7) [28]. PAX7 is functionally involved in craniofacial development [27]. One study investigated seven PAX7 variants in non-syndromic cleft lip/palate case-parent trios from multiple populations, and two PAX7 variants showed a strong parent-of-origin effect [26].

**PAX9**

Paired box 9 (PAX9) belongs to the family of paired-box DNA-binding domain-containing transcription factors, which play key roles in organogenesis [29]. Pax9 gene deletion is well known to induce cleft palate in mice [30]. The deletion causes defects in palatal shelf elevation and extracellular matrix changes (decrease in hyaluronic acid saturation), defective organ development from pharyngeal pouches and tooth development arrest at the bud stage [31, 32]. Pax9 expression needs to be balanced and correctly timed for normal palate development – upregulation is seen during palatal vertical growth and elevation, and downregulation happens before the palatal shelves fuse together [33]. PAX9 downstream affects SHH by promoting growth in anterior-posterior palate axis and rugae formation [34]. PAX9 gene deletion causes a significant loss of BMP pathway signalling (BMP4, MSX1) and causes defects in WNT β-catenin-dependent pathway by the upregulation of genes DKK1 and DKK2, which antagonize WNT signalling, and by the downregulation of WNT7A, WNT3, WNT9B [30,34,35]. PAX9-deficient mice die shortly after birth, exhibiting complete cleft palate [36].
**RYK**

Receptor-like Tyrosine Kinase (RYK) protein has an extracellular domain similar to WIF1 (WNT inhibitory factor 1), a transmembrane domain, and a kinase-dead tyrosine kinase domain, and it is able to bind to WNT5A protein [37, 38]. RYK is involved in multiple molecular events including heterodimerization with other receptor tyrosine kinases (RTKs) [39], activation of Src kinase [40], binding to frizzled (Fz) receptors [38]. WNT can induce the nuclear translocation of the RYK intracellular domain, which promotes neuronal differentiation [37, 41]. RYK is essential for normal development and formation of craniofacial structures like the secondary palate. Mice deficient in RYK have a specific craniofacial appearance, shortening of limbs and increased postnatal mortality caused by feeding and respiratory complications associated with a complete cleft of the secondary palate [39].

**SHH**

One of the signalling pathways involved in craniofacial development is the Hedgehog family, in particular, the Sonic hedgehog (SHH) and the Indian hedgehog (IHH) [42]. IHH is required for normal ossification and bone development of the craniofacial region [42, 43]. IHH null mice exhibit reduced expression of osteogenesis factors and decreased ossification process in the palate [44]. SHH is one of the most studied signalling pathways of lip and palate morphogenesis [45]. SHH is essential for craniofacial development, particularly the palate and frontonasal development, and is predominantly found at sites of epithelial-mesenchymal interactions, inducing mesenchymal cell proliferation [46, 47, 48, 49]. SHH is required for mesenchymal cell survival in early stages of development and for cell proliferation in later stages [19, 49]. Before the palatal shelf elevation and fusion, SHH is found in the oral side of the palatal epithelium, afterwards only in spots of thickened epithelium (rugae) [43, 50]. The enhanced SHH signalling might have a restrictive role in WNT signalling by enhancing WNT antagonist signalling [51]. SHH signalling plays an important role in fusion of facial processes and formation of the upper lip. Enhanced SHH signalling caused by mutated Patched1 during head development can lead to cleft lip with craniofacial abnormalities (hypertelorism) [51, 52].
SOX3
The SRY-Box Transcription Factor 3 (SOX3) gene found in the X-chromosome belongs to the SOXB1 (SOX1-3) subfamily of transcription factors [53, 54]. SOX1, SOX2 and SOX3 are expressed in neural progenitor cells where they help to sustain the undifferentiated state of progenitor cells and counteract the activity of proneural differentiation factors, which is important for the development of the neural tube and various placodes [55]. The SOX2 gene is the closest relative of SOX3 and is one of the main pluripotency factors involved in the regulation of stem cell activity and differentiation [54, 56]. SOX3 is known as one of the earliest neural markers in vertebrates and is currently the most studied functional aspect of SOX3 action [57]. In murine telencephalon, Sox3 is expressed in neural stem/progenitor cells during embryonic development and is later downregulated during neuronal differentiation [58].

WNT3A
The Wingless-Type MMTV Integration Site Family (WNT) genes were discovered in 1982, and similar homologous genes have been reported in other organisms like mice (Int gene) and Drosophila (wingless gene) [59]. The WNT gene family encodes 19 different proteins, including WNT1, WNT2, WNT2b (WNT13), WNT3, WNT3A, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT9A (WNT14), WNT9B (WNT14B), WNT10A, WNT10B, WNT11, and WNT16. These proteins are characterized by being secretory glycoproteins that are rich in cysteine [59, 60]. WNT proteins can bind to cell surface receptors and are important in the process of autocrine and paracrine regulation through the WNT signalling pathway [59]. Wingless-Type MMTV Integration Site Family, Member 3A (WNT3A) together with other WNT genes play an important role in craniofacial morphogenesis, which has been studied in mouse models. WNT3A expression has been detected in the upper lip region, also in the primary and secondary palate, and they play a role in regional specification within the developing face of vertebrates [61]. WNT3A together with WNT11 and WNT8A mediate neural crest cell migration and differentiation within the pharyngeal/branchial arches, contributing to the formation of connective tissue and bone in the head and neck region [61]. WNT genes, including WNT3A, have been suggested as candidate genes for the development of the cleft lip and palate [61, 62].
WNT9B

Wingless-Type MMTV Integration Site Family, Member 9B (WNT9B), belongs to the WNT gene family. It has also been suggested as a potential candidate gene in the formation of the cleft lip/palate [61, 63]. Recessive knockout mutation of WNT9B (WNT9B-/-) gene in murine models showed the formation of the cleft lip with or without the cleft palate with incomplete penetrance [64]. WNT9B together with WNT3 are located in the clf1 region of chromosome 11, which has been associated with the cleft lip and palate [61, 65]. The WNT signalling pathway is essential for the proper development of the craniofacial region. Typically, the loss of function of WNT genes is associated with defects in the craniofacial region, including the cleft lip [65].

BARX1

BarH-like homeobox 1 (BARX1) is a homeobox gene expressed in ectomesenchymal cells of the developing mandibular and maxillary prominences and plays a role in the formation of pharyngeal osteochondrogenic condensation [66, 67, 68]. BARX1 is also expressed in the anterior and posterior palatal shelves and is present in a restricted epithelial localization of the anterior domain [68]. BARX1 mesenchymal expression seen in the posterior palate is complemented by the anterior expression of MSX1 [69]. WNT3A can significantly increase the expression of BARX1 by activating the WNT signalling pathway. Reduced BARX1 expression can potentially cause defects in osteochondrogenic cell condensation and subsequently cause maxillary hypoplasia [66].

CONCLUSION

The amount of information about genes HOXB3, SOX3, WNT9B in the development of the craniofacial region is quite limited. More detailed information is available about SHH and WNT3A about their involvement in craniofacial development and pathogenesis of facial malformations. For genes DLX4, FOXE1, PAX9, RYK, and WNT9B most information about their functionality comes from morphological and genetical research of mice or other model animals, but the information from human studies is limited to genetical studies or specific case studies. This limits the possibility of accurate prediction of the formation of craniofacial pathologies in humans and causes difficulties in understanding the possible mechanisms of cleft and other facial anomaly formation in unclear or multietiological cases.
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