Original Article

Expression of interferon regulatory factor 6, muscle segment homeobox 1, paired box gene 9, homeo box B3, and related to tyrosine kinases in human cleft-affected tissue

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ABSTRACT

Background and Aim: Recent studies demonstrate direct roles of different genes during formation of secondary palate, but there are no still data about local expression and distribution of gene products in cleft palate affected human tissue. Thus, the aim of our study was to investigate cleft disordered cartilage and bone for detection of local expression of key regulators of palatogenesis and its correlations. **Materials and Methods:** The study involved 16 patients with unilateral cleft lip and palate. Tissue samples were proceeded for detection of interferon regulatory factor 6 (IRF6), muscle segment homeobox 1 (MSX1), paired box gene 9 (PAX9), homeo box B3 (HOXB3), and related to tyrosine kinases with biotin-streptavidin immunohistochemistry. Distribution of immunoreactive structures was detected semiquantitatively. Statistical analysis included the Mann–Whitney test and Pearson's correlation test. **Results:** Statistically significant differences were found between expression of IGFR6, MSX1, and HOXB3 in the cartilage and bone. We also detected statistically significant correlation between the expressions of PAX9 and MSX1 in the bone tissue. **Conclusions:** Cleft lip and palate disordered cartilage is characterized by more pronounced expression of IRF6, MSX1, and PAX9. Expression of HOXB3 is more characteristic for cleft lip and palate affected bone. Considered as a whole, our results suggest that the cleft lip and palate affected cartilage seems more plastic in tissue remodeling what can probably result in qualitative postoperative tissue reconstruction.

Key words: Cleft lip and palate, genes, local expression

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Access this article online

Website: www.jofs.in

DOI: 10.4103/0975-8844.181931

Quick Response Code:

INTRODUCTION

Clefts of the lip and/or palate affect approximately 1 in 700 live births, with wide variations across racial and ethnic groups, geographic origin, and socioeconomic status. Native American and Asian populations have the highest reported birth prevalence rates, which are as often as high as 1 in 500.[1] Mentioned congenital craniofacial defect occurs in approximately 1/700-800 newborn infants in Latvia.[2]

Development of secondary palate starts with formation of a pair of outgrowths from the inner part of maxillary

How to cite this article: Krivicka-Uzkurele B, Pilmane M. Expression of interferon regulatory factor 6, muscle segment homeobox 1, paired box gene 9, homeo box B3, and related to tyrosine kinases in human cleft-affected tissue. J Orofac Sci 2016;8:59-65.

processes. Such palatal shelves are composed of a core of neural-crest derives mesenchyme covered by sheet of the cells derived from facial ectoderm.[3] They grow vertically down either side of the tongue, then elevate to a horizontal position above the tongue, gradually contact each other through their medial edge epithelia, and adhere forming transient medial epithelial seam.[4]

Fundamentally, palatogenesis involves epithelial-mesenchymal interactions and extensive remodeling of the extracellular matrix of the palatal shelves. Studies show that mentioned processes are regulated by numerous genes including interferon regulatory factor *6* (*IRF6*), muscle segment homeobox 1 (*MSX1*), paired box gene 9 (*PAX9*), related to tyrosine kinases (*RYK*), and homeo box B3 (*HOXB3*).[4-9]

IRF6 is a member of the IRF family of transcription factors. Currently with nine members, the IRF family plays a critical role in the innate immune response and has been implicated in numerous other cellular processes, including cell cycle regulation and apoptosis.[10] During normal palatal development, *IRF6* coordinates epithelial proliferation and differentiation.[4] *IRF6*-null mice exhibit a hyperproliferative epidermis that fails to undergo final differentiation and leads to severe intraoral epithelial adhesions.[11] Detailed studies show that IRF6 is expressed in the midline seam and epithelial triangles of palatal shelves.[12]

The mammalian *MSX* gene family consists of three members, named *MSX1*, *MSX2*, and *MSX3*. MSX1 is widely expressed in many organs during embryonal development, particularly in the regions where epithelial-mesenchymal interactions take place.[6] Detailed review of Alappat *et al*. reports about diffuse expression of MSX1 in the mesenchyme of the anterior parts of developing palatal shelves.[13]

Analysis of the literature on regionally expressed genes by Smith *et al*. confirms that the main function of *MSX1* is regulation of the cell proliferation in the anterior mesenchyme.[14] Moreover, they report about ability of the cells migrate from the posterior region of the palate to anterior region of the palate and then to the oral region of the anterior palate and thus also plasticity of the cell populations during early stages of palate development. In humans and mice, loss of *MSX1* function results in clefts of secondary palate. In the *MSX1* null mutant embryos, palatal shelves fail to elevate from a vertical to a horizontal position, consequently they do not fuse and remain opened, with the base of the nasal septum exposed in the oral cavity.[15] Failed fusion between palatal primordia is the result of significantly lower levels of cell proliferation in their anterior region leading to growth impairment.[13]

PAX gene family consists of nine members that, based on the presence or absence of structural motifs, are divided into four subgroups. Members of Group I – PAX1 and PAX9 – are expressed in the developing facial processes and influence the formation of lower face. *PAX9*-deficient mice die shortly after birth, exhibiting complete cleft palate.[6] Peters *et al*. already in 1998 demonstrated expression of PAX9 not only in palatal shelves but also in the mesenchyme of the mandibular arch facing the palatal shelves and concluded that mentioned gene is not necessary for the capability of the shelves to elevate but is required to regulate their shape at the critical stage of secondary palate formation.[16] Recent studies indicate that *PAX9* plays a crucial role in patterning the anterior-posterior axis, outgrowth of the developing palatal primordia, and normal palatal shelf elevation.[3] Moreover, Nakatomi *et al*. investigated genetic interactions between *PAX9* and *MSX1* during craniofacial development and found that a combined reduction of *PAX9* and *MSX1* gene dosage in humans may increase the risk for orofacial clefting.[17]

HOX genes encode a family of transcriptional regulators that elicit distinct developmental programs along the head-to-tail axis of animals.[18] Before formation of oral cavity, neural crest cells migrate into the developing pharyngeal arches and provide the precursors of connective tissue, cartilage, and bone of the head and neck. The movement and destination of neural crest cells into the facial primordia are controlled by a number of genes including *HOXB3*. Deficiencies of neural crest cells migration or proliferation lead to abnormal formation of cartilage and bone and accordingly produce an extensive group of craniofacial malformations including orofacial clefts.[8,19]

RYK gene encodes a catalytically inactive member of the receptor protein tyrosine kinase (RTK) family. The RTKs typically are transmembrane signal transduction glycoproteins with an extracellular N-terminal ligand-binding domain and an intracellular C-terminal tyrosine kinase domain. They regulate diverse cellular functions including mitogenesis, differentiation, and morphogenesis.[20] Researches demonstrate that mice homozygous for a null allele of *RYK* have a distinctive craniofacial appearance, shortened limbs, and postnatal mortality due to feeding and respiratory complications associated with a complete cleft of the secondary palate.[21]

However, despite the many researches, there are no still data about local expression and distribution of gene products in cleft palate affected human tissue. Thus,

the aim of our study was to investigate cleft disordered cartilage and bone for detection of local expression of key regulators of palatogenesis and its correlations.

MATERIALS AND METHODS

The research is based on the material of cleft lip and palate patients, which was gathered within a period from 2003 to 2006 at the cleft lip and palate center of the Institute of Stomatology of the Riga Stradins University. This study has been approved by the local Ethical Committee of Riga Stradins University (2003).

The research involved 16 patients with unilateral cleft lip and palate aged from 6 years 3 months to 17 years. Eleven cartilage and ten bone tissue samples were collected during the nasal surgical procedure from the borders of the cleft region. Routine histological staining with hematoxylin and eosin was developed for each case to get review picture of the slide.

Immunohistochemical staining

Tissue were fixed for a day in mixture of 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (ph 7,2). After samples were rinsed in Tyrode's buffer, containing 10% saccharose for 12 h, embedded into paraffin, and cut in 6–7 μm thin sections. Sections were proceeded for detection of IRF6 (IRF6, ab167403, work dilution 1:200, Abcam), *MSX1* (MSX1, orb18823, work dilution 1:200, Biorbyt Ltd.,), PAX9 (PAX9, sc-56823, work dilution 1:100, Santa Cruz Biotechnology, Inc.,), HOXB3 (HOXB3, sc-28606, work dilution 1:50, Santa Cruz Biotechnology, Inc.,), RYK (RYK, orb38371, work dilution 1:100, Biorbyt Ltd.,) by use of biotin-streptavidin immunohistochemical method.

Evaluation of immunohistochemical staining

Distribution of immunoreactive structures was detected semiquantitatively.[22] Using ×200 magnification, the quantity of structures was analyzed in five visual fields of one section. Scale was as follows: "-" – no positive structures found in visual field, "0/+" – occasional positive structures seen in visual field, "+" – few immunoreactive structures seen in visual field, "++" – moderate number of immunoreactive structures seen in visual field, "+++" – numerous immunoreactive structures seen in visual field, and "++++" – abundance of immunoreactive structures seen in visual field.

Results were illustrated using Leica DC 300F camera and the image processing and analysis software, Image-Pro Plus version 6.0. (Media Cybernetics, USA).

Statistical analysis

Statistical analysis was performed with IBM SPSS version 22 (IBM Corp., Armonk, USA) and included the Mann–Whitney test and Pearson's correlation test, $P < 0.05$ being considered statistically significant.

RESULTS

Expression of IRF6 was observed in the cartilage samples of all patients. The relative number of positive cartilage cells varied from few to numerous [Table 1]. Four tissue samples demonstrated few positive cells and five – moderate number of positive cells [Figure 1]. Moderate to numerous (++/+++) and numerous immunoreactive and mostly mature chondrocytes we saw in one case each. By contrast, IRF6 immunostaining was notably reduced in the bone and these difference can be evaluated as statistically significant ($Z = -3,60$; $P < 0.001$). We found a few positive osteocytes in the tissue samples of three patients [Figure 2].

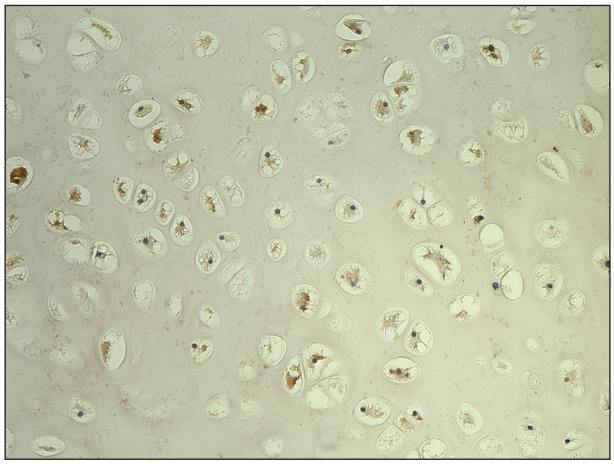

Figure 1: Moderate number of interferon regulatory factor 6-positive chondrocytes in the zone of mature cells. Interferon regulatory factor 6 (IMH, ×200)

Table 1: Semiquantitative distribution of immunoreactive structures in the cartilage and bone in patients with unilateral cleft lip and palate

Tissue/gene products	IGRF6	Msx1	Pax9	Ryk	HoxB3
Cartilage	+-++-+++	0/+-+-++	0-0/+-++	0-0/+-+	0/+-+-++
Bone	0-+	0-0/+-+	0-0/+-+	0-0/+-+	+-++-++/+++

factor, which variations in the tissue groups were statistically plausible, and the relative number of factor positive structures -- no positive structure seen in the visual field; 0/+ rare positive structures seen in the visual field; + few positive structures seen in the visual field; +/++ few to moderate number of positive structures seen in the visual field; ++ moderate number of positive structures seen in the visual field; ++/+++ moderate to numerous positive structures seen in the visual field; +++ numerous positive structures seen in the visual field; ++++ abundance of positive structures seen in the visual field _ - the most frequently observed relative number of positive structures

MSX1-positive cells we detected in the cleft lip and palate affected cartilage of all patients. We saw moderate number of positive cells in six cases, few – in three cases, and occasional – in two cases. The relative number of immunoreactive bone cells was significantly lower ($Z = -3{,}53$; $P < 0.001$). MSX1 immunoreactivity was detected in four cases. Three tissue samples demonstrated occasionally and one – few positive osteocytes.

Expression of PAX9 was observed in the cleft-affected cartilage of seven patients. Mainly, we found occasional positive cells, but two tissue samples demonstrated moderate number of immunoreactive structures. PAX9-containing occasional and few osteocytes were seen in one case each. Interestingly, we found statistically significant correlation between the expressions of PAX9 and MSX1 in the bone tissue ($r = 0.81$; $P = 0.004$).

Slight immunoreactivity of HOXb3 was characteristic for cleft lip and palate affected cartilage. Mainly, we found occasional or few positive cells. Only one tissue sample demonstrated moderate number of positive chondrocytes. Relative number of immunoreactive bone cells was more prominent and this difference can be evaluated as statistically significant ($Z = 0.04$; $P = 0.002$). Thus, we saw moderate to numerous positive osteocytes in five cases, moderate number – in two cases, and few – only in three cases [Figure 3]. Frequently expression was regional.

We detected RYK-positive cells in the three cleft lip and palate affected cartilage samples and four bone materials. There were mostly regionally located occasional or few positive cells [Figure 4].

DISCUSSION

Genetic expressions are clearly predominant during embryonic craniofacial morphogenesis. Genes causing various orofacial defects include IRF6, MSX1, and PAX9 and a lot of publications describe in details expression of gene proteins during development of oral cavity.[6] However, despite the many studies, there are no data about expression of mentioned gene products in human cleft-affected tissue. Thus, we focus on immunohistochemical analysis of human tissue taken from cleft borders during surgery.

IRF6 is highly expressed in epithelial cells and is required for regulation of proliferation and differentiation during epithelial development.[23] Studies indicate that mice with loss of IRF6 functions exhibit cleft palate owing to failure of palatal shelf elevation resulting from adhesion between the palatal shelves and the tongue, following a defect in epithelial differentiation.[24] Despite the fact that the primary defect in IRF6-deficient mice is in keratinocyte differentiation and proliferation, recent studies suggest that IRF6 also guides lingual mesodermal

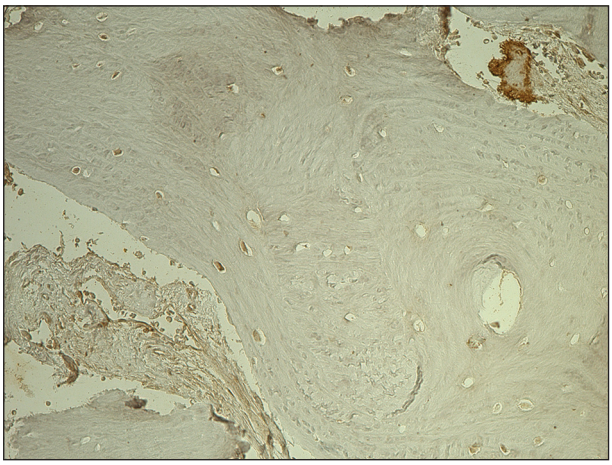

Figure 2: Expression of interferon regulatory factor 6 in few osteocytes. Interferon regulatory factor 6 (IMH, ×200)

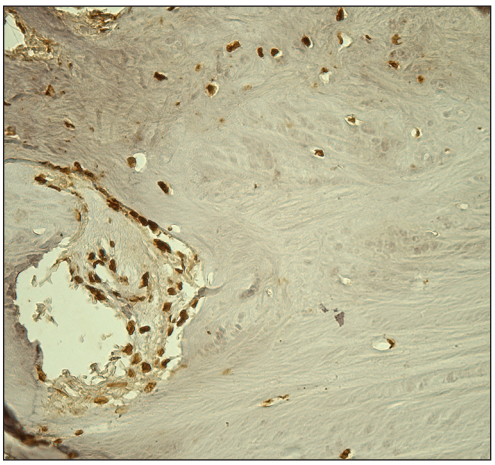

Figure 3: Expression of homeo box B3 in regionally located osteocytes. Homeo box B3 (IMH, ×250)

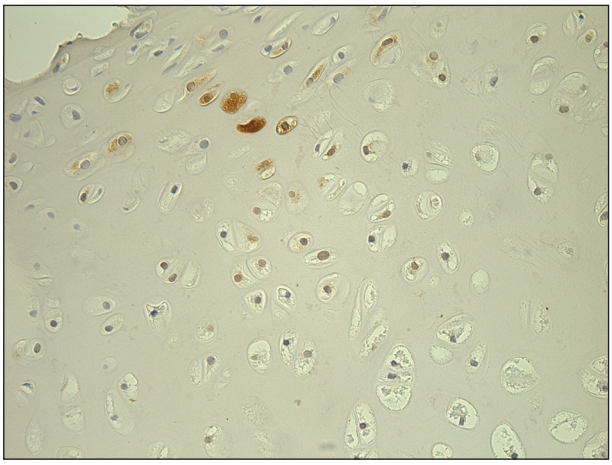

Figure 4: Few related to tyrosine kinases immunoreactive regionally located cartilage cells. Related to tyrosine kinases (IMH, ×250)

and mesenchymal development.[23] During zebrafish palatogenesis, IRF6 is expressed in the bilateral maxillary prominences chondrocytes and required for integration with the median frontonasal prominence.[25] In addition, these mice have skeletal and cutaneous abnormalities, suggesting additional functions for IRF6 in bone and epidermal homeostasis.[26]

Our results showed that expression of IRF6 in the cartilage and bone was clearly different. Although the relative number of positive structures was variable, all cleft-affected cartilage samples demonstrated IRF6 immunoreactivity. Thus, we can suppose that IRF6 promotes and controls proliferation potential of the cartilage cells and is the basis of successful tissue remodeling and repair. Moreover, Jones *et al*. tested and confirmed the hypothesis that children with mutations in IRF6 have more wound complications following cleft repair than in children with nonsyndromic cleft lip and palate.[26] Thus, we can speculate that regenerative abilities of cartilage are more pronounced.

Members of MSX gene family, including MSX1, are among most critical factors involved in the craniofacial development. MSX1 is transcription factor expressed at multiple sites of tissue interactions. Especially, it is associated with epithelial-mesenchymal interactions.[6] Orestes-Cardoso *et al*. investigated the tissue of mice from first postnatal week until 15 months and found maintained expression of MSX1 in the sites where it plays an early morphogenetic role during initial skeletal patterning – cranial sutures, mandible, and alveolar bone. Histological sections demonstrated that progenitor as well as differentiating and differentiated cells of all the bone lineage could express the MSX1 protein.[27] After a year, they published data that suggest that MSX1 play a role in a site-specific manner not only in early development but also in skeletal growth, modeling, and homeostasis throughout the entire lifetime.[28] Our results demonstrated expression of MSX1 in all cartilage tissue samples, while in bone it was found only in four cases. Although relative amount of positive cartilage cells was variable, mainly we detected moderate number of positive structures. Thus, our data suggest that cleft-affected cartilage is still plastic tissue and demonstrates more abilities to proliferate, remodelate, and accordingly heal. Moreover, there are clear data about direct role of MSX1 in the regeneration of chick and mouse limb buds.[29]

PAX genes are important regulators of organogenesis in all species and play important roles during multiple stages of development. Functions of mentioned genes include triggering differentiation processes as well as maintaining proliferative activities.[30] The member of the PAX transcription factor family, PAX9 is a key regulator in mesenchyme during the development of a wide range of organ primordia including the palatal shelf.[31] Recent investigations show that PAX9 is expressed not only in the palatal mesenchyme but also in the posterior palatal epithelium and plays an important role in regulating the mesenchymal-epithelial interactions during palate development.[3] Interestingly, in addition to a cleft secondary palate in PAX9, mutants are defects of formation of alveolar bone.[17] Our results showed that expression of PAX9 is more characteristic for cartilage taken from cleft borders. Variable number of positive cartilage cells was seen in the material of seven patients while slight expression of PAX9 in the bone was detected only in two cases. Further, our results continue to strengthen previous assumptions about better proliferative and regenerative abilities exactly of the cleft-affected cartilage. Statistically significant correlation what was found between expression of the PAX9 and MSX1 in the bone probably is indicator of genetic interactions in postnatal palatogenesis.

HOX genes are the key determinants of different morphogenetic events in all bilaterian animals. They encode a highly conserved family of transcription factors that play fundamental roles in morphogenesis during embryonic development.[32] At the same time, HOX genes were also shown to participate in processes in the adult body. In mammals, HOX genes are expressed in the adult tissues capable of remodeling and/or constant renewal. Thus, in vertebrates, the genes of the HOX cluster are involved in physiological regeneration, for example, cyclic renewal of hair follicles or hematopoiesis. Genes, including HOXB3, were shown to regulate differentiation of mesenchymal stem cells, which play an active role in reparative morphogenesis in vertebrates.[33] Moreover, studies show that several homeoboxs affect angiogenesis and wound healing in adult tissue. HOXB3 belongs to the group of pro-angiogenic homeobox genes and induces capillary morphogenesis.[34] Our results demonstrated that more pronounced expression of HOXB3 is characteristic for cleft-affected bone, where we observed moderate to numerous positive osteocytes in five tissue samples and moderate number of immunoreactive bone cells – in two cases. In contrast to the previously observed trend, relative number of immunoreactive structures in the cartilage was lesser and this difference was evaluated as statistically significant. In situation such as these, we can speculate that bone as well-vascularized tissue demonstrates regeneration abilities, what, probably, will result in qualitative wound healing. In addition, there is opinion that homeobox gene therapy can also be leveraged in the field of regenerative medicine to induce vascularization;[34] however, in these cases, it will be important to know natural expression levels of gene products in tissue.

Investigations clearly demonstrate that RYK-deficient mouse has a completely cleft secondary palate, more rounded and smaller cranial vault, disproportionately shorter limbs, and reduced in size mandible.[21] We detected weak expression of RYK in the cartilage samples of three patients and bone samples of four patients. Thus, our results suggest that mentioned gene products do not operate in postnatal tissue remodeling in cleft palate patients.

CONCLUSIONS

Cleft lip and palate disordered cartilage is characterized by more pronounced expression of IRF6, MSX1, and PAX9, giving evidence to the involvement of this mentioned factors in the postnatal tissue stimulation and remodeling. Expression of HOXB3 is more characteristic for cleft lip and palate affected bone and probably facilitates tissue regeneration. Considered as a whole, our results suggest that the cleft lip and palate affected cartilage seems more plastic in tissue remodeling, what can probably result in qualitative postoperative tissue reconstruction.

Acknowledgment

We would like to thank Professor Ilze Akota (Department of Oral and Maxillofacial Surgery, Institute of Stomatology, Riga Stradins University) for the supply of tissue.

Financial support and sponsorship

This study was funded by the support of Riga Stradins University project "Longitudinal research on cleft morphopathogenesis."

Conflicts of interest

There are no conflicts of interest.

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